THE RAT CORTICOTROPIN RELEASING HORMONE GENE

by

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A DISSERTATION

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(Professor in Charge of Thesis)

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(Chairman, Graduate Council)

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Dedication

I would like to dedicate this thesis to my Dad and Katie.

ACKNOWLEDGEMENTS

Thinking about the number of people who aided the efforts put forth in this thesis was, in the least, humbling. First, I would thank Jimmy D. (Dr. T) for all his time, efforts and thoughtful suggestions in the last four years. Dr. T has been a good friend and caring advisor.

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ABSTRACT

The mammalian stress response is mediated in large part by the hypothalamic-pituitary-adrenal (HPA) axis. The key hypothalamic releasing factor in this axis has been shown to be corticotropin releasing hormone (CRH). CRH stimulates the synthesis and secretion of adrenocorticotropin (ACTH) from the anterior pituitary which in turn stimulates the production and release of glucocorticoids from the adrenal cortex. Glucocorticoids then mediate the body's adaptive response to stress. Many abnormalities in HPA function are due to pituitary or adrenal dysfunction, but recent studies have suggested that other abnormalities, such as depression, involve the hypersecretion of CRH at the hypothalamic level. Thus, CRH production and release represent key control levels at which man's ability to respond physiologically to external stimuli can be regulated.

In 1983, the ovine cDNA and the human gene for CRH were cloned, providing the first descriptions of the CRH precursors in these species. However, specific DNA probes to study CRH gene regulation in the rat were unavailable. The first objective of this thesis was to obtain these molecular biological tools for future CRH biosynthetic studies in the rat. Therefore, I have isolated a rat preproCRH cDNA from a rat hypothalamic cDNA library, permitting the amino acid sequence of the rat CRH precursor to be deduced from the nucleic acid sequences of the cDNA. The second objective of this thesis was to define the tissue specific expression of CRH mRNA in the rat

using the rat CRH cDNA as a hybridization probe. I have demonstrated using Northern blotting techniques that CRH mRNA is expressed in many regions of the brain as well as in peripheral tissues. In some peripheral tissues, the CRH mRNA is larger than the CRH transcript identified in the brain. I have used RNase protections and Northern blotting techniques to investigate the nature of this size variation. The results of these experiments suggest that the CRH mRNA in testis represents an alternatively spliced mRNA arising from a more distant 5' promoter element.

The last objective of this thesis was to examine the mechanisms of transcriptional regulation of the rat CRH gene. To accomplish this objective, I isolated and characterized the rat CRH gene. The nucleotide sequence of the 5' flanking region of this gene demonstrates a rather remarkable level of homology to the same region in the human gene, suggesting that these conserved DNA sequences may represent key transcriptional control elements in both species. Using gene transfer approaches, I demonstrated that this promoter region contains a cis-acting DNA element involved in the cAMP mediated induction of transcription of the rat, and possibly the human, CRH genes in vitro.

INTRODUCTION

Stress induces a wide variety of biological responses including changes in the cardiovascular and immune systems as well as modifications in central nervous system activity and behavior. The diversity of these responses is an indication of their relative importance as physiological stimuli. Additionally, chronic stress has been shown to be associated with several different disease states and neuropsychiatric disorders. The biological mechanisms of stress are poorly understood primarily due to the complex nature of the interacting systems. A primary event in all forms of stress is the secretion of adrenocorticotropin hormone (ACTH) from the anterior pituitary. ACTH, released into the circulatory system, causes the synthesis and secretion of glucocorticoids from the adrenal cortex, which mediate the physiological effects of stress. Because all forms of stress induce the secretion of ACTH, control of this secretion has been the focus of intense research during the past three decades.

Experiments in the late 1890's by Olivier and Schafer demonstrated the powerful pressor (decrease in heart rate) effects of extracts of the adrenal gland (1). The active substance was isolated and characterized by J.J. Abel who named it epinephrine (2). These experiments, along with those of T.R. Elliott studing adrenal extracts and their effects on the sympathetic nervous system (3,4,5), produced the first evidence that substances were released from nerve terminals and thus the concept of chemical neural

transmission was born. The relationship between the role of the sympathoadrenal medullary system and the elaborate control involved in the maintainence of the internal environment was described by W. Cannon in the early 1900's (6,7). Cannon demonstrated that the sympathoadrenal system was responsible for coordinating all of the "fight or flight" responses exhibited by an animal when presented with an external challenge and that emotional as well as physical stimuli produced the same responses.

In 1936, H. Selye first coined the term, "stress", in describing the many biological effects seen in experimentally treated animals including: 1) adrenocortical enlargement with discharge of secretory granules; 2) involution of the thymicolymphatic apparatus; and 3) the appearance of bleeding ulcers in the gut (8). Later that same year, Selye found that the effects on the lymphatic system and the gut could be abolished by adrenalectomy (9) and in 1937 observed that all three effects could be abolished by hypophysectomy (10). Selye erected the General Adaptation Syndrome theory to account for the effect of stress on the organism in which the pituitary and adrenal glands played a central role as mediators of the response to stress (11). However, it was not until 1948 that Harris first proposed that higher brain centers controlled the pituitary's production of many hormones including ACTH (12), whose activity was described many years earlier but whose structure was not defined until 1954 (13). Harris also suggested that these controlling factors could interact with the pituitary via a complex series of portal vessels connecting

the brain with the anterior pituitary. In 1955, Saffran and Schally (14) and Guillemin and Rosenberg (15) obtained the first evidence of the endocrine action of the brain, when they showed that hypothalamic fragments possessed remarkable corticotropin releasing activity (substances which contain ACTH-releasing activity) when incubated with pituicytes in vitro. It was upon this work that Guillemin and Schally based much of their 1976 Nobel Prize winning efforts for determining the structure of two representative hypothalamic-derived anterior pituitary releasing hormones, thyrotropin-releasing hormone (TRH) and luteinizing hormone-releasing hormone (LHRH) (16,17).

Once the sequence of several hypothalamic hormones was determined, it became possible to show that many were synthesized not only in the hypothalamus, but in many regions of the brain. Such widespread distribution of neurohormones in the central nervous system, along with the demonstration that brain hormones exerted relatively long lasting effects at minute concentrations, helped to explain subsequent observations that neuropeptides appeared to organize the activity of multiple nerve cell aggregates located disparately throughout the central nervous system promoting the execution of complex behavioral and physiological processes.

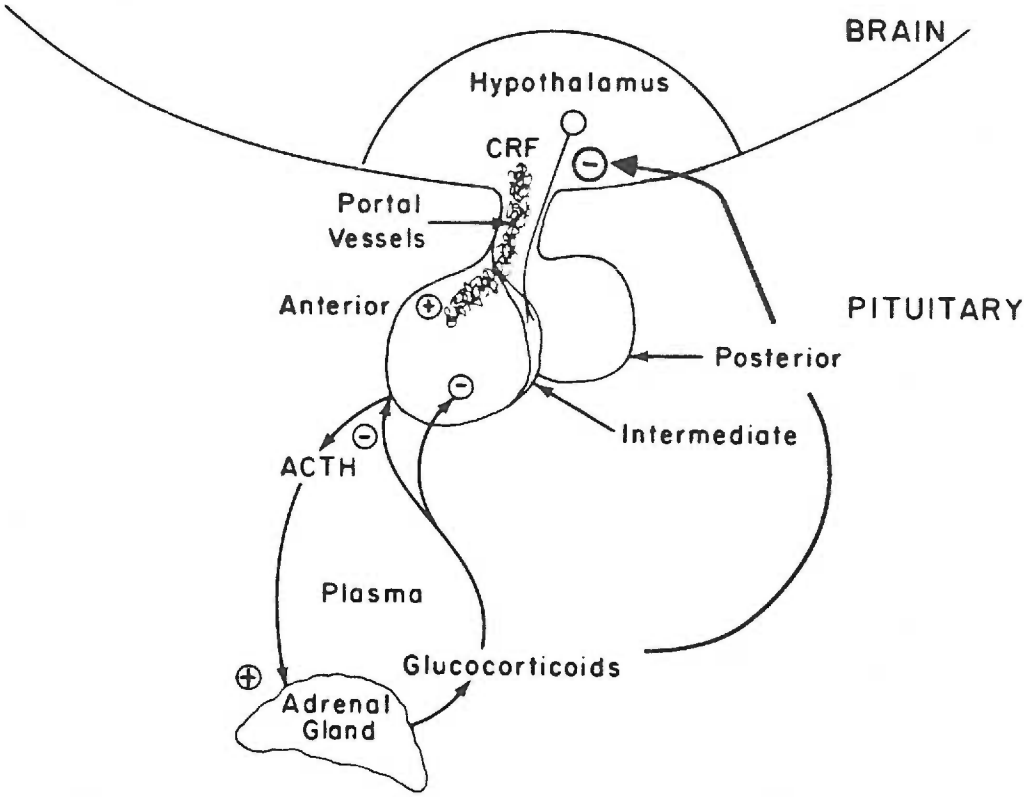
Over the past few years, some of the molecular mechanisms controling ACTH secretion have been determined. These mechanisms have included identification of neurotransmitters, neurohormones, hormones and second messenger systems which effect ACTH

secretion and also ACTH synthesis. ACTH, a 39 amino acid peptide, is synthesized in a larger protein precursor form termed proopiomelanocortin (POMC) which also contains one of the opioid peptides, ß-endorphin (18,19). ACTH is synthesized in corticotroph cells in the anterior pituitary and, when secreted, can enter the general circulatory system by diffusing into adjacent vascular vessels (20). In response to stress, secreted ACTH peptide levels increase dramatically in circulating blood and regulate the synthesis and secretion of adrenal glucocorticoids by binding to specific cell receptors near or on adrenal cell membranes (21,22). Glucocorticoids are synthesized in the adrenal cortex. The major form of glucocorticoids in humans is cortisol but small quantities of corticosterone are also produced. In addition to the myriad of biological effects exerted by glucocorticoids, they also serve as feedback inhibitors to regulate the secretion and synthesis (23) of ACTH from the anterior pituitary and also higher brain centers involved in stimulating ACTH secretion (24). The interactions between the hypothalamus, the pituitary gland, and the adrenal gland are diagrammed in figure 1.

The isolation of corticotropin releasing factors (CRFs) was complicated by the demonstration that many compounds could alter the secretion of ACTH. For example, catecholamines, vasopressin, oxytocin, angiotensin II (25,26), interleukin II, and hepatocyte-stimulating factor (27) have all been shown to influence ACTH secretion. Some of these compounds were tested in whole

Figure 1. The Hypothalamic-Pituitary-Adrenal (HPA) Axis.

A schematic diagramming the interacting tissues of the HPA axis is shown. Corticotropin Releasing Hormone (CRH) produced in the hypothalamus is secreted into the portal blood vessels which subsequently bath the anterior pituitary where ACTH is produced. CRH, interacting via specific membrane receptors on ACTH-producing cells, stimulates the secretion of ACTH into the general circulation. ACTH interacts with the cells of the adrenal cortex and stimulates the synthesis and secretion of glucocorticoids. Glucocorticoids then mediate the vast effects of stress in the body as well as serving as feedback inhibitors at the level of the anterior pituitary and the brain.


BRAIN
Hypothalamus
CRF
Portal
Vessels
Anterior
PITUITARY
Posterior
Intermediate
ACTH
Plasma
Glucocorticoids
Adrenal
Gland

animals and have been suggested to indirectly alter ACTH secretion by affecting CRH secretion from the hypothalamus (28-33). An additional complication to the isolation of CRFs was the lack of a direct assay for ACTH. Most of the ACTH assays available during the early years were bioassays whereby ACTH-activity was measured by indices such as eosinopenia (the number of eosinophils in the blood) (34) and decreases in adrenal ascorbic acid (35). Even Saffran and Schally's work was based on a bioassay measurement for ACTH. During the 1960's and early 1970's many groups published the purifications of various CRF's based on some of these assays. These CRF's included hypothalamic lipid extracts (36), vasopressin (37), and at least two molecular weight pepides (small and large) distinct from vasopressin (38-41).

It was not until the advent of HPLC technology, which aided the discrimination of small molecular weight peptides, that ovine CRH was isolated by Vale et al. in 1981 (42). This 41 amino acid peptide was isolated and sequenced from extracts of 490,000 ovine hypothalami. In this report, they also demonstrated that synthetic ovine CRH peptide was capable of inducing ACTH secretion both in vitro and in vivo. In fact, the synthetic peptide was 10 fold more potent than the isolated form, attributed to the presence of methionine sulfoxide in the latter form of the peptide. The oxidation of the native peptide was prevented in subsequent isolations, and was then shown to be equipotent with the synthetic peptide.

This synthetic CRH peptide was tested for its ability to induce

secretion of ACTH in vitro (42). The ACTH secretory response to maximal concentrations of CRH, as measured from primary cultures of rat anterior pituitary cells, is equal to that due to maximal stimulation of ACTH secretion by cAMP analogs (a known second messenger activator of ACTH secretion). The peptide response is greater than those attributable to maximal ACTH-releasing concentrations of norepinephrine or vasopressin, which exibited 30 to 50 percent and 10 to 20 percent, respectively, of the activity of ovine CRH.

In vivo experiments also demonstrated that the synthetic CRH peptide was a powerful stimulator of ACTH secretion (42). Plasma concentrations of ACTH were elevated 5 - 20 minutes after the intravenous administration of CRH to male rats that were pretreated with chlorpromazine, morphine and nembutal to control for nonspecific stress effects. In these animals, doses ranging from 30 to 3000 ng CRH per kilogram body weight rapidly elevated plasma ACTH levels by 5-20 fold. Additionally, Vale et al. demonstrated that the entire carboxy terminal region of CRH was required for full potency, but the amino terminal region could be modified without altering ACTH-releasing activity (42).

In 1982, Rivier et al. provided the most convincing evidence that CRH peptide is the key regulator of ACTH secretion (43). In those studies, rats were injected intravenously with rabbit antiserum raised against ovine CRH. These intact, nonstressed CRH-antibody treated animals demonstrated markedly reduced

CRH-induced rises of plasma ACTH (1305 pg/ml in normal rabbit serum (NRS) treated versus 182 pg/ml in CRH serum treated). They subsequently evaluated the ability of CRH antibodies to influence the ACTH secreted in response to ether stress. In these studies, antibody-treated animals exhibited a 75 % reduction in the ACTH released in response to ether stress (1729 pg/ml in NRS treated versus 363 pg/ml in CRH serum treated). Additionally, they demonstrated that CRH antibodies inhibited the rise in ACTH levels in adrenalectomized rats (a condition known to cause increased levels of ACTH in plasma). These results demonstrated that administration of CRH antiserum reduced ACTH secretion induced by exogenous synthetic ovine CRH to levels that were statistically comparable to control values, blocked most of the stress-induced ACTH rise in intact animals, and inhibited the increased ACTH secretion observed after removal of corticosteroid feedback.

With the availability of synthetic CRH, many groups produced antibodies against this peptide and utilized these antibodies to determine the anatomical localization of CRH-like peptides in the central nervous system (44-49) as well as in peripheral endocrine tissues (49-57). These anatomical studies will be further discussed in chapters in this thesis. Additionally, these antibodies lead to more precise radioimmunoassays which have been utilized to quantitate CRH-like peptides in tissues under basal and regulated conditions.

As a result of these studies by Vale and coworkers, as well as

others, it is generally accepted that CRH is the key regulator of ACTH secretion from the anterior pituitary. However, these same studies have shown that ACTH secretion is multifactorial, in that many compounds appear to participate in the regulation of ACTH secretion. For example, vasopressin was originally shown to possess ACTH-releasing activity, however this activity was lower than that induced by CRH. In more recent studies, it has been demonstrated that vasopressin significantly potentiates CRH-induced ACTH secretion in vitro and in vivo (25,58). Interestingly, CRH neurons in the hypothalamus appear to co-express vasopressin after long term adrenalectomy (59-63) thereby making it possible for these two peptides to coordinate the secretion of ACTH from the anterior pituitary. This work has lead to suggestions that CRH may be the main activator of ACTH secretion during periods of acute demand but that other agents (catecholamines and vasopressin) may participate in periods of chronic stress (64).

Due to the complexity of the central nervous system, little progress has been made in determining the molecular mechanisms that alter the production of neurohormones produced solely in the brain. Immunoreactive CRH has been identified in many regions of the brain but only in the hypothalamus does this peptide have a described function (influencing ACTH secretion). The site of synthesis and the nature of this immunoreactivity in other regions of the brain has not been fully characterized. These gaps in our knowledge concerning the sites of synthesis of CRH and the

molecular mechanisms which regulate the production of the CRH precursor will be addressed in this thesis through the molecular cloning of the rat CRH cDNA and gene. Additionally, the complete amino acid structure of the rat CRH precursor will be described and will provide the basis for future biosynthetic studies concerning this precursor.

Prior to the start of the work described in this thesis, two significant molecular cloning reports appeared. The first was the isolation of an ovine CRH cDNA from a hypothalamic cDNA library by Furutani et al. (1983) (65). The second, was the isolation and characterization of the human CRH gene by Shibahara et al (1983) (66). These two papers contributed heavily to the design of the work described within this thesis, and will be discussed in detail in appropriate chapters.

The rat CRH cDNA was isolated from a rat hypothalamic cDNA library using a complementary oligonucleotide whose sequence was determined following inspection of the CRH peptide-encoding region of ovine and human CRH nucleotide sequences. The isolation of this cDNA is described in chapter 1. Using the CRH cDNA as a molecular probe, the regional CNS and peripheral tissue distribution of CRH mRNA was determined and is described in chapter 2. These results confirmed earlier immunocytochemical studies in the CNS and periphery, in addition to suggesting novel sites of synthesis in peripheral tissues. One such novel site of synthesis was the testis, however the testicular CRH mRNA is much larger in length than CRH

mRNA from the brain. The nature of this size variation is examined in chapter 4.

In order to obtain the tools necessary to address questions concerning the transcriptional regulation of the rat CRH gene, this gene was isolated and characterized. The structure of the rat CRH gene is described in chapter 3 of this thesis. This work will describe a rather remarkable level of nucleotide homology between the 5' flanking regions of the rat and human CRH genes, suggesting that these two genes may share common transcriptional regulatory themes. The nucleotide sequence of the 5' flanking region of the rat CRH gene was used in gene transfer studies to investigate the potential for second messengers to activate transcription of this gene. These gene transfer experiments will focus on the cAMP regulation of the rat CRH gene and are discussed in chapter 5.

MATERIALS AND METHODS

Radiolabelling of Nucleic Acid Probes

1.Radiolabelling of CRH Deoxyoligonucleotide Probes

The CRH probe was made radioactive by enzymatically labelling the 5' terminus of synthetic oligonucleotides using T4 DNA Polynucleotide Kinase (PNK) (New England Biolabs). Approximately 10 pmoles of deoxyoligonucleotide were incubated with 100 μCi of γ^{32}P-ATP in 10mM Tris (pH 9.0), 50mM Dithiothreitol, 10mM $MgCl_2$, and 5 Units of PNK (New England Biolabs) for 45 minutes at 37°C. Labelled probe was separated from unincorporated label by passing the reaction over a G-50 Sephadex (1 cm X 10 cm) column equilbrated in 2X SSC (1X SSC = 15mM Sodium Citrate, 150mM Sodium Chloride), 0.1 % SDS ($NaDodSO_4$). The labelled probe is found in the void volume fractions.

2. cRNA probe systhesis

During the last few years it has become possible to synthesize radioactive RNA for the purpose of screening cDNA libraries, Southern blots and Northern blots. The single-stranded RNA probes (termed cRNA probes) are synthesized using bacteriophage (SP 6 or T7) DNA dependent RNA polymerase and appropriate vectors containing their promoters (67). 0.5μg of restriction endonuclease-linearized plasmid containing a specific cDNA insert was added to a reaction mix containing 40 mM Tris (pH 7); 6 mM $MgCl_2$; 2 mM spermidine; 10 mM NaCl; 10 mM DTT; 500 mM rGTP,

rATP, rCTP (Pharmacia); 10 uM rUTP (cold) (Pharmacia); 100 μCi α^{32}P rUTP (New England Nuclear, 800 Ci/mmole); 1 unit RNasin (Promega Biotec). 10 units SP 6 or T7 RNA Polymerase (Boehringer Mannheim) was added (the choice of polymerase was dependent upon the orientation of the cDNA insert in the vector) to start the reaction, which was incubated at 40°C for 60 minutes. The probe synthesis was terminated by phenol-chloroform extraction; ethanol precipitation was performed at room temperature, in the presence of 1.4M NH_4OAc and 2.5 volumes of ethanol. Following centrifugation, the pellet was washed extensively with 70 % ethanol, lightly dried, and resuspended in 80 % formamide. The probes were prepared the day they were to be used.

Samples (approximately $1X10^6$ cpms) of all cRNA probes were analyzed on denaturing polyacrylamide gels to determine the quality of RNA transcripts synthesized. Transcripts which were judged to be >90% full length were used for RNase protection assays and Northern blot analysis.

3. Nick translation of isolated DNA fragments

Typically, 100 ng of insert was nick translated with the use of BRL Nick Translation Reagent Kit (Cat. #8160 SB). In these reactions, 50 μCi α^{32}P dCTP was added to a final reaction volume of 15 ul. Reactions were performed at 14°C for 45 minutes. The reaction was stopped by the addition of 50 μl 2X SSC and the sample was immediately applied to a 1 X 10 cm G-50 Sephadex column equilibrated in 2X SSC, 0.1 % SDS. The labelled fragments were found

in the void volume. Typical incorporations from these reactions generated radiolabelled DNA which was approximately 1 X 10^9 cpm/µg.

In Situ Screening of cDNA Library

The hypothalamic cDNA library was titered and approximately 40,000 plaques were plated on LB plates. This was performed by combining the proper dilution of the phage library with 350 µl of $MgSO_4$/CaCl (10mM) and 350 µl of an overnight E. coli C600 HflA bacterial culture. This infection was allowed to proceed for 10-15 minutes at 37°C. Liquid top agar (52°C) was then added, and the infections were mixed and poured onto warm LB plates. These plates were cooled to room temperature and then grown overnight at 37°C (approximately 12 hours). The plates were grown to near confluency and then placed at 4°C for 30 minutes. Replicate nitrocellulose filters (Schleicher and Schuell) were utilized to lift these plates. The filters were then denatured, neutralized, air dried, and baked for 2 hours at 80°C under vacuum.

The filters were prehybridized and hybridized with standard buffers without formamide (68). Prehybridization and hybridization temperatures were 45°C for the original oligonucleotide (#140) screening and 65°C for the rescreening of the library using the partial cDNA insert from pCRF-D1. Final wash conditions for all library filters were 4X SSC at 50°C for the oligonucleotide screening and 0.1X SSC at 58°C for the cDNA screening. Positive phage were

picked into 100mM NaCl, 10mM Tris (pH 7.5), 10mM $MgCl_2$ and stored at 4°C over chloroform. Phage were plaque purified by repeated screening at reduced densities.

Preparation of Lambda DNA

High titer stocks of positive lambda clones produced from plate lysates were used to obtain large quantities (typically 50 to 200 μg) of λ DNA from 40 ml infection cultures. Approximately 1 X 10^6 pfu were added to approximately 10^8 E. coli C600 HflA for lambda gt10 or LE-392 for EMBL-3 (about 150 ul of an overnight culture) in 40 ml LB media containing 0.2% maltose and 5 mM CaCl. The infections were allowed to proceed for 4-6 hours at 37°C or until bacterial lysis was apparent. This culture was then cleared by centrifugation and the supernatant collected. RNase A and DNase I were added to 25 μg/ml to digest bacterial RNA and DNA. After 30 minutes at 37°C, 0.2 volumes of 20% PEG, 2.5 M NaCl were added, and the resultant mixture was stored at 4°C overnight to precipitate the phage particules. The PEG precipitated phage particles were collected by centrifugation and resuspended in 1X Proteinase K buffer (100 mM Tris (pH 8), 150 mM NaCl, 10 mM EDTA, 0.2% SDS, 100 μg/ml Proteinase K (Boerhringer Mannheim)). The phage coats were digested at 55°C for 45 minutes, phenol/chloroform extracted, 2X chloroform extracted and ethanol precipitated. Typical yields from this procedure were approximately 50-200 μg of phage DNA.

Southern Blot Analysis

Approximately 2-5 μg of lambda DNA from cDNA and genomic clones or 20 μg of rat liver genomic DNA were digested with restriction enzymes according to manufacturers recommendations. Typically these digests were performed at 37°C for 2-4 hours. These digests were then electrophoresed on agarose gels (0.7%- 1.5%) in 1X TBE (90mM Tris (pH 7.5), 80mM Boric Acid, 2.5mM EDTA). The gel was denatured (in 1.5M NaCl, 0.5M NaOH) for approximately 30 minutes (dependent upon the thickness of the gel) and then neutralized (in 0.5M Tris (pH 7.5), 3M NaCl) for 1 hour. The gel was then transferred to nitrocellulose membranes by capillary action in 20 X SSC (0.3M NaCitrate (pH 7.5),3M NaCl) overnight. The nitrocellulose membrane was removed, baked at 80°C under vacuum, prehybridized and hybridized with cDNA probes at 65°C or 45°C for deoxyoligonucleotide probes. The cDNA hybridized blots were washed in 0.1X SSC, 0.1% SDS, 5mM EDTA at 58°C and deoxyoligonucleotide hybridized blots were washed in 4X SSC, 0.1% SDS, 5mM EDTA at 50°C.

Plate Lysate Preparation

Plate lysates were obtained by plating plaque-pure phage individually at a density of 20,000 to 40,000 plaques per plate and eluting the phage from the plates overnight in SM solution (50mM Tris (pH 7.5), 100mM NaCl, 8.1mM $MgSO_4$). The SM was then removed from the plates, cleared of bacteria with chloroform, centrifuged and

stored at 4°C (they are stable for months). The phage titer from these preparations was typically in the 10^9 -10^{10} pfu (plague forming units) / ml.

DNA fragment Insert Isolation Procedure

In these procedures, approximately 5-30 μg of plasmid or lambda DNA were digested with appropriate restriction enzymes according to the manufacturer's recommendations. These digests were then electrophoresed on low melting temperature (LMT) agarose gels ranging from 0.7 % to 1.2 % agarose dependent upon the size of the fragment to be isolated (the smaller the fragment, the greater the concentration of agarose). The gel was stained with ethidium bromide, the insert identified and excised from the gel with a razor blade. The gel slice was melted and diluted in low salt buffer in preparation for concentration on Elutips (Schieicher & Schuell) according to the manufacturers recommendations. The concentrated fragment was then ethanol precipitated and resuspended in water at approximately 50 ng/μl. Microgram quantities of inserts could be prepared in this fashion (particularly from plasmids) and easily subcloned or radiolabelled for use in further experiments.

RNA extraction from tissue

Tissue from which RNA was to be extracted was frozen on dry ice (large chunks of tissue were pulverized with a mortar and pestle in liquid nitrogen). RNAs were extracted by the method of Chirgwin

(69) as briefly described here. The tissue was added to 18 ml of breaking buffer (per 4 g tissue) consisting of 6M Guanidinium Isothiocyanate, 20mM Tris (pH 7.5), 10mM EDTA (pH 8), 5% ß-mercaptoethanol (BME), and homogenized with a polytron (for approximately 30 seconds). Sarkosyl was then added to a final concentration of 3% and the solution rehomogenized for approximately one minute. 7.2 grams of optical grade CsCl were added to give 0.2 g/ml and the solution was heated to 65°C for 10 minutes. This was followed by centrifugation at 10,000 rpms for 10 minutes in a Sorval SS34 rotor. The supernatant was then loaded onto an 8 ml cushion of 5.7 M CsCl, 100mM EDTA in a polyallomer tube and centrifuged in a Ti60 rotor at 33,000 rpms for 16 hours. The resulting total RNA pellet was resuspended in autoclaved dH_2O, phenol-chloroform extracted, made 0.3M in NaOAc (pH 4.8) and stored under 2.5 volumes of ethanol at -70°C.

Oligo dT Cellulose Chromatography

Poly (A) RNA was isolated by oligo d(T) chromatography. Poly (A) RNA was utilized on all Northern blots to enrich for the CRH mRNA. A column was prepared by plugging a 3 ml sterile syringe with glass wool which had been rinsed in 2% dichlorodimethylsilane, rinsed with diethylpolycarbonate (DEP)-treated dH_2O, and autoclaved. 50 mg oligo d(T) cellulose resin (Type 7, Collaborative Research) was hydrated in the column with 1X HSB (1M NaCl; 40 mM Tris (pH 7.5); 2 mM EDTA (pH 8); 0.2% SDS), and washed with 5 ml of the same buffer.

The resin was then treated with 2 ml of 0.1M KOH, 5mM EDTA (pH 8), followed by treatment with 5 ml of 1X LSB (40 mM Tris (pH 7.5), 2 mM EDTA (pH 8), 0.2% SDS) and 5 ml 1X HSB. The pH was checked and found to be approximately 7.5.

Total RNA (approximately 1 mg) which had been stored under ethanol was pelleted by centrifugation, washed with 70 % ethanol, lightly dried, and resuspended in 500 µl DEP dH_2O. The RNA was denatured by incubation at 70°C for 10 minutes and quenched on ice. 500 µl 2X HSB was added and the RNA was loaded onto the column. The flow through was reloaded a total of 5 times. The column was then washed with 10 ml of 1X HSB, 10 ml of 1X ISB (0.5M NaCl, 40 mM Tris (pH 7.5), 2 mM EDTA (pH 8), 0.2% SDS) or until no further material was detected at A_{260} nm, and the poly (A) RNA was eluted with 1.5 ml of 1X LSB. The poly A+ fraction was quantified spectrophotometrically, and stored under ethanol at -70°C.

M13 subcloning and DNA template preparation

DNA fragments to be sequenced were either gel isolated or randomly inserted into M13 vectors. A typical ligation consisted of 10 ng of M13 (mp18 or mp19) RF DNA, digested with the appropriate restriction endonuclease(s), and a 10 fold molar excess of the fragment to be cloned, in 50 mM Tris (pH 8), 10mM $MgCl_2$, 10mM DTT, and 200 units of T4 DNA ligase (New England Biolabs). Ligations were carried out at 25°C for a minimum of 1 hour or at 14°C for a minimum of 10 hours. The ligated DNAs were transformed into E.

coli JPA101 which were rendered competent by standard treatment in 20mM KMES (pH 6.2), 60mM $CaCl_2$, 80mM KCl, 10mM $MnCl_2$, and 5mM $MgCl_2$. This bacterial strain is lambda resistant and can therefore be used to randomly clone lambda phage inserts without purification, as religated (viable) lambda DNA does not produce a productive infection in these cells. JPA101 also yields 10-20 fold more M13 phage than the parental JM101. Transformations were plated to the same strain in the presence of IPTG and X-gal, and recombinants were identified by color.

Template DNA was prepared by picking plaques into 2 ml of 2X YT broth which had been seeded with E. coli JPA101 and grown for 6-8 hours at 37°C. The cells were removed by centrifugation and the supernatant was adjusted to 4% PEG-6000, 0.5M NaCl. After 10 minutes at 4°C, the phage were pelleted by centrifugation in a microfuge at room temperature and resuspended in 200 µl of dH_2O. The DNA was released by phenol-chloroform extraction (twice) and concentrated by ethanol precipitation and centrifugation. The template DNA was washed twice with 70% ethanol, lightly dried, and resuspended in 15 µl dH_2O.

DNA sequencing reactions

DNA sequencing was performed by the dideoxy chain termination method essentially as described (70). Reaction mixes for each template were assembled on ice and contained 0.5 pmoles primer, 1µl template, 10µCi α^{32}P dCTP, 10mM Tris (pH 8.0), 10mM

KCl, 7mM $MgCl_2$, 0.1mM EDTA, 5mM DTT. The mix was heated to 55°C for 10 minutes, 2.5 units of E. coli DNA Polymerase Klenow fragment (Boehringer Mannheim) were added and 3µl of this solution was added to 2µl of each of the ddNTP mixes. The polymerization reaction proceeded for 15 minutes at 37°C at which time 1.5 µl of chase (2.5mM dNTPs, in reaction buffer) and 0.5 units of Klenow enzyme was added, and the reactions proceeded for an additional 10 minutes at 37°C. At this time, 5µl of 0.1% xylene cyanol, 0.1% bromphenol blue, 5mM EDTA in 98% formamide was added to each reaction. The tubes were heated to 100°C for 5 minutes and quenched on ice. 1 µl of each reaction was then loaded on a standard 5% acrylamide/7M urea sequencing gel. The gel and running buffer was 1X TBE (89mM Tris (pH 8), 89mM boric acid, 2mM EDTA). Gels were prerun for at least 30 minutes at 1000V, and the samples were electrophoresed at 1500-2000V at 20 mA. After electrophoresis, the gels were dried and autoradiographed.

Tissue Dissections

Animals were anesthetized under CO_2 for 1-3 minutes, then decapitated via guillotine. Visceral organs were removed from the body cavity as quickly as possible and frozen immediately on dry ice. Cranial cavities were opened using Rusking bone rangeurs to an extent which allowed the whole brain (less olfactory bulbs), from frontal lobes to cervical spinal cord, to be removed in one piece. Pituitary glands could then be removed from the base of the cranial

cavity and frozen on dry ice. Whole brains were subjected to a gross dissection into seven "regions" which accounted for all brain material except thalamus, which was discarded. These seven regions were as follows: brainstem, consisting of medulla oblongata and pons; midbrain; hypothalamus, consisting of a cube of tissue defined by the arterial circle of Willis on the inferior surface of the brain and deep into the brain as far as the superior limit of the third ventricle; hippocampus; striatum; cerebral cortex; and cerebellum. All brain tissues were frozen on dry ice immediately following dissection. Tissues were stored wrapped in foil at -80°C until processed for RNA extraction.

Northern Blot Analysis

Several technological advances in Northern blotting procedures have taken place in the last few years. The first was availability of positively charged nylon membranes which were not only physically tougher than nitrocellulose but claimed to bind more nucleic acid per unit area. The second advance was the ability to conviently synthesize large quantities of radioactively labelled RNA. These new cRNA probes were capable of forming very specific and stable hybrids with complimentary RNA in solution or on membranes. Making use of these new advances in technology and reagents, we were able to detect CRH mRNA by Northern blot analysis when other groups had reported great difficultly.

Denaturing formaldehyde/agarose gels were used to size

separate the RNAs from various brain regions. These 3mm thick gels were prepared as follows. 1.4 g of agarose were melted with 73.8 ml of water and 10 ml of 10X MOPS buffer (0.4 M MOPS (pH 7.0), 100mM NaAcetate,10mM EDTA). The solution was then cooled to 60°C, 16.2 ml of 37% formaldehyde was added, and the gel was poured into a vertical gel apparatus with an agarose plug. The gel was allowed to cool for at least one hour prior to use.

RNA samples, stored as ethanol precipitates, were centrifuged, washed with 70% ethanol, and dried. The RNA pellet was resuspended in 5.5 µl water, 1µl 10X MOPS buffer, 3.5 µl 37% formaldehyde and 10 µl formamide. These samples were denatured at 65°C for 15 minutes and 3µl of filter sterilized 10X RNA dye (25% Ficoll 400, 0.2% bromphenol blue, 0.2% xylene cyanol) was added prior to electrophoresis. The gels were electrophoresed at 120 volts (constant voltage) until the bromphenol blue traveled approximately 3/4 the length of the gel (approximately 10 cm).

These gels were transferred onto Nytran Membranes (Schleicher & Schuell) overnight in 20 X SSC by capillary action. These membranes were baked at 80°C for two hours and prehybridized for two to four hours in formamide/2X DNA Hybrid Buffer (10X SSC, 2X Denhardts, 50mM NaPhosphate (pH 6.5), 0.2% SDS, 0.2% $NaPPO_4$, 200 µg/ml sonicated salmon sperm DNA) mixed 50:50 at 60°C. Single stranded cRNA probes (approximately 1-10 X 10^6 cpms per ml of hybridization buffer) were mixed with fresh prehybridization buffer and formamide (50:50) and the blots were hybridized for 16-18 hours

at 60°C. These blots were then washed in 2X SSC for 30 minutes at room temperature and then 0.1X SSC, 0.1% SDS, 5mM EDTA (pH 8) for 2X 45 minutes at 72°C (high stringency). The blots were then air dried and autoradiographed.

RNase Protection Assays

Full length cRNA probes were synthesized as previously described (see Radiolabelling Nucleic Acid Probes). RNA samples were stored under ethanol. These samples were pelleted by centrifugation, washed with 70% ethanol, lightly dried and resuspended in 5.0 µl sterile dH_2O. To each RNA sample, the following reagents were added: 6.0 µl 5X Hybridization buffer (0.2M PIPES (pH 6.4), 2.0M NaCl, 5.0mM EDTA), 15.0 µl formamide, 3 µl Yeast RNA (6.6 µg/ml), and 1 µl cRNA probe (approximately $1X10^6$ cpms in 80% formamide). The hybridization mix is denatured at 90°C for 15 minutes, followed by incubation at 60°C overnight (at least 16 hours).

To this reaction 0.2ml of RNase digestion buffer (10mM Tris (pH 7.5), 5.0mM EDTA, 0.2M NaCl, 0.1M LiCl, 5µg/ml RNase A (Sigma), 130 U/ml RNase T1(BRL)) is added and incubated at room temperature for 30 minutes. The RNase digestion is terminated by the addition of 10µl of 20% SDS and 10µl of 10mg/ml Proteinase K (Sigma) and incubated at 37°C for 30 minutes. The reactions are then phenol/chloroform extracted, chloroform extracted and ethanol precipitated with the addition of 1µg Yeast RNA/sample. The

precipitates are centrifuged, washed with 70% ethanol, and resuspended 4 µl sterile, dH_2O. To these samples, 4 µl 10X formamide dye (0.1% bromphenol blue, 0.1% xylene cyanol, 98% formamide) is added prior to denaturing at 90°C for 5 minutes and electrophoresis on denaturing 5% acrylamide gels.

$CaPO_4$ Transfection of Plasmid DNA into Eukaryotic Cells

CsCl purified plasmid DNA (approximately 5-10 µg/ 6 cm plate) is transfected into mammalian tissue culture cells as follows. Plasmid DNA (in 10 µl) is added to 31 µl 2M $CaCl_2$ and 209 µl dH_2O. This DNA solution is then added dropwise to 0.25 ml of 2X HBS (280mM NaCl, 50mM Hepes (pH 7.1), 1.5mM Na_2HPO_4) and placed onto tissue culture cells that were plated the day before. The cells were exposed to the precipitated DNA for 4 hours followed by a 2 minute glycerol shock (15% glycerol in 1X HBS). The glycerol solution is then removed and replaced with fresh media. Forty hours later, fresh media or fresh media plus regulators was added. Five to six hours later the cells were harvested and cell extracts were prepared as is discussed in the CAT assay section of Methods.

CAT assay and ß-Galactosidase assay

Cells were collected with a rubber policeman in phophate-buffered saline. Extracts were prepared by lysis of cells in 70 µl of 0.25M Tris-HCl (pH 7.5), 0.5% Triton X-100 followed by centrifugation at 15,000 x g for 10 minutes. Supernatants were

removed and aliquots were heat inactivated at 60°C for 10 minutes before CAT assay. CAT enzyme reaction were performed at 37°C in 192 µl final volume containing 100 µl 0.25M Tris-HCl (pH 7.5), 0.2 µCi ^{14}C-chloramphenicol, 10 µl 30mM acetyl coenzyme A, and 35 µl of heat inactivated cell extract. The reaction was stopped with 500 µl ethyl acetate which was used to extract the chloramphenicol and acetylated products. The organic phase was dried and resuspended in 10 µl of ethyl acetate, spotted on silica gel IB-F TLC plates (Baker), and chromatographed with $CHCl_3$/MeOH (95:5 ascending). After autoradiographic exposure, the acetylated products were excised from the TLC plates and counted in a liquid scintillation counter.

ß-galactosidase assays were performed as previously described (71). The counts per minute (cpm) of acetylated chloramphenicol product were normalized to the ß-galactosidase activity in each extract to yield relative CAT activity.

RESULTS AND DISCUSSION

Chapter 1: Isolation and Characterization of a Hypothalamic cDNA Encoding the Rat Corticotropin Releasing Hormone Presursor

Summary

DNA complementary to the rat hypothalamic mRNA coding for the corticotropin-releasing hormone (CRH) precursor has been cloned from a rat hypothalamic lambda gt10 cDNA library using a synthetic deoxyoligonucleotide complementary to 20 nucleotides from the CRH peptide-encoded region of the human CRH gene (66). Several cDNA clones were isolated and characterized, the longest of which was shown to encode the entire rat CRH protein precursor. Homology studies of the rat, human and ovine amino acid sequences have shown the CRH peptide and putative signal peptide regions to be more highly conserved than the middle portion of the protein precursor.

Introduction

In 1983, Furutani et al. reported the nucleotide sequence of an ovine hypothalamic cDNA encoding the CRH protein precursor (65). This data provided the first information on the structure of the CRH protein precursor which was shown to consist of 190 amino acids including a putative signal peptide. The carboxy terminal region of the precursor, as deduced from the cDNA, contained the 41 amino acid sequence preceded by the proteolytic cleavage site, Arg-Lys-Arg-Arg, corresponding precisely to the amino acid sequence determined for

the ovine CRH peptide by Vale et al. in 1981 (42). Additional pairs of basic amino acids were also found within the ovine prohormone which led to speculation that another novel peptide may be produced from this precursor. Later that same year, Shibahara et al. (66) published the nucleotide sequence of the human gene encoding the CRH protein precursor. Comparisons between the ovine and human sequences revealed many interesting findings. The ovine and human prohormones contained 190 and 196 amino acids, respectively, and both precursors contained the CRH peptide at the carboxy-terminus, preceded by a pair of basic amino acids and followed by a Gly-Lys (suggesting that the CRH peptide could be amidated). The human and ovine signal peptide regions were highly homologous at both the amino acid (91.6 %) and nucleotide (92 %) levels. However, the nucleotide sequences analysis revealed that the human CRH peptide contained seven amino acid substitutions from the ovine sequence, all of which represented changes among chemically similar amino acids and were caused by single nucleotide substitutions except for the Ile/Ala replacement at the carboxy terminus of CRH.

The nucleotide analysis of the human CRH genomic DNA sequences also provided the first description of the structural organization of this gene, including the promoter sequences and intron/exon boundaries. This gene was shown to contain two exons, separated by an 800 bp intron, with the second exon containing the entire human CRH protein precursor. The cap site marking the 5' limit of the mRNA sequence was putatively assigned to nucleotide -985 (55

bp upstream of the site corresponding to the 5' end of the cloned ovine cDNA), because putative TATA and CAAT boxes are found 23 and 58 bp upstream of the putative capping sites, respectively. Although no human CRH cDNA sequence was reported to define the intron/exon boundaries, the first exon was described to contain 169 bp of the 5' untranslated region of the mRNA, with the second exon containing 14 bp of the 5' untranslated region, the protein coding region and the 3'untranslated region of the mRNA. The 3' untranslated region of the human CRH gene contains two copies of the poly(A) addition signal (AATAAA), whereas the ovine cDNA has three signals, suggesting that the human CRH mRNA may be polyadenylated at multiple sites as was the ovine CRH mRNA. Shibahara et al. also utilized Southern blotting techniques to analyze human genomic DNA to demonstrate that the CRH gene appears to be represented in a single copy in the human genome.

Additionally in 1983, the rat CRH peptide was isolated and sequenced by Rivier et al (72). These studies demonstrated that the rat CRH peptide was identical in amino acid sequence to the human CRH peptide. However, no rat CRH specific DNA probes were as yet available.

For many years our laboratory has been interested in the identification of mechanisms which regulate neuropeptide gene expression within the pituitary and brain. Our studies have concentrated on the rat as a model animal system. Some of the first studies determined the structure of the large polyprotein precursor,

proopiomelanocortin (POMC) which contained both ACTH and ß-endorphin molecules (18,19). It was during this time that the 41 amino acid CRH peptide was isolated by Vale and coworkers and shown to be the key regulator of both ACTH secretion and POMC synthesis (73). We therefore set out to determine the complete protein structure of this key regulatory factor of POMC gene expression, corticotropin-releasing hormone (CRH). It was of interest to determine the precise structure of this important regulator for the following reasons. First, the rat system has been studied most extensively in paradigms aimed at understanding the control of the hypothalamic-pituitary-adrenal axis. Therefore, this animal system has provided the best available information on the anatomy and regulation of CRH. Secondly, it was necessary to confirm the precise sequence of the CRH peptide and precursor of this species for future biosynthetic and regulatory studies.

Towards this goal, a deoxyoligonucleotide probe (#140) was synthesized which was complementary to a region of the previously published human CRH gene which encoded the CRH peptide. The nucleotide sequence and its relationship to the ovine and human sequences is shown in figure 2. This sequence was chosen for two reasons; 1) this nucleotide region was previously shown to be highly conserved between ovine and human sequences; and 2) the rat and human peptides were previously shown to be identical. This deoxyoligonucleotide probe was radiolabelled and used to screen and characterize cDNA clones from a λ gt10 rat hypothalamic cDNA

library.

Results and Discussion

Rat poly A+ RNA was isolated from rat hypothalami by guanidium isothiocyanate extraction (69) and subsequently selected by chromatography on oligo (dT) cellulose (see Methods). A hypothalamic cDNA library was constructed from this mRNA by Dr. J. Adelman in λ gt10. A library of approximately 3.0 X 10^6 clones was obtained. Approximately 600,000 lambda plaques were plated and screened in situ for the presence of cDNA clones which hybridized to the P^{32}-radiolabelled deoxyoligonucleotide probe (#140) described in figure 2.

Several hybridization positive cDNA clones were isolated (lambda clones 1-7) and subjected to plaque purification. Once purified, these phage were used to produce high titer plate lysates as described in Methods. Deoxynucleotide positive lambda DNA corresponding to lambda clones 1-7 (prepared as described in Methods) was digested with Eco RI and analyzed by Southern blotting using the deoxyoligonucleotide #140 as a hybridization probe. The results of this analysis is shown in figure 3.

From this analysis there appeared to be several different sizes of inserts with variable hybridization intensities. The largest of these inserts was found in clone 1 which contained an insert of approximately 2000 bp in length. The smallest of these inserts was found in clone 3 which contained a 500 bp insert. DNA inserts from

Figure 2. Nucleotide Sequence Encoding the Human and Ovine CRH Peptides.

The 41 amino acids of the human CRH peptide are shown with the corresponding nucleotide sequence derived from the sequencing of the human CRH gene (upper nucleotide sequence). Nucleotide differences that are found in the ovine CRH encoded region are noted below the human sequence (amino acid sequence of the ovine CRH peptide is not shown). The underlined human/ovine nucleotide sequence is complementary to the nucleotide sequence for deoxyoligonucleotide #140 which was used to screen the rat hypothalamic cDNA library.

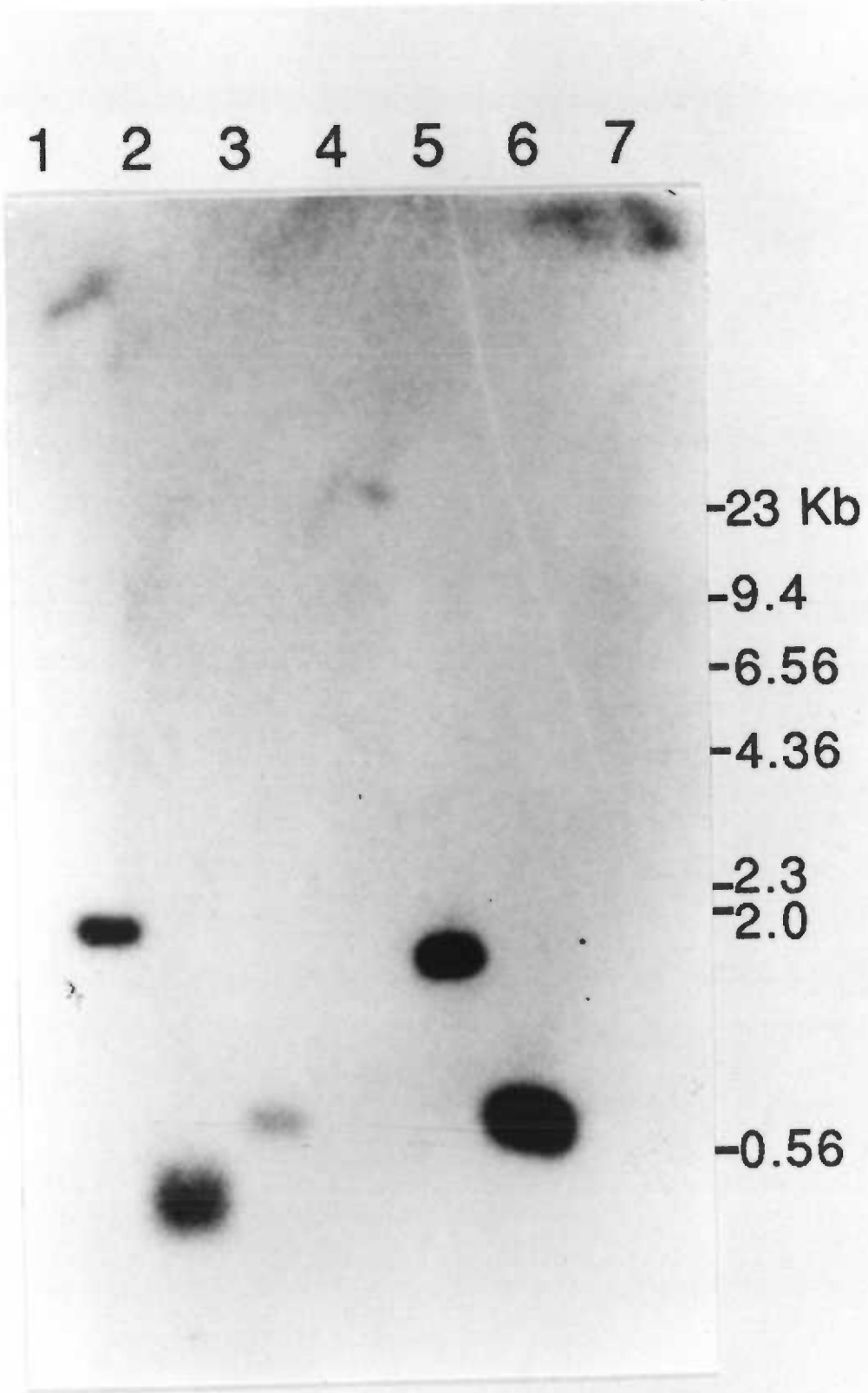
CRH Peptide Nucleotide Sequence

```
SerGluGluProProIleSerLeuAspLeuThrPheHisLeuLeuArgGluValLeuGluMetAla
TCCGAGGAGCCTCCCATCTCCCTGGATCTCACCTTCCACCTCCTCCGGGAAGTCTTGGAAATGGCC
   C    A        T                              A                A

ArgAlaGluGlnLeuAlaGlnGlnAlaHisSerAsnArgLysLeuMetGluIleIle
AGGGCCGAGCAGTTAGCACAGCAAGCTCACAGCAACAGGAAACTCATGGAGATTATT
 A      T        G             T     T        GT    C   GC
```

Figure 3. Southern Blot Analysis of Rat Hypothalamic cDNA Clones Isolated on First Screening.

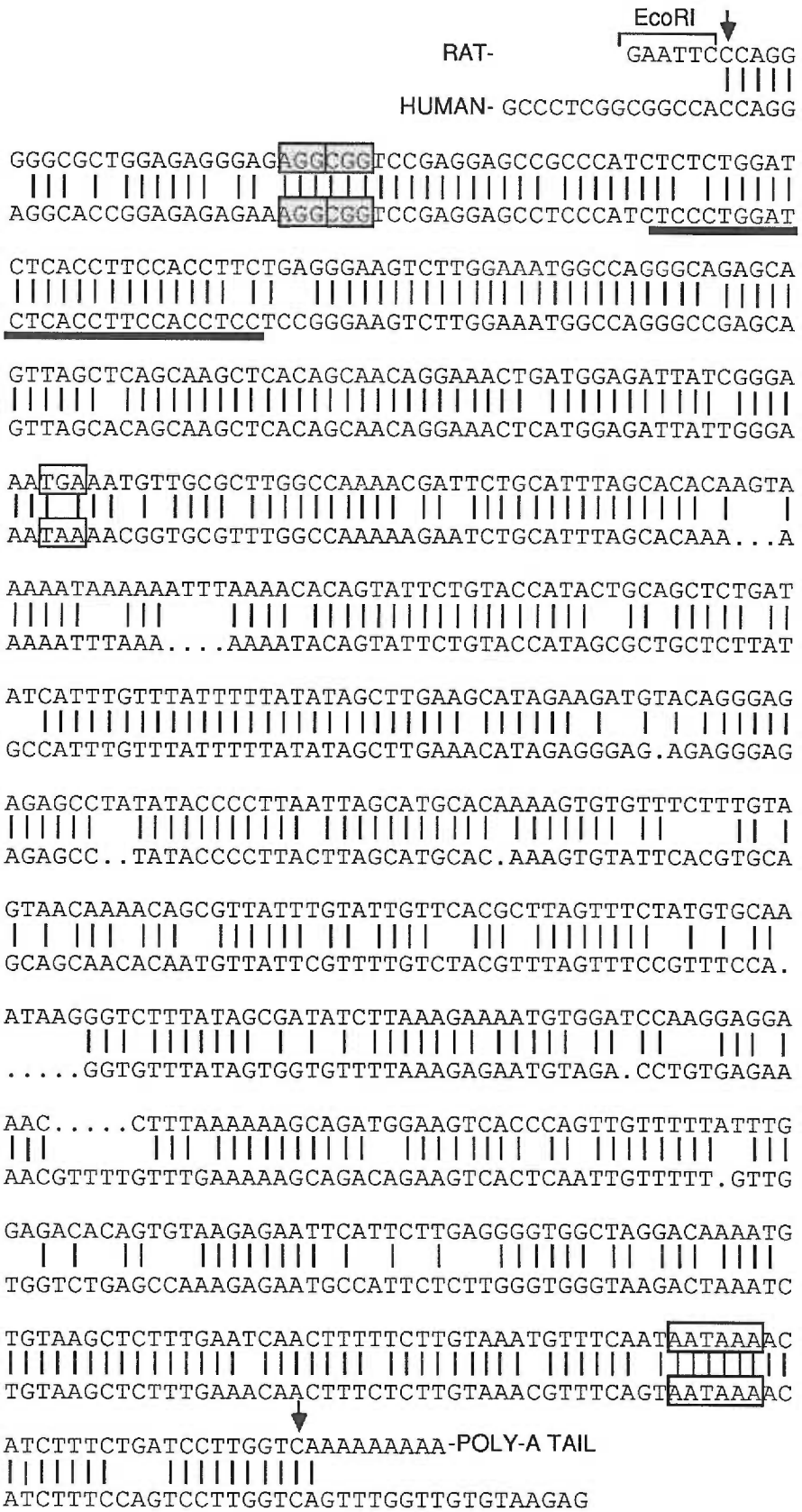
Hypothalamic cDNA clones that were hybridization-positive to radiolabelled oligonucleotide #140 (see text) were used to produce λ DNA. This DNA was subjected to restriction enzyme digests with EcoRI (a restriction site found in the linker used to clone the original hypothalamic cDNA), electrophoresis, and blotting to nitrocellulose. This nitrocellulose was prehybridized, hybridized with radiolabelled oligonucleotide #140, and washed as described in Methods. Approximately 2 μg of λ DNA is found in each lane. The numbers on the top of the figure represent the respective clone numbers (lane 1 = clone 1, etc.) The autoradiogram was exposed overnight at -70°C with an intensifying screen. The numbers (in kilobases) found on the right edge of the photograph represent the positions of DNA bands from a λ–HindIII digest used for molecular weight determinations.


1 2 3 4 5 6 7
-23 Kb
-9.4
-6.56
-4.36
-2.3
-2.0
-0.56

clones 1-7 were isolated on 1% low-melting temperature (LMT) agarose and subcloned into M13 mp18 DNA for DNA sequence analysis. Lambda clone #6 appeared to demonstrate a significant homology to the 3' untranslated region of the human gene and upon complete sequence analysis was shown to be a partial cDNA clone for rat CRH mRNA. The nucleotide sequence of the other six clones were unrelated to CRH as determined by the lack of apparent homology to the human gene sequence. This partial cDNA from clone #6 appeared to include the C-terminal portion of the CRH protein presursor and the 3'untranslated region of the corresponding mRNA. The nucleotide sequence of this clone and the homology to the human CRH gene sequence is shown in figure 4. This lambda cDNA clone terminated at approximately nucleotide number +435 of the human CRH gene and number +412 of the ovine cDNA. The cDNA nucleotide sequence corresponding to the deoxynucleotide probe was found near the 5' end of the sequence and demonstrated that there was a single nucleotide mismatch at position #18 of the synthetic probe between the human/ovine and rat sequence. Because clone #6 did not contain the nucleotide sequences which encode the entire CRH protein precursor, this insert from clone #6 was used to re-screen the hypothalamic cDNA library to obtain a full length cDNA clone.

In order to use this insert as a hybridization probe to re-screen the hypothalamic cDNA library, the insert was removed from lambda gt10 (an Eco RI restriction fragment) and subcloned into the pUC-19 vector. Plasmid clones containing the 620 bp Eco RI insert were

Figure 4. Nucleotide Sequence of the Partial Rat CRH cDNA (pCRF-D1). The nucleotide sequence of the 620 bp insert derived from λ clone #6 is shown in the upper sequence. The lower sequence is the corresponding region of the human CRH gene. The poly A addition signal sequence (AATAAA) is boxed and the site of addition of the poly A tail is indicated with an arrow. The translation terminator (TGA or TAA) for both CRH protein precursors is indicated with an open box. The nucleotide codons encoding the putative proteolytic cleavage site (Arg-Arg) are indicated by shaded boxes and mark the position of the N-terminus of the CRH peptides. The nucleotide sequence of deoxyoligonucleotide #140, used to isolate this cDNA, is underlined with a bold line.


EcoRI
RAT- GAATTCCCAGG
HUMAN- GCCCTCGGCGGCCACCAGG
GGGCGCTGGAGAGGGAGAGGCGGTCCGAGGAGCCGCCCATCTCTCTGGAT
AGGCACCGGAGAGAGAAAGGCGGTCCGAGGAGCCTCCCATCTCCCTGGAT
CTCACCTTCCACCTTCTGAGGGAAGTCTTGGAAATGGCCAGGGCAGAGCA
CTCACCTTCCACCTCCTCCGGGAAGTCTTGGAAATGGCCAGGGCCGAGCA
GTTAGCTCAGCAAGCTCACAGCAACAGGAAACTGATGGAGATTATCGGGA
GTTAGCACAGCAAGCTCACAGCAACAGGAAACTCATGGAGATTATTGGGA
AATGAAATGTTGCGCTTGGCCAAAACGATTCTGCATTTAGCACACAAGTA
AATAAAACGGTGCGTTTGGCCAAAAAGAATCTGCATTTAGCACAAA...A
AAAATAAAAAATTTAAAACACAGTATTCTGTACCATACTGCAGCTCTGAT
AAAATTTAAA....AAAATACAGTATTCTGTACCATAGCGCTGCTCTTAT
ATCATTTGTTTATTTTTATATAGCTTGAAGCATAGAAGATGTACAGGGAG
GCCATTTGTTTATTTTTATATAGCTTGAAACATAGAGGGAG.AGAGGGAG
AGAGCCTATATACCCCTTAATTAGCATGCACAAAAGTGTGTTTCTTTGTA
AGAGCC..TATACCCCTTACTTAGCATGCAC.AAAGTGTATTCACGTGCA
GTAACAAAACAGCGTTATTTGTATTGTTCACGCTTAGTTTCTATGTGCAA
GCAGCAACACAATGTTATTCGTTTTGTCTACGTTTAGTTTCCGTTTCCA.
ATAAGGGTCTTTATAGCGATATCTTAAAGAAAATGTGGATCCAAGGAGGA
.....GGTGTTTATAGTGGTGTTTTAAAGAGAATGTAGA.CCTGTGAGAA
AAC.....CTTTAAAAAAGCAGATGGAAGTCACCCAGTTGTTTTTATTTG
AACGTTTTGTTTGAAAAAGCAGACAGAAGTCACTCAATTGTTTTT.GTTG
GAGACACAGTGTAAGAGAATTCATTCTTGAGGGGTGGCTAGGACAAAATG
TGGTCTGAGCCAAAGAGAATGCCATTCTCTTGGGTGGGTAAGACTAAATC
TGTAAGCTCTTTGAATCAACTTTTTCTTGTAAATGTTTCAATAATAAAAC
TGTAAGCTCTTTGAAACAACTTTCTCTTGTAAACGTTTCAGTAATAAAAC
ATCTTTCTGATCCTTGGTCAAAAAAAAA-POLY-A TAIL
ATCTTTCCAGTCCTTGGTCAGTTTGGTTGTGTAAGAG

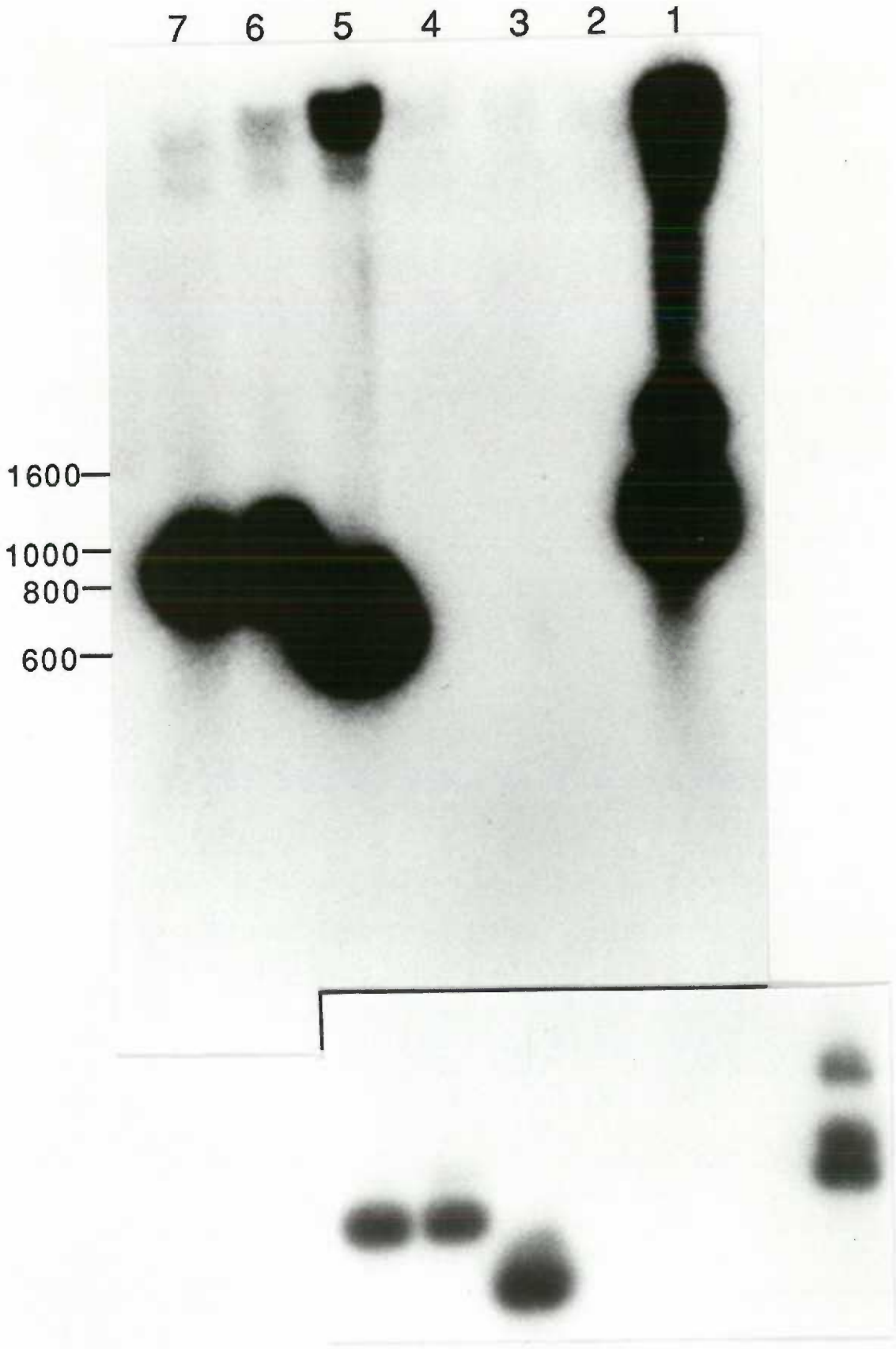
labelled, pCRF-D1. Radioactive labelling of the pCRF-D1 insert was performed by nick-translation of the insert DNA (described in Methods). Typically, 100ng of insert was used to prepare radiolabelled hybridization probe for rescreening the rat hypothalamic cDNA library. After replating 450,000 plaques, five positively hybridizing plaques were isolated in duplicate (L1b-5b).

These new clones were plaque purified and lambda DNA prepared. This DNA was digested with Eco RI and analyzed by Southern blotting using radiolabelled pCRF-D1 insert as the hybridization probe. The autoradiogram of this Southern blot is shown in figure 5. Again, several clones containing various sized inserts were obtained. From this analysis, Lambda 1b (in lane #1) appeared to contain the largest positively hybridizing insert of approximately 1.6 kilobases (Kb). However, this lambda clone appeared to contain at least 5 Eco RI inserts. Three of these hybridization positive Eco RI inserts are shown in the insert in Figure 5. A 150 bp hybridization positive fragment was subsequently identified and is not shown in figure 5 (this fragment was electrophoresed off the bottom of the gel in figure 5). Additionally, a 400 bp Eco RI insert was identified by ethidium bromide staining that was not hybridization positive (not shown). These different fragments are discussed from the smallest (number 1= 150 bp fragment) to the largest (number 5= 1.6 Kb fragment). These inserts were isolated individually, subcloned into M13 vectors and sequenced.

Fragment 1 (approximately 150 bp) was determined to be a 3'

Figure 5. Southern Blot Analysis of Rat Hypothalamic cDNA Clones Isolated on Second Screening.

Hypothalamic cDNA clones that were hybridization-positive to radiolabelled cDNA insert from pCRF-D1 (see text) were used to produce λ DNA. This DNA was subjected to restriction digests with EcoRI (a restriction site found in the linker used to clone the original hypothalamic cDNA), agarose gel electrophoresis, and subsequent blotting to nitrocellulose. This nitrocellulose was prehybridized, hybridized with nick-translated pCRF-D1 insert (620 bp), and washed, as described in Methods. Approximately 2 μg of λ DNA is found in each lane as follows: Lane 1= clone 1b, lane 2= clone 2b, lane 3= λ/Hind III digest, lane 4= 1 Kb Ladder (BRL), lane 5= clone 3b, lane 6=clone 4b, and lane 7= clone 5b. The autoradiogram was exposed overnight at -70°C with an intensifying screen. The numbers (in kilobases) found on the right edge of the photograph represent the approximate molecular weights of hybridizing signals as determined by the 1Kb Ladder (BRL). The inset photograph represents a portion of the same autoradiogram exposed for a shorter period of time so that the three bands in lane 1 are apparent.


7
6
5
4
3
2
1
1600
1000
800
600

untranslated portion of the CRH mRNA based upon significant homology to the human gene. Fragment 2 was a 400 bp cDNA sequence unrelated to rat CRH mRNA. Fragment 3 (approximately 800 bp) was shown to contain all of the CRH protein precursor sequence, again based upon homology with the human and ovine precursors. Fragment 4 was shown to be a partial restriction enzyme digest of the lambda clone in that it contained both fragments 1 and 3. Fragment 5 (the largest insert) was also shown to be a partial restriction enzyme digest containing fragments 1, 2, and 3. Fragment 2 (the 400 bp cDNA sequence unrelated to rat CRH mRNA) contained EcoR I linker sequences on both the 3' and 5' ends and was fused to the 5' end of the CRH sequence in this λ clone. The other hybridizing inserts (lanes 5, 6, and 7) were isolated, sequenced and shown to be authentic, but shorter rat CRH cDNAs. Non-hybridizing clones were not persued.

Fragments related to rat CRH were isolated and subcloned into pUC-19 (pCRH 1= fragment 1, pCRH 2= fragment 3, and pCRH 3= fragment 4). pCRH 3 was used for detailed restriction mapping and complete DNA sequencing. The results of the restriction mapping, DNA sequencing strategy and DNA sequence are shown in figure 6. This cDNA clone contained a different 3' poly A addition site than that found in the CRF-D1 insert. The full length cDNA appeared to utilize a more 3' poly A addition signal.

Nucleotide sequence analysis demonstrated that this insert contained an open reading frame of an 187 amino acid protein with the CRH peptide located at the extreme carboxy terminus of the

Figure 6. Nucleotide Sequence, Restriction Map, and Sequencing Strategy of a Rat Hypothalamic CRH cDNA.

The complete nucleotide sequence of a rat hypothalamic CRH cDNA isolated from λ clone #1 (upon second screening) is shown in this figure. The open reading frame starts with a Methionine (Met) residue and continues as a 187 amino acid precursor. The putative signal peptide cleavage residue (Ala) is indicated by a shaded box. Two potential sites for N-linked glycosylation (Asn-X-Ser/Thr) are indicated by open boxes. The position of a pair of basic amino acids (Arg-Arg), marking the putative site of proteolytic processing necessary to liberate the 41-amino acid CRH peptide, is indicated by the hatched box. The translational termination codon (TGA) is indicated (End). A schematic diagram of this rat CRH cDNA indicating several restriction sites is shown below. The hatched boxes indicate the Eco RI DNA linkers that were attached to cDNA in the hypothalamic cDNA library. The protein coding region of the CRH precursor is indicated by a larger open box with the position of the CRH peptide (CRH) shown. Solid boxes indicate the position of poly A addition (AAUAAA) sequences. Horizontal arrows, shown below the restriction map, indicate the direction and extent of nucleotides sequence determinations.

```
GCAGAACAACAGTGCGGGCTCACCTGCCAAGGGAGGAGAAGAGAGCGCCCCTAAAC

ATGCGGCTGCGGCTGCTGGTGTCCGCGGGCATGCTGCTGGTGGCTCTGTCGCCCTGTCTG
MetArgLeuArgLeuLeuValSerAlaGlyMetLeuLeuValAlaLeuSerProCysLeu

CCTTGCAGGGCCCTGCTGAGCAGGGGATCCGTCTCTGGAGCGCCGCGGGCCCCGCAGCCG
ProCysArgAlaLeuLeuSerArgGlySerValSerGlyAlaProArgAlaProGlnPro

TTGAATTTCTTGCAACCGGAGCAGCCCCAGCAACCTCAGCCGATTCTGATCCGCATGGGT
LeuAsnPheLeuGlnProGluGlnProGlnGlnProGlnProIleLeuIleArgMetGly

GAAGAATACTTCCTCCGCCTGGGGAACCTCAACAGAAGTCCCGCTGCTCGGCTGTCCCCC
GluGluTyrPheLeuArgLeuGlyAsnLeuAsnArgSerProAlaAlaArgLeuSerPro

AACTCCACGCCCCTCACCGCGGGTCGCGGCAGCCGCCCCTCGCACGACCAGGCTGCGGCT
AsnSerThrProLeuThrAlaGlyArgGlySerArgProSerHisAspGlnAlaAlaAla

AACTTTTTCCGCGTGTTGCTGCAGCAGCTGCAGATGCCTCAGCGCCCGCTCGACAGCAGC
AsnPhePheArgValLeuLeuGlnGlnLeuGlnMetProGlnArgProLeuAspSerSer

ACGGAGCTGGCGGAACGCGGCGCCGAGGATGCCCTCGGTGGCCACCAGGGGGCGCTGGAG
ThrGluLeuAlaGluArgGlyAlaGluAspAlaLeuGlyGlyHisGlnGlyAlaLeuGlu

AGGGAGAGGCCGGTCCGAGGAGCCGCCCATCTCTCTGGATCTCACCTTCCACCTTCTGAGG
ArgGluArgArgSerGluGluProProIleSerLeuAspLeuThrPheHisLeuLeuArg

GAAGTCTTGGAAATGGCCAGGGCAGAGCAGTTAGCTCAGCAAGCTCACAGCAACAGGAAA
GluValLeuGluMetAlaArgAlaGluGlnLeuAlaGlnGlnAlaHisSerAsnArgLys

CTGATGGAGATTATCGGGAAATGAAATGTTGCGCTTGGCCAAAACGATTCTGCATTTAGC
LeuMetGluIleIleGlyLysEnd

ACACAAGTAAAAATAAAAAATTTAAAACACAGTATTCTGTACCATACTGCAGCTCTGATA

TCATTTGTTTATTTTTATATAGCTTGAAGCATAGAAGATGTACAGGGAGAGAGCCTATAT

ACCCCTTAATTAGCATGCACAAAGTGTGTTTCTTTGTAGTAACAAAACAGCGTTATTTGT

ATTGTTCACGCTTAGTTTCTATGTGCAAATAAGTGTCTTTATAGCGATATCTTAAAGAAA

ATGTGGATCCAAGGAGGAAACCTTTAAAAAAGCAGATGGAAGTCACCCAGTTGTTTTTAT

TTGGAGACACAGTGTAAGAGAATTCATTCTTGAGGGGTGGCTAGGACAAAATGTGTAAGC

TCTTTGAATCAACTTTTTCTTGTAAATGTTTCAATAATAAAACATCTTTCTGATCCTTGG

TCAATTTGGTTGTGTCAGAGAACGTTGACTATATTTTTAATAAAATCTGAAAGGTT
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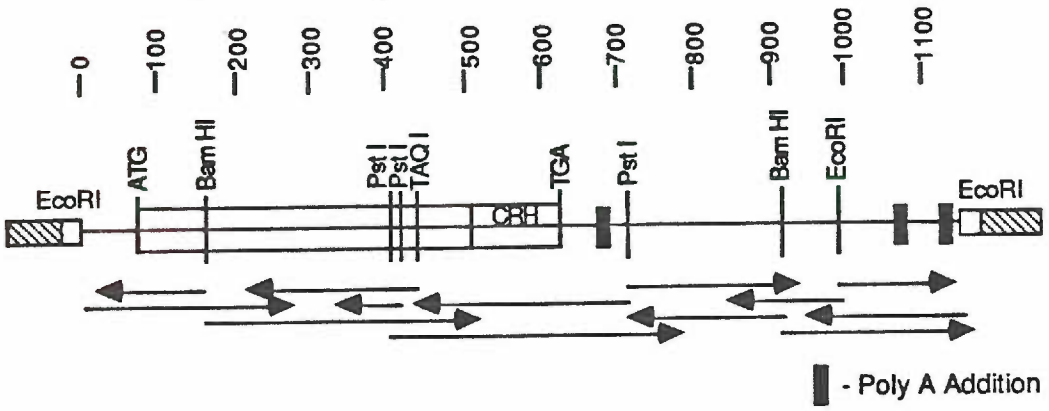


precursor. The sequence also contained the previously sequenced CRF-D1 insert. The open reading frame begins with a methionine residue (AUG), with the first 24 amino acids exhibiting features characteristic of signal peptides of secretory proteins. A possible cleavage site of the signal peptide appears to be located after the alanine residue specified by the 24th codon. A translational termination codon (UGA) was found in frame after the 187th codon which specified a Lysine residue. The deduced amino acid sequence for residues 145-185 agrees precisely with the sequence of the rat CRF peptide determined by peptide analysis. Within the rat CRH precursor, as is the case with the ovine and human precursors, the CRH peptide is flanked on the N-terminus by a pair of basic amino acid residues, Arg-Arg, which, as with many neuropeptide precursors, represents a proteolytic processing site. The carboxy-terminus of rat CRH is followed by the residues Gly-Lys, which implies that the CRH peptide is amidated.

In the ovine, human, and rat precursors, the putative proteolytic cleavage site which would serve to liberate the CRH peptide appears well conserved. However, other putative proteolytic cleavage sites at pairs of basic amino acids are not. The ovine and human proteins share an additional pair of basic amino acids at positions 116-117 and 123-124, respectively, found 30 amino acids in the ovine and 29 amino acids in the human from the amino-terminus of the CRH peptide. However, this pair of basic amino acids is not found in the rat protein precursor, being changed from an

Arg-Arg (in both ovine and human) to an Gln-Arg. In addition, potential post-translational modification sites exist in some of these precursors. For example, two N-linked glycosylation site (Asn-X-Ser/Thr) are found in the rat precursor (at amino acid 71-73 and 81-83), one in the human (at amino acid 80-82) and none in the ovine. These protein precursors also contain a relatively high percentage of proline residues (9.1% in the rat precursor, 12.8% in the human, and 12.1% in the ovine). This high percentage of proline residues may reflect considerable structural constraints on the three dimensional configuration of these precursors. Structural inflexibility could serve to influence the proteolytic processing of the protein precursor making the CRH peptide sequence or other amino acid sequences more or less available to processing enzymes.

The amino acid sequence of the CRH precursor across species is highly conserved; being 80 % homologous between rat/human, 65 % homologous between rat/ovine and 76 % homologous between human/ovine. The CRH peptide region (83-100%) and the putative signal peptide region (75-92%) are more highly conserved amoung the three species than is the middle portion of the precursor (55-72%).

RESULTS AND DISCUSSION

Chapter 2: Tissue Specific Expression of CRH mRNA

Summary

The CNS and peripheral distribution of CRH mRNA was determined by performing Northern blot analysis. CRH mRNA was identified in many regions of the CNS supporting previously published CRH immunohistochemistry. Additionally, several peripheral sites of CRH synthesis were demonstrated and potential roles of peripheral CRH discussed. This vast distribution of CRH synthesis has prompted suggestions that a network of CRH containing neurons and endocrine cells could provide a mechanism for the CRH peptide to coordinate many different biological functions in an organisms response to environmental stimuli.

Brain Distribution

Introduction

The CRH peptide has been detected immunologically in many regions of the central nervous system (44-49) as well as several peripheral tissues (49-57). In the central nervous system of the rat, CRH immunoreactive material has been detected in almost every major division of the brain, including hypothalamus, thalamus, cerebral cortex, hippocampus, amygdala, striatum, midbrain, and several pons and medulla regions. As shown in figure 7 as modified from reference 74, the highest concentration of CRH peptide (39.27

Figure 7. Concentrations of CRH Peptide in the Rat Brain.

CRH peptide concentrations were assayed in 1 mm punches of tissue from various regions of the rat CNS by Palkovits et al. Tissue from each microdissected brain area from three animals was pooled and homogenized in 100µl of 2N Acetic acid, boiled, centrifuged, and the supernatant lyophilized. The limit of detection of the radioimmunoassay used to detect CRH peptide was 0.3 ng/mg protein. (Data in this table is from Palkovits, Brownstein, and Vale (1985), Federation Proceedings, **44**: 215-219).

CRH Peptide in Rat Brain

	Concentration ng/mg protein (mean +/-SEM)
Hypothalamus	
Median eminence	39.27+/- 9.5
Paraventricular N.	0.58 +/- 0.09
Arcuate N.	1.46 +/- 0.33
Ventromedial N.	0.31 +/- 0.15
Dorsomedial N.	0.59 +/- 0.17
Ventral premammillary N.	<0.3
Dorsal premammillary N.	<0.3
Posterior pituitary	0.34 +/- 0.01
Cerebral (frontal) Cortex	<0.3
Globus pallidus	<0.3
Caudate N.	<0.3
Thalamus	
Periventricular N.	0.42 +/- 0.16
Ventral N.	<0.3
Midbrain	
Substantia Nigra	<0.3
Ventral tegmental area	0.47 +/- 0.13
Superior/Inferior colliculi	<0.3
Central Grey	0.35 +/- 0.14
Pons	
Dorsal raphe	0.81 +/- 0.39
Pontine Nuclei	0.38 +/- 0.23
Reticular formation	0.39 +/- 0.20
Parabrachial N.	0.83 +/- 0.26
Locus ceruleus	0.76 +/- 0.27
Dorsal tegmental N.	<0.3
Cerebellum	
Cortex	<0.3
Nuclei	0.30 +/- 0.15
Medulla oblongata	
Vestibular N.	<0.3
Spinal N.	<0.3
Inferior olive	1.72 +/- 0.29
Nucleus of the solitary tract	0.39 +/- 0.22

ng/mg protein) was found in the median eminence of the basal hypothalamus. The source of most of this immunoreactive material appears to be the paraventricular nucleus of the hypothalamus, where the cell bodies of several hundred CRH neurons which project to the median eminence are found (45,46). It is here in the median eminence that CRH peptide is thought to enter the portal vasculature that subsequently influences anterior pituitary secretion of ACTH and ß-endorphin. Most other hypothalamic nuclei also contain CRH immunoreactive material except for the premammillary and mammillary regions and the supraoptic nuclei (74-76) (although the latter observation appears controversial).

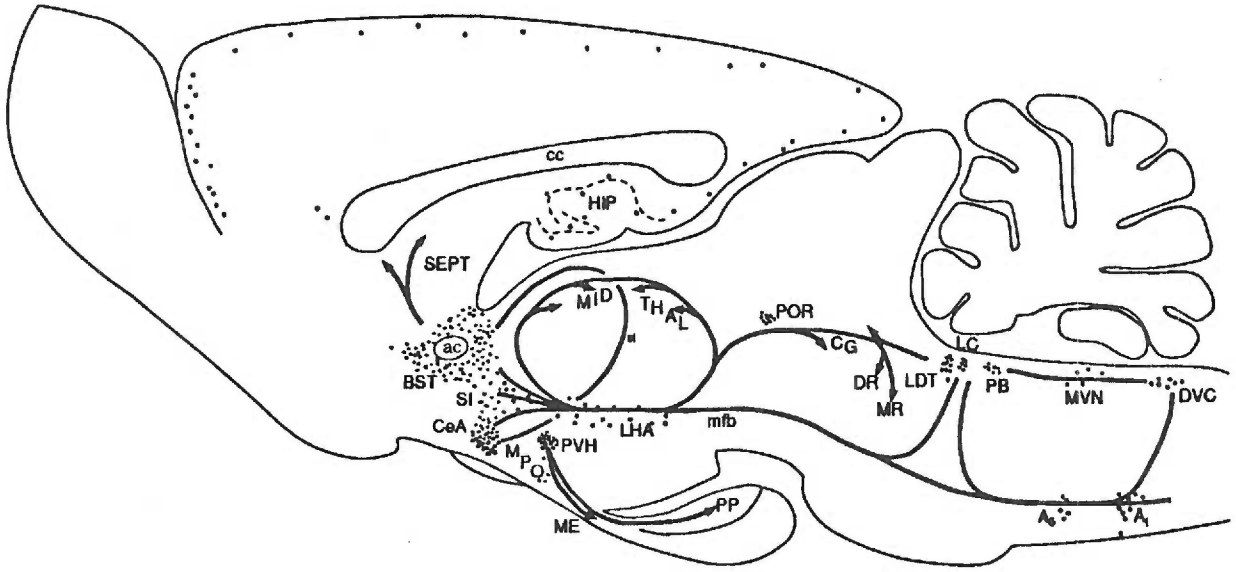
Several regions of the limbic system, in addition to the hypothalamus, also appear to contain moderate quantities of CRH peptides (74), including the bed nucleus of the stria terminalis, the central nucleus of the amgydala, and the CA1 and CA3 areas of the hippocampus. In brainstem structures, CRH peptides are found in the periaqueductal gray, midbrain and dorsal raphe, locus ceruleus, parabrachial nuclei, inferior olive, and nuclei of the solitary tract. In general, these distributions of immunoreactivity are associated predominately with limbic and brain stem areas that are thought to play an important role in neuroendocrine responses and autonomic mechanisms. Two CRH containing regions will be discussed as examples of these potential roles in neuroendocrine and autonomic mechanisms. The hippocampus, a limbic structure, is generally thought to be involved with memory as well as contributing to the

emotional status of the animal. This structure has close ties to the hypothalamus via the fornix (a myelinated axonal fiber pathway) and has been implicated as the CNS site sensitive to the negative effects of adrenal steroids which feedback to regulate of certain hypothalamic neurohormones (77-79). CRH acting as a neurohormone/neurotransmitter in this region could modify the hippocampal regulation of hypothalamic neurohormones. Interestingly, Aldenhoff et al. have shown that CRH excites hippocampal pyramidal neurons in vitro (80). In the brainstem, CRH peptide is found in the nucleus of the tractis solitarius (NTS), a nucleus involved in the transmission of visceral information to higher brain centers important in the reflex control of cardiovascular, respiratory and alimentary functions. This nucleus receives afferent information from specialized receptors such as baroreceptors of the carotid sinus (which monitor arterial blood pressure) and chemoreceptors of the carotid body (which monitor oxygen tension in circulating blood) via the glossopharyngeal and vagus nerves. CRH peptide function in the NTS is presently unclear but could modify visceral afferent information and thus visceral reflexes during periods of stress. Fisher et al. have shown that central administration of CRH increases mean arterial blood pressure and heart rate in the dog and rat (81,82). An anatomical diagram which summarizes the location of CRH immunoreactivity in the rat (modified from reference 46) is shown in figure 8.

The CRH peptide has been injected into the CNS of many species

Figure 8. Schematic Sagittal View of the CRH neurons and fibers in the Rat Brain.

The major CRH-stained cell groups (black dots) and fiber systems (arrows) are indicated. Abbreviations used in this figure are: A_1,A_5= Noradrenergic cell groups; ac= anterior commissure; BST= bed nucleus of the stria terminalis; cc= corpus callosum; CeA= Central Nucleus of the Amygdala; CG= central gray; DR= dorsal raphe; DVC= dorsal vagal complex; HIP= hippocampus; LC= locus coeruleus; LDT= laterodorsal tegmental nucleus; LHA= lateral hypothalamic area; ME= median eminence; MID THAL= midline thalamic nuclei; MPO= medial preoptic area; MR= median raphe; MVN= medial vestibular nucleus; PB= parabrachial nucleus; POR= perioculomotor nucleus; PP= posterior pituitary; PVH= paraventricular nucleus of the hypothalamus; SI= substantia innominata; SEPT= septal region. Modified from reference 46.


cc
HIP
SEPT
MID
TH
AL
POR
CG
LC
ac
BST
SI
DR
LDT
PB
MVN
DVC
MR
CeA
LHA
mfb
MP
PVH
PP
ME
A2
A1

to determine whether it could alter any measurable behavioral or physiological responses. For example, CRH injected intracerebroventricularly (i.c.v.) has been shown to alter states of behavioral activation (83), increase sympathetic activity (84), and inhibit feeding (85) and sexual behaviors (86). Electrophysiological studies have suggested that CRH has potent excitatory actions on the electrical activity of central neurons. Aldenhoff et al. have shown that CRH decreases post-burst hyperpolarizations and excites hippocampal pyramidal neurons in vitro (80). Additionally, CRH has been applied by pressure through three-barrel micropipettes to brainstem slice preparations and was shown to activate the noradrenergic neurons of the locus coeruleus (87). The effective dose in these studies is comparable to that reported to cause the behavioral and autonomic activations as seen in i.c.v. administration of CRH. Thus, it appears that CRH peptide may play a role in modifying the CNS and autonomic nervous systems in a fashion similar to that seen during stress.

In order to determine whether this material is synthesized at these sites or transported to these sites from other regions of synthesis, Northern blot analysis was used to detect the presence of CRH mRNA in a variety of brain regions dissected as described in Methods.

Results and Discussion

Brain tissue was dissected from adult male Sprague Dawley

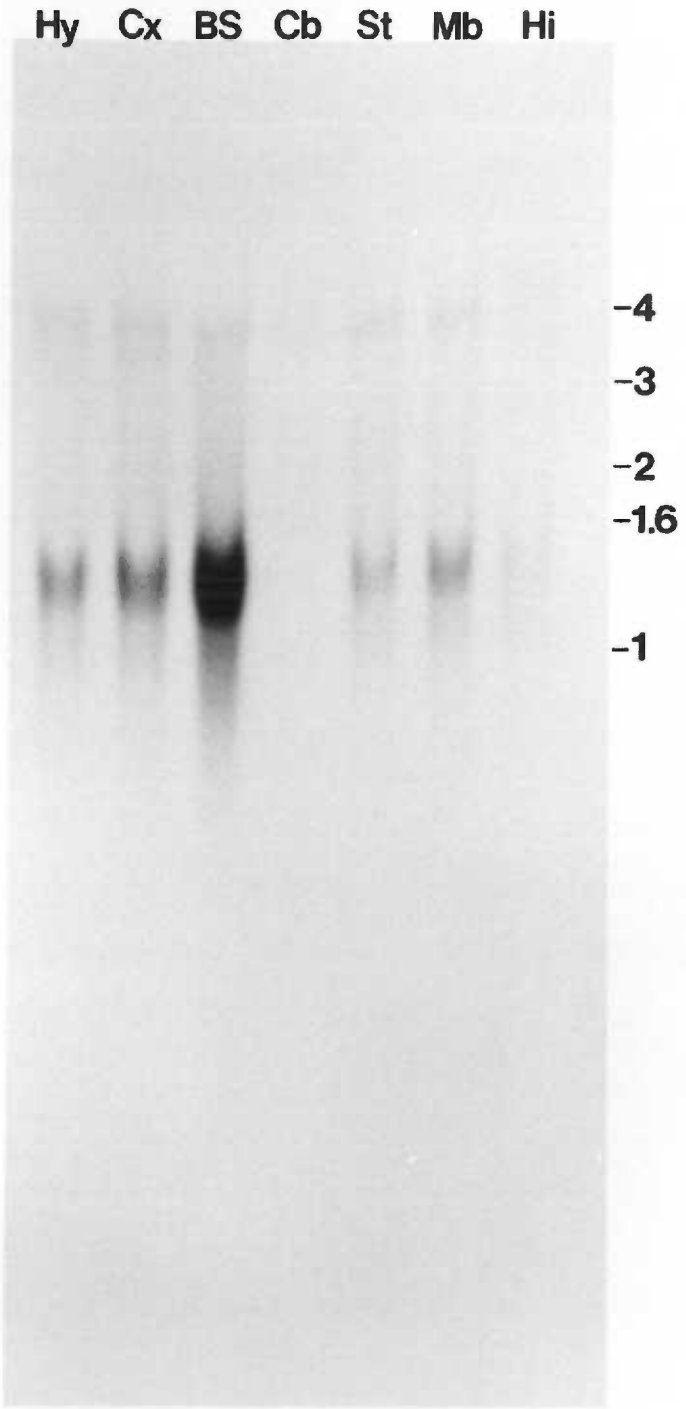
rats as described in Methods. RNA was isolated by the guanidinium isothiocyanate/CsCl procedure (69) and poly (A) RNA selected by oligo d(T) cellulose chromatography. Poly (A) RNA was size separated on denaturing formaldehyde/agarose gels and tranferred to nlyon membranes as described in Methods. Radiolabelled complementary RNA probes were ulitized as hybridization probes on these blots. The Northern blot (hybridized with a 976 nucleotide ^{32}P-cRNA probe) autoradiogram investigating the presence of CRH mRNA in several brain regions is shown in figure 9.

Even though varying quantities of poly (A) RNA were loaded in each lane of the Northern blot, it appears that the highest concentration of CRH mRNA is found in the brainstem (medulla/pons) region of the rat brain. CRH mRNA is also detected in the cerebral cortex, hypothalamus, midbrain, striatum, and hippocampus. The only brain region tested that contained undetectable levels of the CRH mRNA was the cerebellum (even on much longer exposures of this blot). The CRH mRNA content of brain regions estimated as the percentage of the total poly A+ RNA content may thus be represented as follows: brainstem >> cerebral cortex = hypothalamus > midbrain > striatum > hippocampus.

In general, the presence of CRH mRNA corresponds to the presence of immunorective CRH in the brain. However, the levels of the mRNA do not appear to correspond to the concentrations of CRH immunoreactivity. For example, the hypothalamus has the greatest concentration of immunoreative CRH, whereas the CRH mRNA is only

Figure 9. Distribution of CRH mRNA in Rat Central Nervous System.

Poly A RNA was isolated from various rat brain tissues, denatured with formaldehyde, and electrophoresed through a 1.4% agarose gel containing 2.2M formaldehyde. After transfer, the blot was hybridized with a ^{32}P-radiolabelled CRH cRNA probe with a specific activity of approximately 2.4 X 10^9 cpm/μg. The autoradiogram shown was exposed for 48 hours at -70°C with an intensifying screen. The numbers on the right edge of the photograph represent the approximate positions of denatured DNA molecular weight markers in kilobases. The poly A RNA samples (amounts noted) were derived from the following rat tissues: 1) hypothalamus, 8.0 μg; 2) cerebral cortex, 8.8 μg; 3) brainstem (medulla/pons), 13.9 μg; 4) cerebellum, 7.2 μg; 5) striatum, 12 μg; 6) midbrain, 11 μg; 7) hippocampus, 7.8 μg. The low intensity bands at approximately 1.7 kb and 3.9 kb represent the positions of 18 S and 28 S rRNA, respectively.


Hy
Cx
BS
Cb
St
Mb
Hi
-4
-3
-2
-1.6
-1

of moderate to low level when compared to other brain regions. The inferior olive has been shown to contain a large number of CRH immunoreactive neurons with axonal projection to the purkinje cell layer of the cerebellar cortex. Thus even though the cerebellum contains significant levels of the mature CRH peptide, it is, in fact, synthesized in the inferior olive which is spatially quite distant.

Factors such as tissue specific CRH precursor processing, secretion, transport, or rate of turnover of CRH immunoreactivity may account for these findings. This point of discrepancy highlights the need to perform Northern and/or in situ hybridization analysis to confirm sites of synthesis of proteins in complex tissues such as the central nervous system.

Additionally, the size of the brain CRH mRNA was found to be similar in all regions tested, being approximately 1400 nucleotides in length. This size was slightly larger than expected, as the previously reported ovine cDNA was only about 1100 nucleotides in length. However, no Northern blot data was presented in this ovine cloning report to verify the size of the hypothalamic ovine CRH mRNA.

Non-Brain Distribution

Introduction

The presence of CRH immunoreactivity in non-brain tissues is not as well characterized, but CRH-IR has been reported in the spinal cord (49,50), adrenal gland (49,51), pancreas (49,52), duodenum (49) and testis (56,57). CRH immunoreactivity in the spinal cord was

localized to lamina I and II of the dorsal horn and appeared continuous anteriorly with the spinal trigeminal nucleus. When rats were treated with capsaicin (an agent which causes selective depletion of small diameter afferents of the C-fiber type, a fiber type which has been shown to transmit diffuse pain sensations) it caused a depletion of CRH immunoreactivity, which has prompted suggestions that CRH peptides are found in primary sensory pathways (88).

In the dog adrenal, CRH immunoreactivity is found in medullary cells adjacent to blood vessels at the junction of the medulla and the cortex. The secretion of CRH-like material from dog adrenal appears to be regulated through sympathetic mechanisms as suggested by evidence demonstrating a 3 fold increase of CRH in response to 20 % hemorrhage (a hemodynamic stimulus known to activate a sympathetic adrenal response) (51). This anatomical localization of CRH like material and its regulation by hemorrhage has prompted suggestions that CRH peptides may play a role in influencing adrenal blood flow. Investigations into the potential roles of CRH in the adrenal have also shown that the CRH peptide appears to be a weak catecholamine secretagogue (89). Recently, CRH receptors have been characterized in rat, bovine and monkey adrenal medullary tissue (90), further suggesting that CRH peptides could act locally, possibly in a paracrine fashion.

CRH peptides have also been detected in the pancreas of many species including rat, mouse, cat, monkey, chicken, toad, lizard and man (52). Although the colocalization studies were not complete, the

authors concluded that CRH appeared to be in a subpopulation of glucagon secreting cells (A cells) within the endocrine portion of the pancreas. Various forms of stress may be associated with decreased glucose tolerance, hyperglycemia, and elevated glucagon concentration. These effects are generally regarded as neurogenic and are thought to be mediated by the rich autonomic innervation of islet cells (91). Local paracrine regulation of glucagon release by CRH may represent a complementary mechanism, which may be the stimulatory counterpart to the inhibitory effects of somatostatin (92). Other possible roles of CRH in the pancreas include the regulation of secretion of the exocrine portion of the pancreas and regulation of blood flow within the pancreas due to CRH's significant effects on vasomotor activity.

The presence of testicular CRH peptides has been recently published (56,57). In these studies (in ovine and rat), CRH immunoreactivity appears to be produced in Leydig cells (testosterone-producing) and in advanced germ cells. The CRH peptide levels were also shown to fluctuate with age, and these fluctuations appeared to correlate with the developmental patterns of Sertoli and Leydig cell functions. For example, immunoreactive CRH was found to increase at 60 and 90 days of age which corresponds to a stage of full spermatogenic activity and sperm production. However, no direct evidence for a functional role of CRH peptides in this organ has been reported.

In the gastrointestinal tract, CRH immunoreactivity has been

localized to epithelial (endocrine) cells in the stomach and small intestine of the monkey, the cat and occasionally the rat (93). These cells were most frequent in the gastric antrum and became less numerous in the duodenum, jejunum, and ileum, and none were found in the colon. CRH-containing cells within these regions were shown to be distinct from glucagon and gastrin-containing cells.

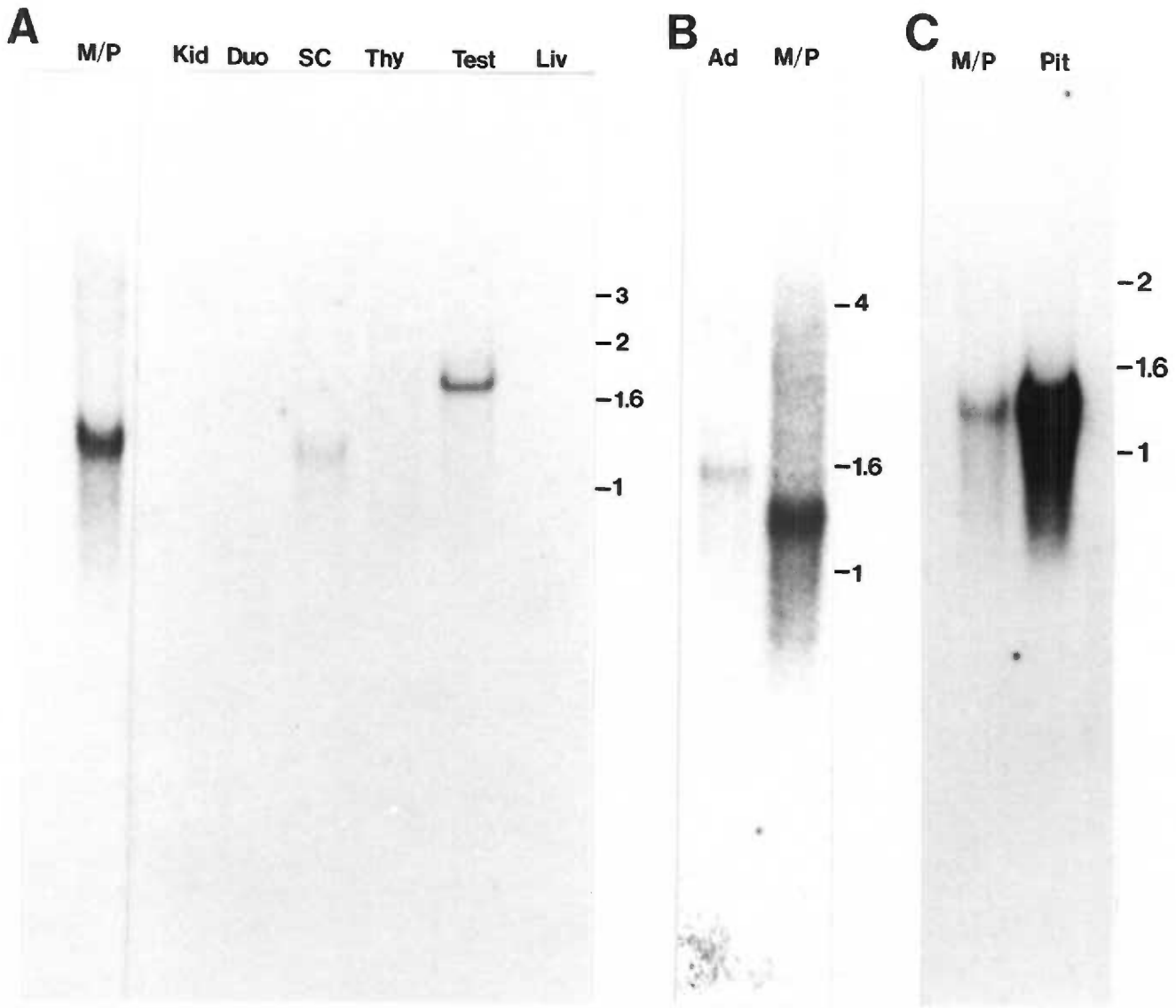
Results and Discussion

To determine whether the CRH immunoreactivity was synthesized in these peripheral tissues, samples were analyzed for the presence of CRH mRNA by Northern blot analysis. Tissue from adult male Sprague Dawley rats was removed as quickly as possible and frozen on dry ice. Total RNA was extracted, poly (A) RNA purified, size separated on denaturing formaldehyde/agarose gels and transferred to nylon membranes as previously described (in Methods). The autoradiograms of these Northern blots of RNA from non-brain tissues are shown in figure 10.

CRH mRNA was detected in the adrenal gland (whole adrenal), spinal cord, testis and pituitary gland (whole pituitary). CRH mRNA was undetectable in the liver, thymus, duodenum and kidney. In general, the presence of CRH mRNA in these peripheral regions corresponds to the presence of CRH immunoreactivity, with the exceptions of the duodenum and pituitary. The inability to detect duodenum CRH mRNA may be due to the fact that there are very few CRH positive cells in this region of the gut, and thus gut levels of CRH

Figure 10. Distribution of CRH mRNA in Rat Peripheral Tissues

Poly A RNA was isolated, electrophoresed, blotted, and probed as described in the legend to fig. 4. The numbers on the right edges of all photographs represent the approximate positions of denatured DNA molecular weight markers in kilobases. A) The RNA samples (amount noted), were derived from the following rat tissues: 1) brainstem (medulla/pons) total RNA, 20 μg; 2) kidney poly A RNA, 2 μg; 3) duodenum poly A RNA, 3.3 μg; 4) spinal cord poly A RNA, 5.5 μg; 5) thymus poly A RNA, 10 μg; 6) testis poly A RNA, 8.5 μg; 7) liver poly A RNA, 5 μg. The autoradiogram was exposed for 90 hours at -70°C with an intensifying screen. B) The RNA samples (amounts noted) were derived from the following tissues: 1) adrenal poly A RNA, 10.5 μg; 2) brainstem total RNA, 20 μg. The autoradiogram was exposed for 72 hours at -70°C with an intensifying screen. C) The RNA samples (amounts noted) were derived from the following tissues: 1) brainstem (medulla/pons) total RNA, 5 μg; 2) total pituitary poly A RNA, 20 μg. The autoradiogram was exposed for 48 hours at -70°C with an intensifying screen.


A
M/P
Kid
Duo
SC
Thy
Test
Liv
-3
-2
-1.6
-1
B
Ad
M/P
-4
-1.6
-1
C
M/P
Pit
-2
-1.6
-1

mRNA may lie below the sensitivity of the Northern blot analysis. Alternatively, the antibodies used to detect CRH in the duodenum may in fact recognize a related antigen and not authentic CRH peptide. The presence of CRH mRNA in the pituitary is quite an interesting finding in light of the dogma which states that the anterior pituitary is the target gland of this neuropeptide releasing factor. It is not known whether this transcript is located in only one or all three lobes of the rat pituitary gland. This localization may represent an example of autocrine or paracrine regulatory mechanisms influencing hormone secretion. Alternatively, this transcript may represent a novel mRNA which shares significant homology with CRH mRNA. Interestingly, there have been two reports of CRH immunoreactive material in the anterior pituitary (54,55). In these reports on rat and human tissues, the cell types which contained the CRH-like material were different, which has prompted suggestions that this material may not be authentic CRH peptide but a related molecule with shared epitopes.

Additionally, CRH mRNA in the adrenal gland and testis was shown to be variable in length. The adrenal transcript was approximately 200 nucleotides larger than the CNS transcript, while the testis form was approximately 700 nucleotides larger. This difference in length may be due to a varibility in the size of the 3' untranslated region, alternate splicing, or to aberrant initiation of transcription. Tissue specific differences in transcript size have been reported for a number of other genes including the proopiomelanocortin gene, proenkephalin gene and the rat

prodynorphin gene (94-96). In the testis, the rat prodynorphin transcript has been shown to represent an alternatively spliced RNA which removes a 5' untranslated exon found in the striatal form of the dynorphin mRNA (J. Garrett, unpublished observation).

RESULTS AND DISCUSSION

Chapter 3: Isolation, Characterization and Sequence of the Rat CRH Gene.

Summary

The regulatory mechanisms and factors which act as modulators of cellular CRH mRNA levels remain to be fully characterized. The molecular mechanisms underlying the expression of the CRH precursor and its gene are essential to an understanding of the regulation within the hypothalamic-pituitary-adrenal axis. Towards this end, the gene encoding the rat CRH precursor was isolated and characterized. The structure of this gene and its relationship to the human CRH gene are described.

Introduction

In 1983, Shibahara et al. isolated and sequenced the human CRH gene (66). In this report, the structure of the human gene was described to contain two exons separated by an 800 bp intron based upon comparisons with the previously published ovine CRH cDNA nucleotide sequence (65). Southern blot analysis of human placental DNA hybridized with genomic fragments derived from the human CRH gene suggested that the cloned CRH gene is present in one copy in the human genome.

Based upon nucleotide comparisons between the ovine CRH cDNA and the human CRH gene, the first exon was suggested to contain

approximately 170 nucleotides of the 5' untranslated region of the mRNA. The second exon was suggested to contain 15 bp of the 5' untranslated region, the entire protein coding region and the 3' untranslated region of the CRH mRNA. TATAA and CAAT boxes were found 23 and 58 bp upstream of a putative cap site (the cap site was assigned based upon nucleotide sequence homology between the 5' end of the ovine CRH cDNA sequence and the nucleotide sequence found 3' to the TATAA box of the human CRH gene). The translation initiation site was assigned to the AUG codon in frame with an amino acid sequence with a high degree of homology to the ovine CRH peptide sequence. The 3' untranslated region of the human CRH gene contained two copies of the sequence, AAUAAA, which is involved in poly A addition following transcription termination.

The assignment of the initiating methionine residue (AUG) suggested that the human proCRH protein precursor contained 196 amino acids. The amino acid sequence of the amino-terminal 24 amino acid residues exhibited characteristics of signal peptides of secretory proteins. The deduced structure of human CRH peptide exhibited seven amino acid changes from the ovine CRH peptide.

In late1985, Jingami et al. reported the isolation and characterization of a rat hypothalamic CRH cDNA (97). This sequence information demonstrated that our longest cDNA was in fact incomplete, lacking approximately 120 nucleotides of the 5' untranslated region of the CRH mRNA. The nucleotide sequence of the remaining portions of the rat CRH mRNA reported in this paper

matched our CRH cDNA sequence precisely.

Results and Discussion

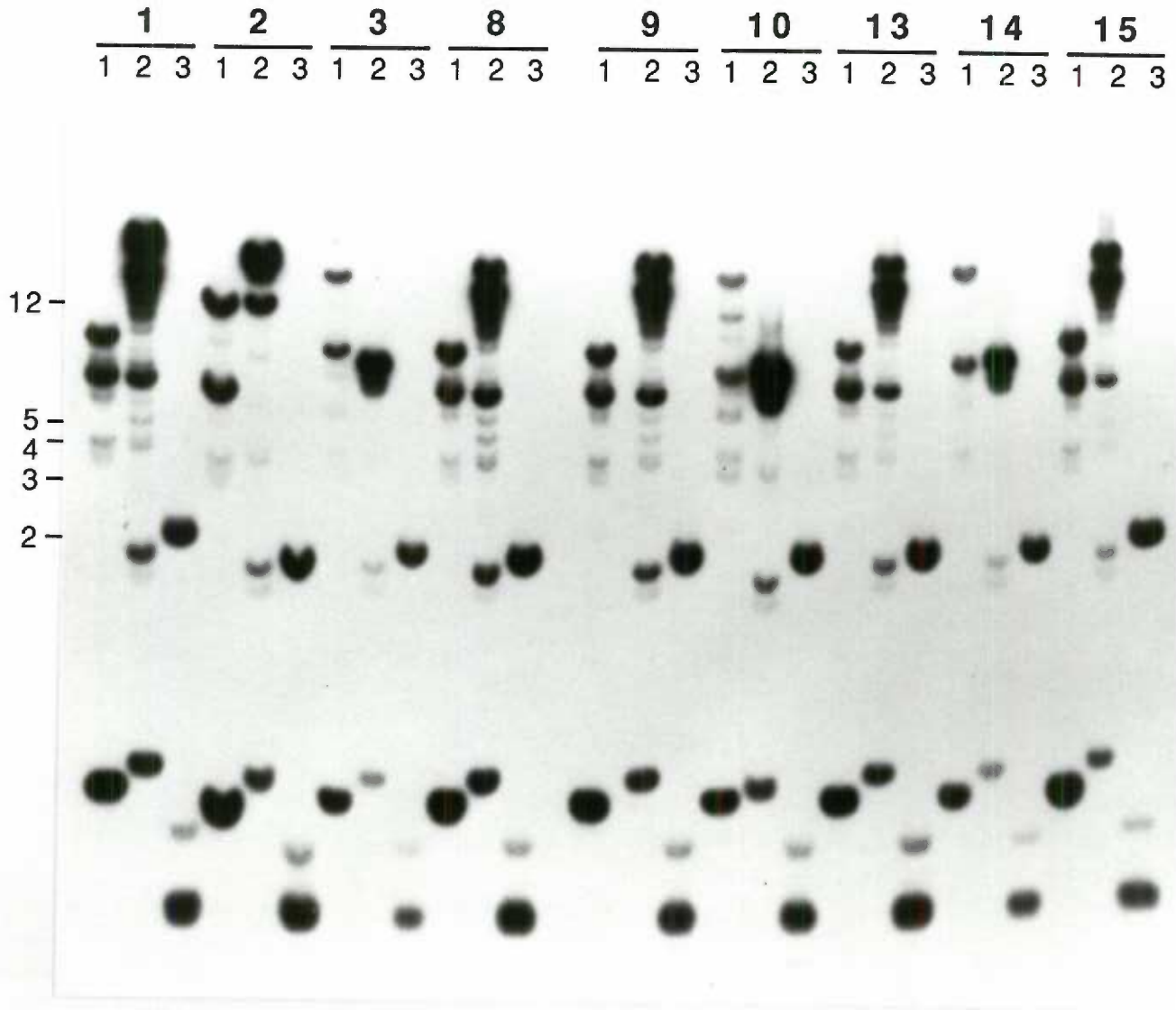
A rat lambda (EMBL-3) genomic library was screened using a 976 bp rat CRH cDNA insert (fragment 3, as described in chapter 1) as a hybridization probe, yielding fifteen hybridization positives in duplicate. The DNA from these recombinant phage was purified and analyzed by Southern hybridization using probes prepared from the rat cDNA sequence. An example of such a Southern blot analysis is shown in figure 11.

In this figure, there are three classes of clones, with all clones containing common Pst I, Sal I and Eco RI restriction fragments which hybridized to the full length cDNA probe described in chapter 1. These classes are most easily distinguished by their Sal I restriction patterns. The first class of clones is represented by clones 1, 8, 9, 13, and 15 containing three cDNA-hybridization positive bands in the Sal I digest of 10, 7 and 0.5 kb. The second class of clones is represented by clones 3,10, and 14 containing three cDNA-hybridization positive bands in the Sal I digest of 14, 8.5 and 0.5 kb. The third class includes clone 2 which contains three cDNA-hybridization positive bands in the Sal I digest of 11, 6.5 and 0.5 kb. The remaining six positives also fell into one of these three classes. This data suggests that the three classes of clones contain different genomic DNA fragments representing the same proCRH gene.

To confirm the number of CRH genes present in the rat genome,

Figure 11. Southern Blot Analysis of Rat Genomic Clones Hybridized With the Full Length CRH cDNA.

Rat genomic DNA (isolated from clones 1, 2, 3, 8, 9, 10, 13, 14, and 15; in large numbers at the top of the figure) was subjected to three restriction digests (1= Sal I, 2= EcoR I, 3= Pst I; smaller numbers at the top of the figure), agarose gel electrophoresis, and blotting to nitrocellulose. The nitrocellulose was prehybridized, hybridized with nick-translated pFrag2 DNA insert and washed (identical conditions to those for nick-translated pCRF-D1 DNA insert were used and are described in Methods). Approximately 2 μg of λ DNA is found in each lane. The autoradiogram was exposed at room temperature. The numbers (in kilobases) found on the left edge of the photograph represent the positions of DNA bands from the 1Kb Ladder (BRL) used for molecular weight determinations.


1 2 3 8 9 10 13 14 15
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
12
5
4
3
2

rat liver DNA was digested with various restriction enzymes and subjected to Southern blot analysis using the 976 bp rat CRH cDNA insert as a hybridization probe. The autoradiogram of the genomic Southern blot is shown in figure 12. This analysis demonstrated the presence of a unique 6.0 kb Eco RI fragment and a greater than 10 kb Hind III fragment after hybridization. As expected, two BamH I (6.5 kb and 770 bp) and Pvu II (2.2 kb and 450 bp) restriction fragments hybridized to this probe due to the presence of internal BamH I and Pvu II restriction enzyme recognition sites in the CRH cDNA sequence. The presence of unique Eco RI and Hind III fragments and appropriate sized BamH I and Pvu II fragments, along with the evidence that all positively hybridizing lambda genomic clones shared similar restriction patterns, supports the hypothesis that a single CRH gene is present in the rat genome.

Once confidence was established that a single CRH gene is present in the rat genome, the complete nucleotide sequence of the rat CRH gene was determined. Figure 13 is an autoradiogram of the λ DNA Southern blot shown in figure 9, but washed and re-hybridized with an oligonucleotide probe complementary to the 5' end of the rat CRH cDNA sequence (#228) (This oligonucleotide correspondes to the 25 nucleotides found 11 bp downstream from the 5' end of a rat hypothalamic cDNA sequence reported by Jingami et al. (97) and its position is diagrammed in figure 14). A schematic diagram of the relative positions of the oligonucleotides used to analyze genomic clones is shown in figure 14. From this analysis, all of the clones

Figure 12. Southern Blot Analysis of Rat Genomic DNA

Rat liver DNA (20μg/lane) was digested with EcoRI (lane 1), BamHI (lane 2), PvuII (lane 3), and HindIII (lane 4), and electrophoresed through a 0.8% agarose gel, transferred to nitrocellulose, and hybridized with a nick translated 976 bp EcoRI insert isolated from a full length rat CRH cDNA. The autoradiogram shown was exposed for 16 hours at -70°C with an intensifying screen. The sizes (in kilobases) of various DNA fragments are shown to the right of the figure.

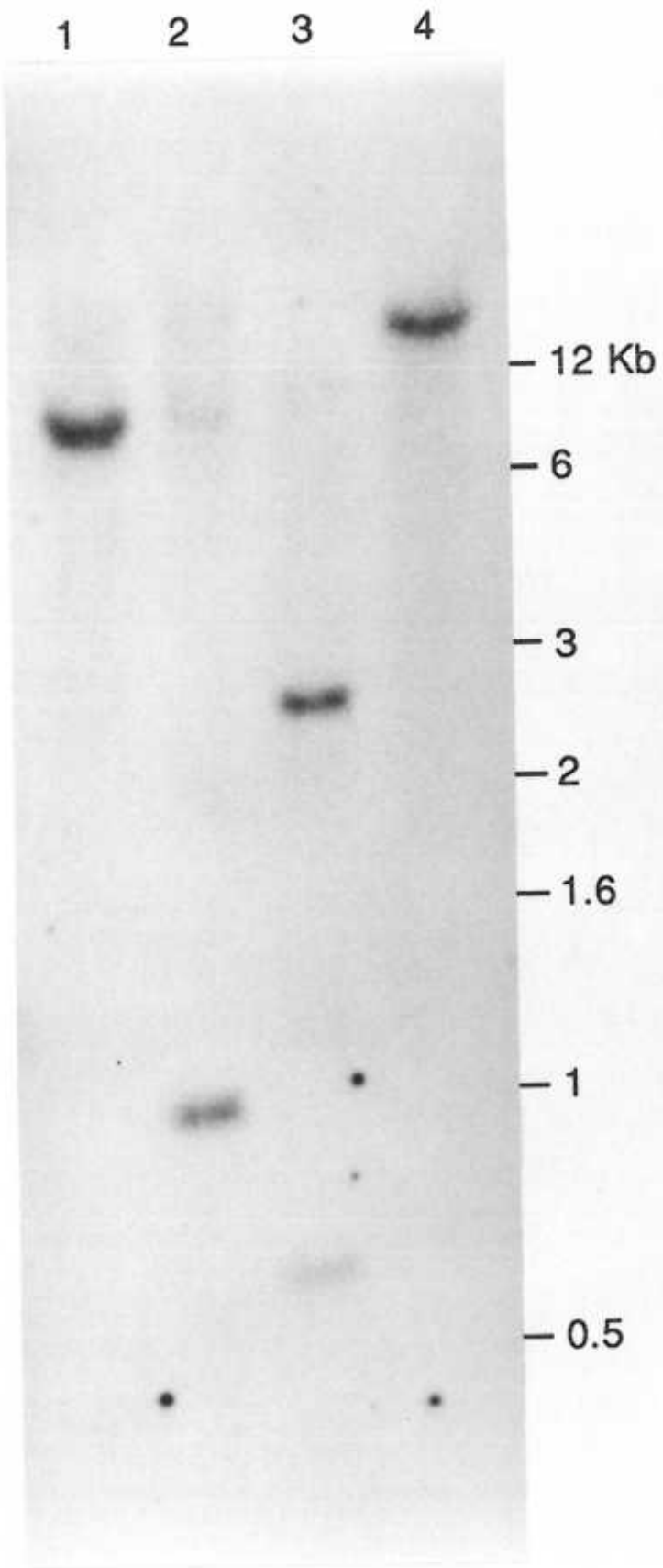
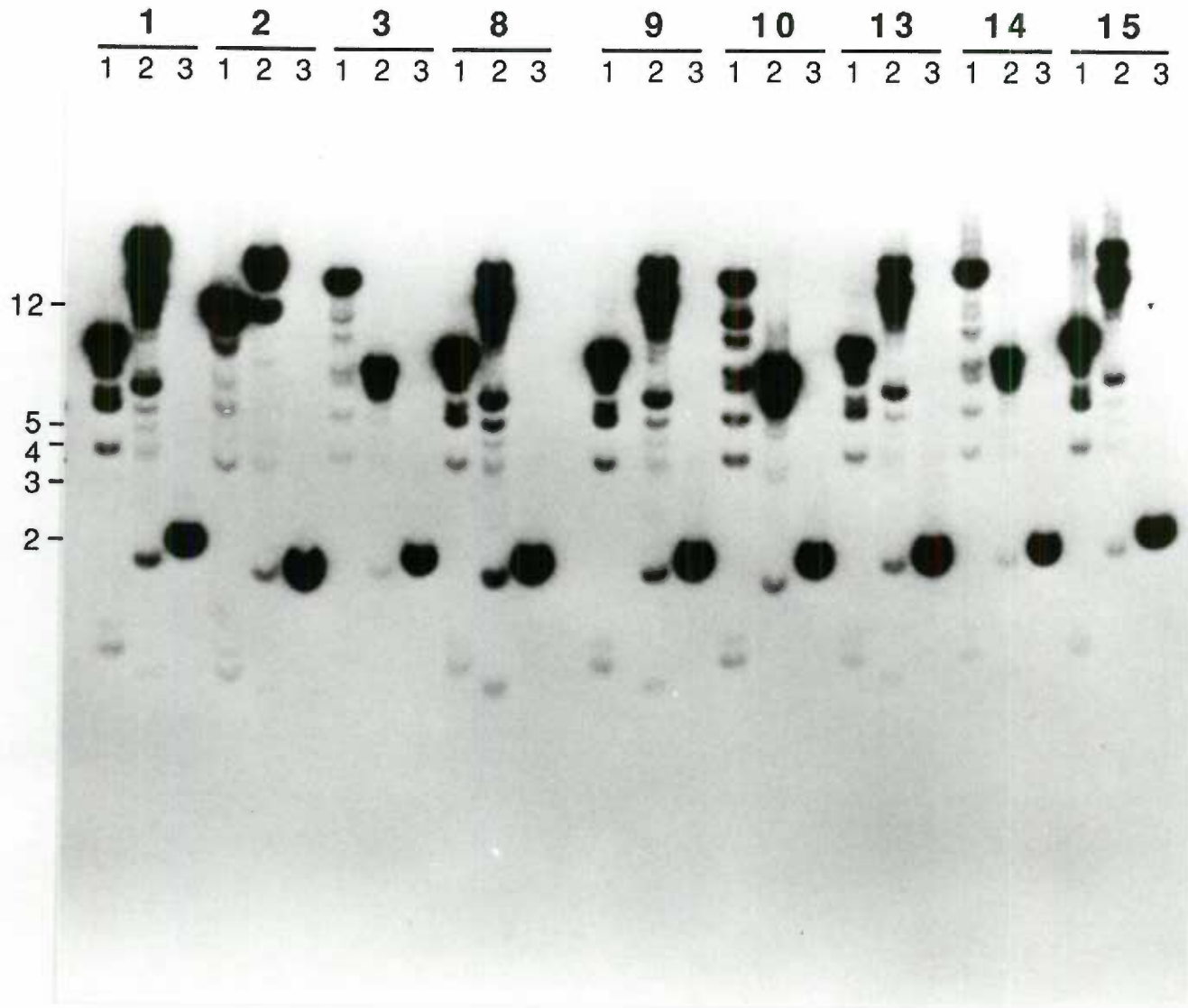

1
2
3
4
12 Kb
6
3
2
1.6
1
0.5

Figure 13. Southern Blot Analysis of Rat Genomic Clones Hybridized With Oligonucleotide #228.

Rat genomic DNA (isolated from clones 1, 2, 3, 8, 9, 10, 13, 14, and 15; in large numbers at the top of the figure) was subjected to three restriction digests (1= Sal I, 2= EcoR I, 3= Pst I; smaller numbers at the top of the figure), agarose gel electrophoresis, and blotting to nitrocellulose. The nitrocellulose was prehybridized, hybridized with oligonucleotide #228 (corresponding to the 5' end of the CRH mRNA), and washed (as described in Methods for oligonucleotide #140). Approximately 2 μg of λ DNA is found in each lane. The autoradiogram was exposed overnight at -70°C with an intensifying screen. The numbers (in kilobases) found on the left edge of the photograph represent the positions of DNA bands from the 1Kb Ladder (BRL) used for molecular weight determinations.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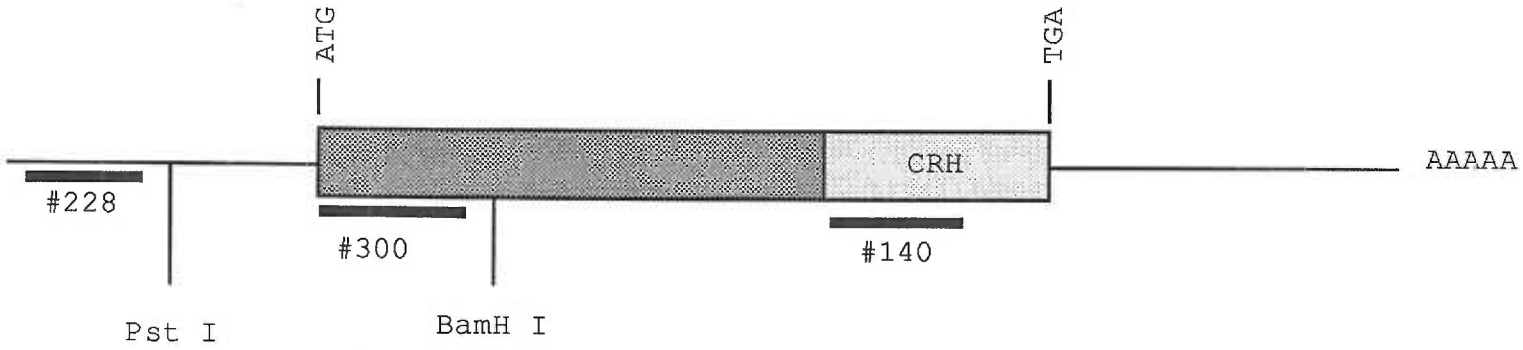
14
15
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
12
5
4
3
2

Figure 14. Deoxyoligonucleotides Used to Isolate CRH cDNA and Genomic Clones.

The position of deoxyoligonucleotides #140 (used to isolate the rat hypothalamic cDNA), #228 (used to identify 5' flanking fragments of the rat CRH gene) and #300 (used to identify the intron fragments of the rat CRH gene) are indicated. Key restriction enzyme sites are noted. The translation intiaition codon (ATG), translation termination codon (TGA) and the CRH peptide (CRH) are indicated.


ATG
TGA
CRH
AAAAA
#228
#300
#140
Pst I
BamH I

appeared to contain a 2 kb Pst I fragment in common. This fragment was interesting due to the presence of a Pst I restriction site present in the 5' untranslated region of the CRH cDNA, 49 bp 3' to the complementary sequence of the oligonucleotide #228. It thus appeared likely that this Pst I fragment would contain the promotor element of this gene. Therefore, a Pst I restriction digest was performed on clone number 9 and this 2 kb DNA fragment was isolated on LMT agarose gels. This fragment was subcloned into pUC-18, hybridization tested for the oligonucleotide (#228), and restriction mapped. From this pUC-18 clone, a Bgl II - Pst I restriction fragment (592 bp) was isolated, subcloned into M13 mp18 and mp19 and sequenced. This Bgl II- Pst I fragment was indeed shown to include characteristic promoter-like elements. A Bgl II - BamH I fragment (approximately 1.4 kb) was isolated (from clone 9) which was hybridization positive to both the deoxyoligonucleotide#228 and another deoxyoligonucleotide (#300, see figure 14) which corresponded to the signal peptide region of the CRH mRNA (shown to be on Exon II in the human gene). This fragment was isolated due to the presence of a BamH I restriction enzyme site found 3' to the position of oligonucleotide #300. This hybridization pattern and the presence of a BamH I restriction site in the signal peptide region of the cDNA suggested that this Bgl II/ BamH I fragment might span an intron. This fragment was subcloned into pUC-18, restriction mapped, and sequenced. From this analysis, the fragment was shown to contain a 700 bp intron, as determined by a comparison to the

nucleotide sequence of the hypothalamic cDNA.

The remainder of the CRH protein coding and 3' untranslated sequences were identified and shown to reside in a single exon uninterupted by intronic sequences. The complete nucleotide sequence and sequencing strategy of the entire rat CRH gene are shown in figures 15 and 16, respectively.

Many structural similarities are evident between the human and rat CRH genes. First, the overall structure of the two genes is quite similar in that both CRH genes contain two similarly sized exons, with a single intron interupting the 5' untranslated region of the respective mRNAs. As shown in figure 15, the rat CRH gene Exon I contains approximately 160 bp (dependent on the placement of the cap site) of the 5' untranslated region of the mRNA. Exon II contains 15 bp of the 5' untranslated region of the mRNA, the entire protein coding region, and the complete 3' untranslated region of the mRNA.

To date, no human CRH cDNA sequence has been reported. Therefore, no data exists on the precise sequence of the human CRH mRNA with regards to the splicing pattern of the human gene. However, the human CRH gene does appear to be capable of generating a hypothalamic CRH mRNA that would be quite homologous to the rat hypothalamic CRH mRNA and therefore utilize similar splice sites in the human gene. The rat CRH gene splice junctions are well conserved from the human gene and follow the donor and acceptor rules as described by Breathnach et al. (98). A general splicing scheme as determined from the hypothalamic CRH cDNA is shown in figure 17.

Figure 15. Nucleotide Sequence of the Rat CRH Gene

The nucleotide sequence of the message strand is shown, along with the deduced amino acid sequence. The sequence of the CRH peptide is boxed. The putative cap site is indicated by an asterisk. The TATAA and CAAT boxes are outlined by solid boxes; additional TATAA and CAAT sequences are indicated by dotted boxes. The exon/intron junctions are indicated by vertical lines. The poly A addition sequences (AATAAA) are underlined and the points of addition of poly A tails from isolated hypothalamic cDNA clones are indicated by arrowheads.

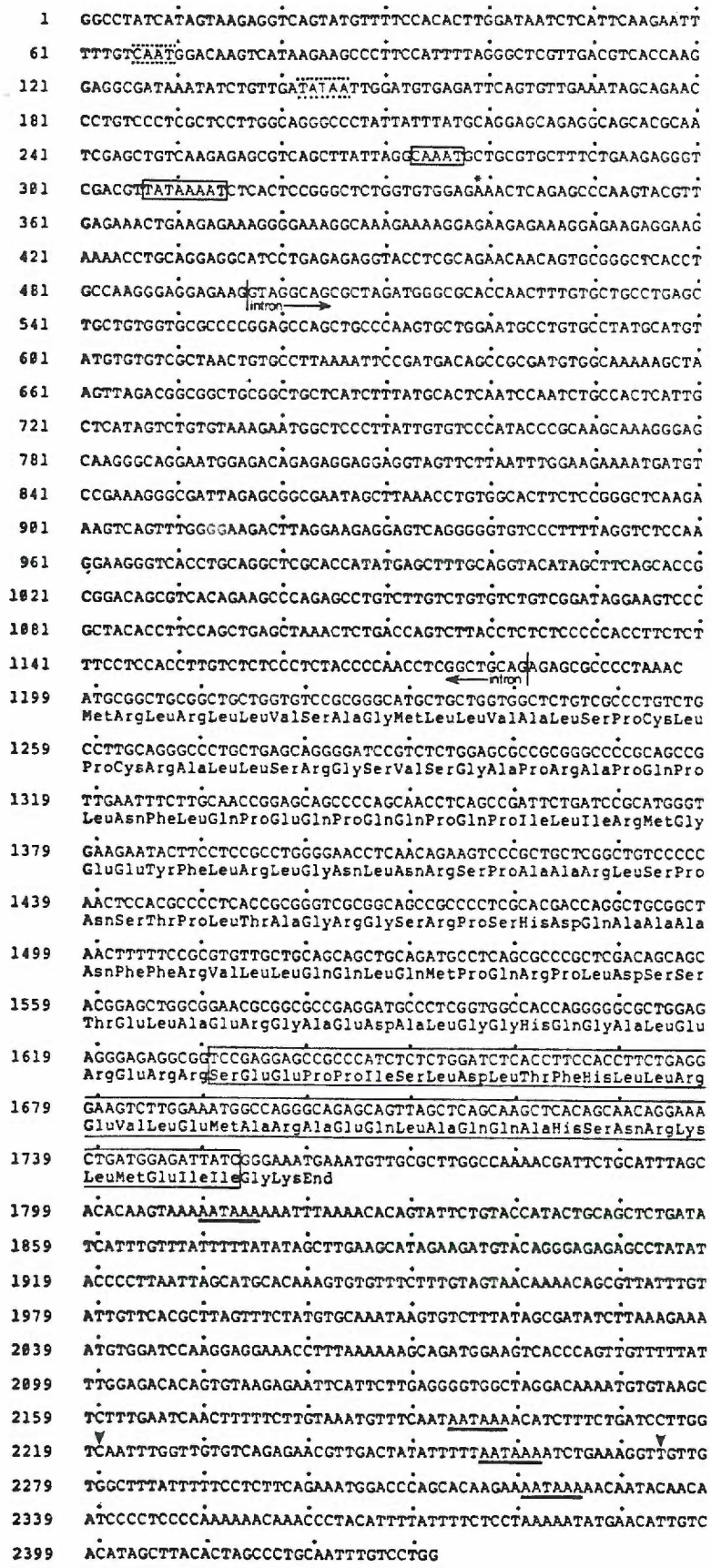

1 GGCCTATCATAGTAAGAGGTCAGTATGTTTTCCACACTTGGATAATCTCATTCAAGAATT
61 TTTGTCAATGGACAAGTCATAAGAAGCCCTTCCATTTTAGGGCTCGTTGACGTCACCAAG
121 GAGGCGATAAATATCTGTTGATATAATTGGATGTGAGATTCAGTGTTGAAATAGCAGAAC
181 CCTGTCCCTCGCTCCTTGGCAGGGCCCTATTATTTATGCAGGAGCAGAGGCAGCACGCAA
241 TCGAGCTGTCAAGAGAGCGTCAGCTTATTAGGCAAATGCTGCGTGCTTTCTGAAGAGGGT
301 CGACGTTATAAAATCTCACTCCGGGCTCTGGTGTGGAGAAACTCAGAGCCCAAGTACGTT
361 GAGAAACTGAAGAGAAAGGGGAAAGGCAAAGAAAAGGAGAAGAGAAAGGAGAAGAGGAAG
421 AAAACCTGCAGGAGGCATCCTGAGAGAGGTACCTCGCAGAACAACAGTGCGGGCTCACCT
481 GCCAAGGGAGGAGAAGGTAGGCAGCGCTAGATGGGCGCACCAACTTTGTGCTGCCTGAGC
intron
541 TGCTGTGGTGCGCCCCGGAGCCAGCTGCCCAAGTGCTGGAATGCCTGTGCCTATGCATGT
601 ATGTGTGTCGCTAACTGTGCCTTAAAATTCCGATGACAGCCGCGATGTGGCAAAAAGCTA
661 AGTTAGACGGCGGCTGCGGCTGCTCATCTTTATGCACTCAATCCAATCTGCCACTCATTG
721 CTCATAGTCTGTGTAAAGAATGGCTCCCTTATTGTGTCCCATACCCGCAAGCAAAGGGAG
781 CAAGGGCAGGAATGGAGACAGAGAGGAGGAGGTAGTTCTTAATTTGGAAGAAAATGATGT
841 CCGAAAGGGCGATTAGAGCGGCGAATAGCTTAAACCTGTGGCACTTCTCCGGGCTCAAGA
901 AAGTCAGTTTGGGGAAGACTTAGGAAGAGGAGTCAGGGGGTGTCCCTTTTAGGTCTCCAA
961 GGAAGGGTCACCTGCAGGCTCGCACCATATGAGCTTTGCAGGTACATAGCTTCAGCACCG
1021 CGGACAGCGTCACAGAAGCCCAGAGCCTGTCTTGTCTGTGTCTGTCGGATAGGAAGTCCC
1081 GCTACACCTTCCAGCTGAGCTAAACTCTGACCAGTCTTACCTCTCTCCCCCACCTTCTCT
1141 TTCCTCCACCTTGTCTCTCCCTCTACCCCAACCTCGGCTGCAGAGAGCGCCCCTAAAC
intron
1199 ATGCGGCTGCGGCTGCTGGTGTCCGCGGGCATGCTGCTGGTGGCTCTGTCGCCCTGTCTG
MetArgLeuArgLeuLeuValSerAlaGlyMetLeuLeuValAlaLeuSerProCysLeu
1259 CCTTGCAGGGCCCTGCTGAGCAGGGGATCCGTCTCTGGAGCGCCGCGGGCCCCGCAGCCG
ProCysArgAlaLeuLeuSerArgGlySerValSerGlyAlaProArgAlaProGlnPro
1319 TTGAATTTCTTGCAACCGGAGCAGCCCCAGCAACCTCAGCCGATTCTGATCCGCATGGGT
LeuAsnPheLeuGlnProGluGlnProGlnGlnProGlnProIleLeuIleArgMetGly
1379 GAAGAATACTTCCTCCGCCTGGGGAACCTCAACAGAAGTCCCGCTGCTCGGCTGTCCCCC
GluGluTyrPheLeuArgLeuGlyAsnLeuAsnArgSerProAlaAlaArgLeuSerPro
1439 AACTCCACGCCCCTCACCGCGGGTCGCGGCAGCCGCCCCTCGCACGACCAGGCTGCGGCT
AsnSerThrProLeuThrAlaGlyArgGlySerArgProSerHisAspGlnAlaAlaAla
1499 AACTTTTTCCGCGTGTTGCTGCAGCAGCTGCAGATGCCTCAGCGCCCGCTCGACAGCAGC
AsnPhePheArgValLeuLeuGlnGlnLeuGlnMetProGlnArgProLeuAspSerSer
1559 ACGGAGCTGGCGGAACGCGGCGCCGAGGATGCCCTCGGTGGCCACCAGGGGGCGCTGGAG
ThrGluLeuAlaGluArgGlyAlaGluAspAlaLeuGlyGlyHisGlnGlyAlaLeuGlu
1619 AGGGAGAGGCGGTCCGAGGAGCCGCCCATCTCTCTGGATCTCACCTTCCACCTTCTGAGG
ArgGluArgArgSerGluGluProProIleSerLeuAspLeuThrPheHisLeuLeuArg
1679 GAAGTCTTGGAAATGGCCAGGGCAGAGCAGTTAGCTCAGCAAGCTCACAGCAACAGGAAA
GluValLeuGluMetAlaArgAlaGluGlnLeuAlaGlnGlnAlaHisSerAsnArgLys
1739 CTGATGGAGATTATCCGGAAATGAAATGTTGCGCTTGGCCAAAACGATTCTGCATTTAGC
LeuMetGluIleIleGlyLysEnd
1799 ACACAAGTAAAAATAAAAAATTTAAAACACAGTATTCTGTACCATACTGCAGCTCTGATA
1859 TCATTTGTTTATTTTTATATAGCTTGAAGCATAGAAGATGTACAGGGAGAGAGCCTATAT
1919 ACCCCTTAATTAGCATGCACAAAGTGTGTTTCTTTGTAGTAACAAAACAGCGTTATTTGT
1979 ATTGTTCACGCTTAGTTTCTATGTGCAAATAAGTGTCTTTATAGCGATATCTTAAAGAAA
2039 ATGTGGATCCAAGGAGGAAACCTTTAAAAAAGCAGATGGAAGTCACCCAGTTGTTTTTAT
2099 TTGGAGACACAGTGTAAGAGAATTCATTCTTGAGGGGTGGCTAGGACAAAATGTGTAAGC
2159 TCTTTGAATCAACTTTTTCTTGTAAATGTTTCAATAATAAAACATCTTTCTGATCCTTGG
2219 TCAATTTGGTTGTGTCAGAGAACGTTGACTATATTTTTAATAAAATCTGAAAGGTTGTTG
2279 TGGCTTTATTTTTCCTCTTCAGAAATGGACCCAGCACAAGAAAATAAAAACAATACAACA
2339 ATCCCCTCCCCAAAAAACAAACCCTACATTTTATTTTCTCCTAAAAATATGAACATTGTC
2399 ACATAGCTTACACTAGCCCTGCAATTTGTCCTGG

Figure 16. Restriction Map and Nucleotide Sequencing Strategy for the Rat CRH Gene.

A limited restriction enzyme map of the rat CRH gene is diagrammed. The 5' limit of CRH mRNA from hypothalamus (Cap) and exon/intron boundaries are indicated (E/I and I/E). The approximate position of restriction enzyme sites and landmarks are represented by the nucleotide numbers shown (0= the cap site). The scale of the diagram is shown in the lower right portion of the figure. Horizontal arrows shown below the restriction map indicate the direction and extent of nucleotide sequence determinations.

Rat CRH Gene Sequencing Strategy

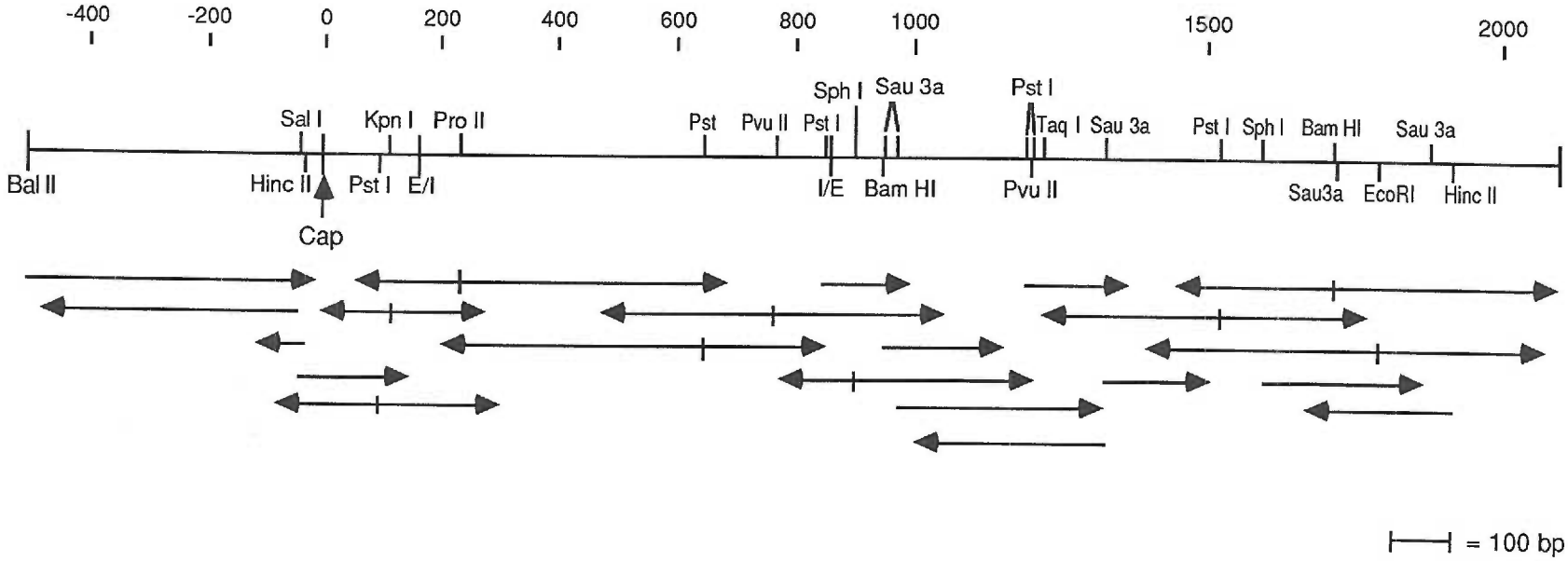
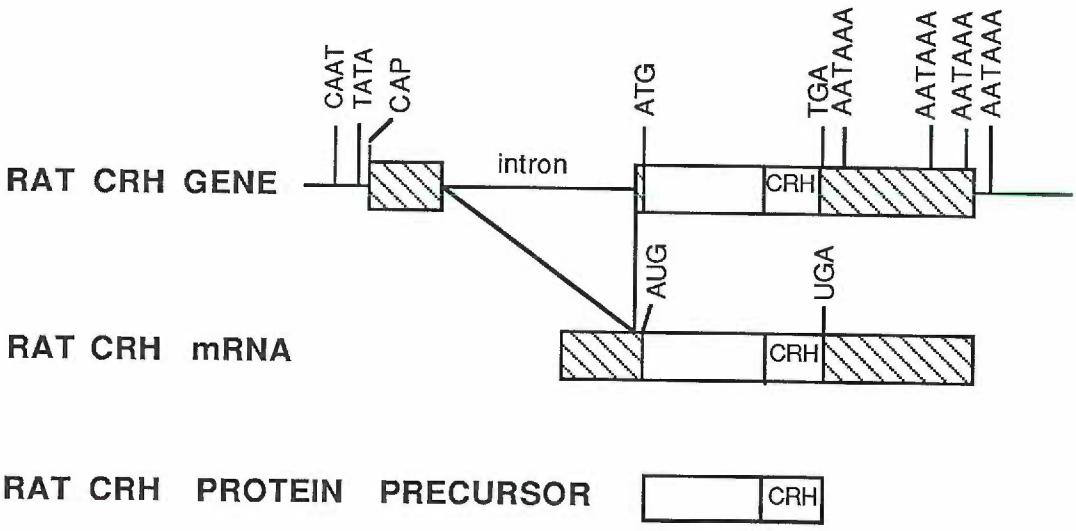


Figure 17. Splicing Scheme of the Rat CRH Gene.

This diagram depicts the splicing pattern of the rat CRH gene in the hypothalamus. This splicing pattern was determined from the sequence of hypothalamic cDNA clones. The rat CRH gene is shown in the top portion of the figure. The TATAA and CAAT boxes, the putative cap site, translation initiation ATG, translation termination TGA, and poly A addition signals (AATAAA) are indicated. The intron is drawn as a line. The rat CRH hypothalamic mRNA is shown in the middle of the figure and demonstrates the removal of a 700 bp intronic region of the gene. The rat CRH protein precursor (187 amino acids) is shown on the bottom of the figure. "CRH" denotes the position of the CRH peptide throughout the figure.


CAAT
TATA
CAP
ATG
TGA
AATAAA
AATAAA
AATAAA
AATAAA
RAT CRH GENE
intron
CRH
AUG
UGA
RAT CRH mRNA
CRH
RAT CRH PROTEIN PRECURSOR
CRH

Within the 5' flanking DNA, TATAA and CAAT boxes are found 23 bp and 60 bp, respectively, 5' to the putative cap site. There are additional TATAA and CAAT boxes found at 191 bp and 268 bp, repsectively, 5' to the putative cap sites. The occurence of multiple TATAA and CAAT boxes (which are both precisely conserved from rat to human) may indicate that this gene contains multiple Pol II transcription initiation sites, thereby generating mRNA with different 5' untranslated regions. This capacity to generate different mRNAs may provide another level of regulation to the CRH gene. These mRNAs may have varied stabilities, rates of transport to the cytoplasm, or rates of translatability.

The 3' untranslated region of the gene has four poly A addition signals (AATAAA) at positions 1810, 2194, 2257, and 2321. All cDNA clones isolated from the hypothalamus used the poly A addition signals at 2194 and 2257. It is interesting to note that the post-transcriptional regulation of the cFos gene appears to involve a AU-rich nucleotide sequences found in the 3'untranslated region of the c-fos mRNA that effects stability (99). It is suggested by these authors that this mRNA sequence may be recognized by a cytoplasmic RNase which subsequently degrades this mRNA and accounts for the 9-10 minute $t_{1/2}$ of this mRNA in vitro. Several AU-rich sequences are found within the 3' untranslated region of the CRH gene, some of which are placed between poly A addition signals. Although no data exists on the stability of the rat CRH mRNA, selective utilization of poly A addition signals may provide a post-transcriptional mechanism

to alter the stability of CRH mRNA in vivo.

Figure 18 highlights the regions of homology between the rat and human CRH genes at the nucleic acid level. The highest degree of homology (94 %) exists in two main regions: the 5' flanking DNA and the CRH peptide-encoding region of the gene. Several other genomic regions share slightly lower degrees of homology (80-90 %). The first of these sequences is represented by the protein coding region outside the CRH peptide exhibiting approximately 84 % homology. The second region represents the DNA sequences coding for the 3' untranslated regions of the mRNA which are approximately 81 % homologous. The 5' untranslated region, the 3' flanking DNA, and the intron are the most divergent sequences. However, the introns are still relatively homologous, showing a 71 % homology at the nucleic acid level. The high level of homology between the rat and human genes suggests that these two genes have been highly conserved though evolution.

The extremely high level of homology found in the CRH peptide-encoding region was expected due to a previous report which demonstrated that the CRH amino acid sequences between these two species were identical and that this neurohormone is very important to the physiology of the organism. However, the 94 % homology found between the 5' flanking regions of the rat and human genes was quite striking. The nucleotide sequence homology of the first 350 bp of the 5' flanking DNA region of the rat and human CRH genes is shown in figure 19. In many genes, DNA sequence elements responsible for

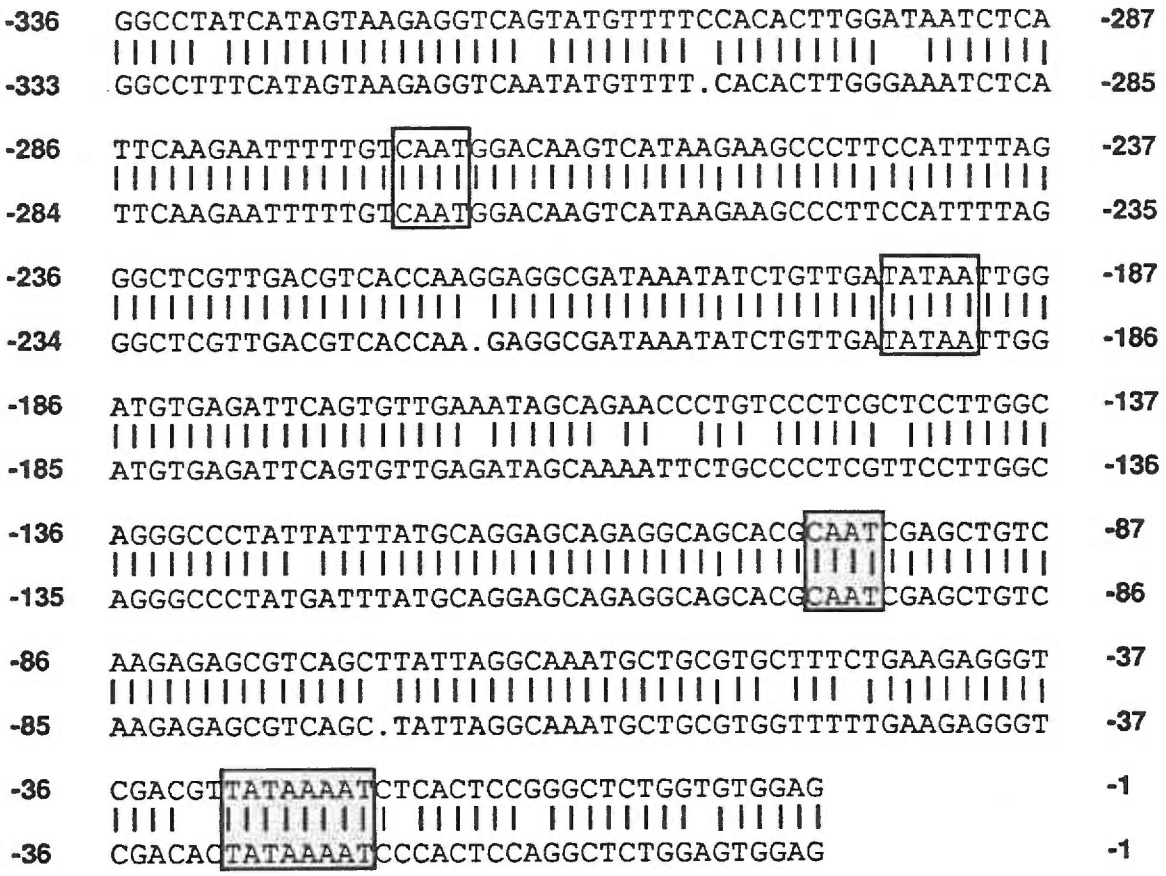
Figure 18. Nucleic Acid Sequence Homology between the Human and Rat proCRH Genes.

A schematic representation of the rat proCRH gene is shown at the top. The exons are shown as blocks, and the intron by a line. The TATAA and CAAT sequence, putative cap site, translation initiation ATG, translation terminator TGA, and poly A addition signals (AATAAA) are indicated. The location of the CRH peptide is indicated by CRH. Percentage homology is as noted in the figure.

> 90 %
80-90 %
70-80 %
-CAAT
-TATA
CAP
intron
-ATG
CRH
-TGA
-AATAAA
-AATAAA
-AATAAA
-AATAAA

Figure 19. Nucleotide Sequence of the 5' Flanking DNA of the Rat and Human CRH Genes.

The nucleotide sequences of the 5' flanking DNA from the rat (upper sequence, -336 bp) and human (lower sequence, -333 bp) CRH genes are shown. The vertical lines denote conserved nucleotides. The major TATAA and CAAT boxes are outlined and shaded (-23 to -30, and -93 to -96, respectively, rat gene numbers). The position of additional TATAA and CAAT boxes are outlined in open boxes (-195 to -191, and -271 to -268, respectively, rat gene numbers). Nucleotide -1 corresponds to the first nucleotide 5' to the putative CRH mRNA cap site. This 5' flanking region of the rat CRH gene displays a 94.6% homology to the same region of the human gene.


-336 GGCCTATCATAGTAAGAGGTCAGTATGTTTTCCACACTTGGATAATCTCA -287
-333 GGCCTTTCATAGTAAGAGGTCAATATGTTTT.CACACTTGGGAAATCTCA -285
-286 TTCAAGAATTTTTGTCAATGGACAAGTCATAAGAAGCCCTTCCATTTTAG -237
-284 TTCAAGAATTTTTGTCAATGGACAAGTCATAAGAAGCCCTTCCATTTTAG -235
-236 GGCTCGTTGACGTCACCAAGGAGGCGATAAATATCTGTTGATATAATTGG -187
-234 GGCTCGTTGACGTCACCAA.GAGGCGATAAATATCTGTTGATATAATTGG -186
-186 ATGTGAGATTCAGTGTTGAAATAGCAGAACCCTGTCCCTCGCTCCTTGGC -137
-185 ATGTGAGATTCAGTGTTGAGATAGCAAAATTCTGCCCCTCGTTCCTTGGC -136
-136 AGGGCCCTATTATTTATGCAGGAGCAGAGGCAGCACGCAATCGAGCTGTC -87
-135 AGGGCCCTATGATTTATGCAGGAGCAGAGGCAGCACGCAATCGAGCTGTC -86
-86 AAGAGAGCGTCAGCTTATTAGGCAAATGCTGCGTGCTTTCTGAAGAGGGT -37
-85 AAGAGAGCGTCAGC.TATTAGGCAAATGCTGCGTGGTTTTTGAAGAGGGT -37
-36 CGACGTTATAAAATCTCACTCCGGGCTCTGGTGTGGAG -1
-36 CGACACTATAAAATCCCACTCCAGGCTCTGGAGTGGAG -1

glucocorticoid regulation, tissue specific expression, cAMP and phorbol ester regulation, and enhancer activity have all been localized to 5' flanking DNA sequences. These highly conserved 5' flanking sequences may therefore represent DNA regulatory elements for CRH gene expression. Additionally, this conserved homology in this region of the two genes may provide significant transcriptional regulatory information on both the rat and human genes. It is interesting to speculate that since the DNA sequences in this region of the gene are quite similar, that the DNA-binding proteins which may regulate the transcription of the gene may also be quite similar. To date, we have also isolated and sequenced a portion of the human CRH gene in the 5' flanking region to identify the 5' limit of this >90 % homology. It appears that this high level of homology drops to approximately 75 % at the -380 bp position. However, there are small regions (30 - 60 nucleotides) of increased homology found 5' to -380 but these regions appear to be separated by regions of low levels of homology. We are pursuing the possibility that these additional regions of increased homology, more distantly separated between the rat and human genes, may also represent common DNA control elements involved in the transcriptional regulation of both the rat and human CRH gene.

RESULTS AND DISCUSSION

Chapter 4: Structural Analysis of the mRNA encoding the CRH precursor in the Testis.

Summary

Immunocytochemical techniques have suggested that the rat testis produces CRH-like material in Leydig cells and a population of germ cells. Northern blot analysis was performed on mRNA extracted from adult rat testis (chapter 2) detected a CRH transcript. This RNA transcript was much larger than the CRH mRNA detected in brain regions. The nature of this size variation was analyzed in this chapter through the use of RNase protection and Northern blotting techniques. The testicular CRH mRNA was shown to lack significant portions of Exon I, the intron and the 5' end of Exon II. The testicular CRH mRNA was demonstrated to contain a region with a high degree of homology to a portion of Exon II. The potential functional significance of these results is discussed.

Introduction

Northern blot data presented in Chapter 2 demonstrated the presence of a transcript in rat testis which appears to be highly homologous to the rat CRH cRNA probe (this transcript will be referred to as a testicular CRH mRNA although this remains to be conclusively demonstrated). In support of this observation, Audhya et al. have isolated and sequenced CRH peptide from ovine testis (57).

Additionally, they have demonstrated that this CRH immunoreactivity appears to be localized to the Leydig cells (the source of gonadal steroids) by immunohistochemistry. Yoon et al. extended this observation by examining CRH immunoreactivity in the rat testis (56). Through immunocytochemistry, they found CRH immunoreactivity in the seminiferous tubules, Leydig cells and in the spermatozoa of the epididymus. Interestingly, Dave et al. has demonstrated the presence of high affinity CRH receptors in membranes prepared from whole rat testis (100). Taken together, these results suggest that CRH may function as an important modulator of testicular physiology by autocrine or paracrine mechanisms.

The testicular CRH RNA transcript detected by Northern blot analysis was shown to be approximately 500-700 nucleotides larger than the CRH transcripts detected in the brain. Potential sources of RNA transcript size variation include altered points of trancriptional initiation, alternate splicing, and/or variable utilization of poly A addition signals. These alterations in RNA structure could regulate the rate of transport from the nucleus to the cytoplasm, stability, and/or translatability of the RNA's. Additionally, if alternate splicing is involved, a different protein could be generated as a result of the altered RNA structure (sequence). Variable sizes of RNA transcripts have been reported for other neuropeptide precursor genes, including the proenkephalin and prodynorphin genes, particularly in rat testis (101,94). The nature of the size variation of the CRH transcript detected in the rat testis will be pursued in this

chapter by RNase protection assays and Northern blots using various genomic fragments as hybridization probes.

Results and Discussion

1. RNase Protection Assays

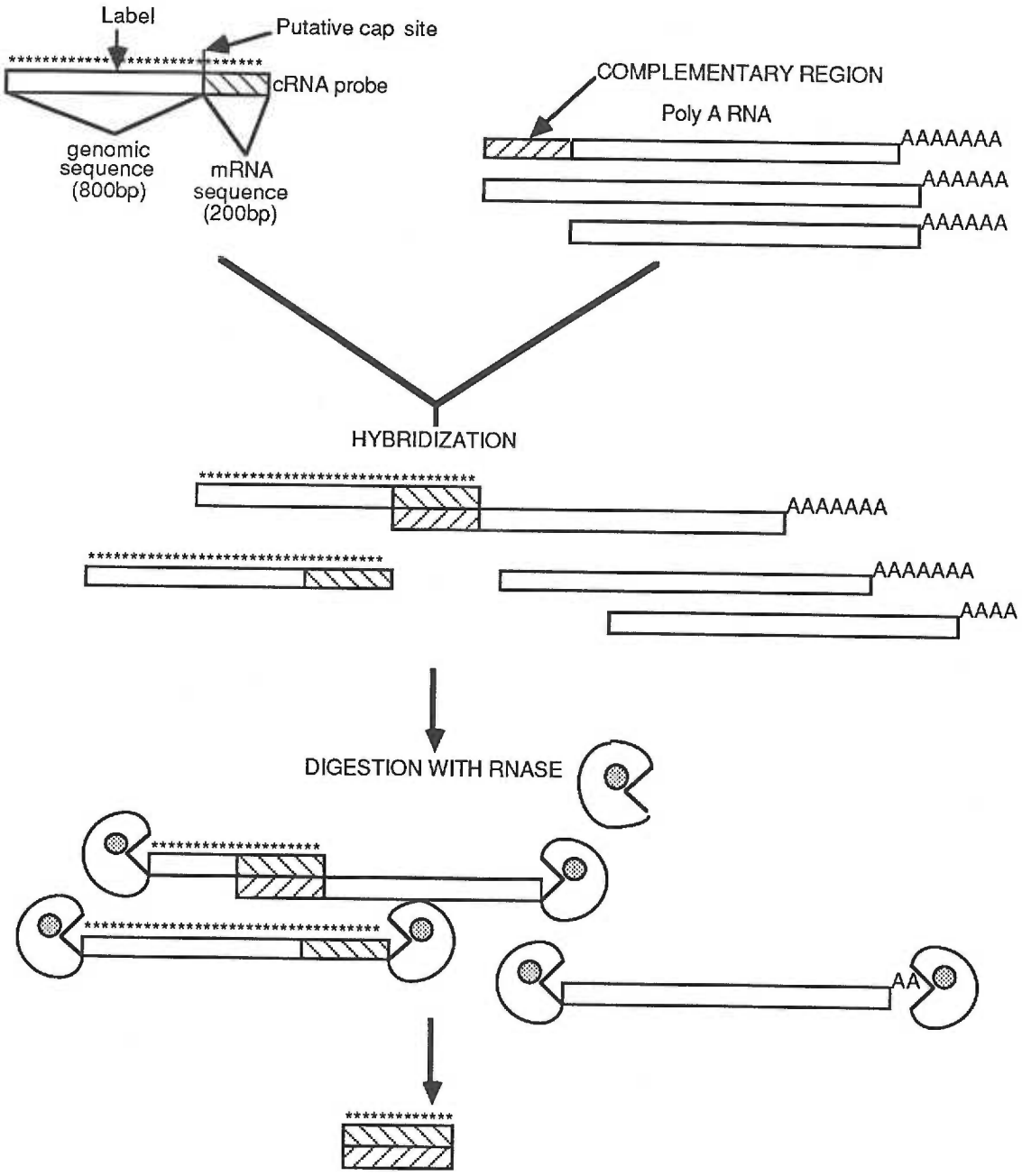
RNase protection assays are used to analyze, in detail, the structure and quantity of specific mRNA's. For these assays, genomic DNA fragments must be isolated which span potential regions of transcript variation. These fragments are subcloned into cRNA-generating plasmids and radioactive single stranded cRNA is synthesized. This radiolabelled cRNA is then hybridized to poly A RNA from various tissues (67). The hybridization reactions are treated with single-stranded ribonucleases (RNases A and T1) to remove excess probe and single stranded ends of hybrids, leaving intact double-stranded RNA fragments of probe annealed to complementary sequences in the sample RNA. These complementary hybrids are then size separated on denaturing polyacrylamide/urea gels, dried and autoradiographed. This scheme is diagramed in figure 20.

RNase protection assays have been used to define the cap sites and the 3' intron boundary of the CRH mRNA in several brain regions (brainstem, hypothalamus, and cerebral cortex) which contained the 1400 nucleotide CRH transcript. These results are compared with those obtained with testis mRNA where the CRH transcript is much larger in size in an attempt to localize the differences in the testicular CRH transcript.

Figure 20. RNase Protection Assay For Mapping RNA Structures.

cRNA probes which correspond to genomic regions of potential mRNA variation (in this figure the cap site is determined) are radiolabelled. The genomic sequence (open box), putative mRNA sequence (hatched box) and putative cap site are labelled (top left portion of the figure). This cRNA probe is mixed and hybridized (Hybridization) with sample mRNA containing mRNA with complementary regions. The hybridization reactions are treated with single-stranded ribonucleases (RNases A and T1) (Packman symbols) to remove excess probe and single stranded ends of hybrids, leaving intact double-stranded RNA fragments of probe annealed to complementary sequences in the sample RNA (two oppositely hatched boxes). These complementary hybrids are then size separated on denaturing polyacrylamide/urea gels, dried and autoradiographed.

RNASE PROTECTION ASSAY



Size fractionate protected fragments on

Polyacrylamide gels &

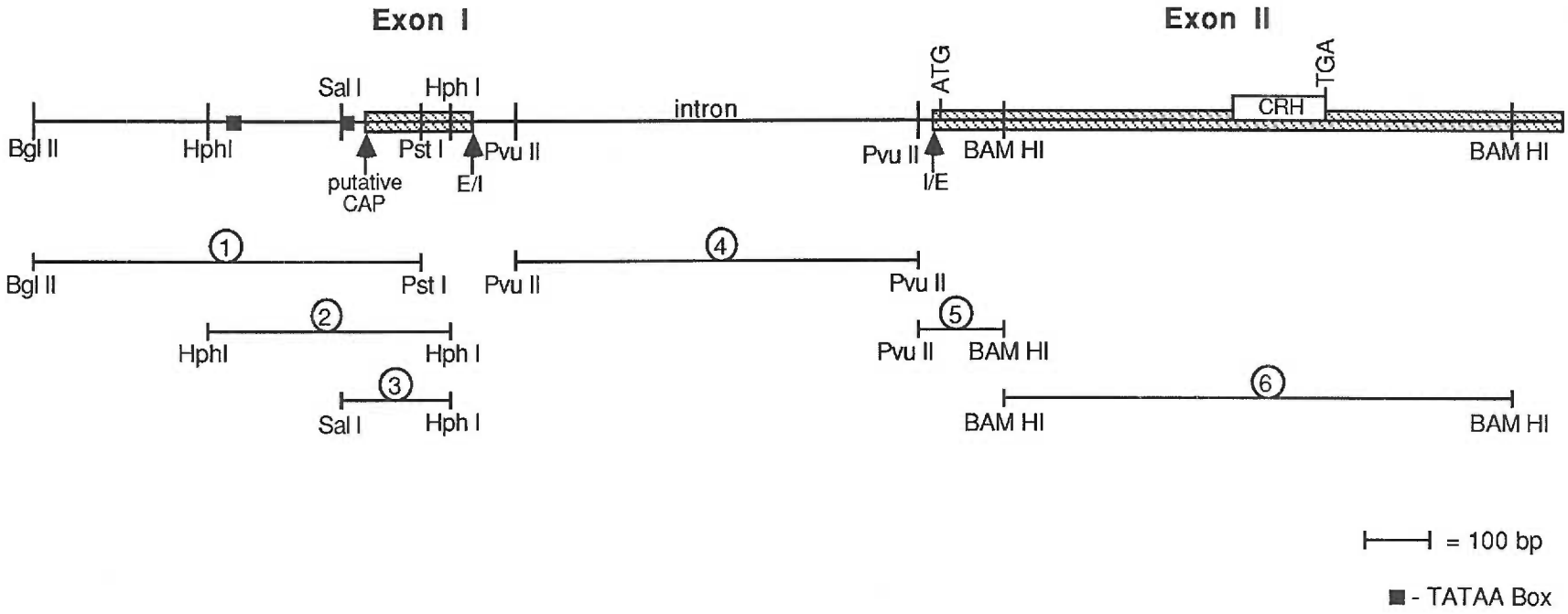
Autoradiography

The first potential point of variation to be analyzed between the CNS and testicular CRH mRNAs was the cap site which defined the point of transcriptional initiation. The promoter sequence of the rat CRH gene was previously determined and described in Chapter 3. Within this DNA sequence, at least two TATAA boxes (DNA sequences shown to be involved in fixing the point of transcriptional initiation by RNA Polymerase II) were identified. Additionally, a hypothalamic cDNA sequence was published whose 5' end maps 28 nucleotides downstream from the most 3' TATAA box. The position of the 5' end of this cDNA will be referred to in this chapter as the putative cap site. In order to determine whether one or both of these TATAA boxes were active in vivo, genomic DNA fragments which span both of these TATAA boxes were utilized in RNase protection cap site determination experiments. The first of these fragments was a 369 bp Hph I fragment which would yield a 128 bp protected hybrid if the putative cap site was indeed the endogenous point of transcriptional initiation. A second genomic fragment was used to confirm the Hph results. This second fragment was a Bgl II - Pst I fragment which would yield a 92 bp protected hybrid, again based upon the assignment of the putative cap site. These fragments are labelled #2 and #1, respectively, in figure 21 which demonstrates their relative positions on the CRH gene.

The autoradiogram shown in figure 22 depicts the cap site results of these RNase protection assays. In this assay, both the Hph I and Bgl II/ Pst I cRNA probes were utilized. Many protected

Figure 21. Region-Specific DNA Fragments from the Rat CRH Gene.

A schematic diagram of the rat CRH gene is shown on the top portion of the figure with several relevant restriction enzyme sites indicated. The putative cap site (putative CAP) and exon/intron (E/I or I/E) boundaries are indicated. Exon I and Exon II are indicated by hatched boxes. The position of the 41-amino acid CRH peptide is indicate by an open box. The intron is shown as a line between Exon I and Exon II. The numbered region-specific DNA fragments were isolated from the gene and subcloned into plasmids for cRNA production.


Exon I
Exon II
Sal I
Hph I
intron
ATG
TGA
CRH
Bgl II
HphI
Pst I
Pvu II
putative CAP
E/I
Pvu II
I/E
BAM HI
BAM HI
1
Bgl II
Pst I
4
Pvu II
Pvu II
2
HphI
Hph I
5
Pvu II
BAM HI
3
Sal I
Hph I
6
BAM HI
BAM HI
= 100 bp
- TATAA Box

fragments were detected. However, a major protected band was detected with the Hph I probe at 130 bp which appears to represent >50 % of the protected fragments. This protected fragment maps to nucleotide # 337 of the rat CRH gene sequence (see figure 15, chapter 3) which is two nucleotides 5' to the position of the putative cap site as previously discussed. Interestingly, several other protected bands were evident and appear to represent additional points of transcriptional initiation. These additional protected fragments appear with both the Hph I and Bgl II/ Pst I cRNA probes as shown in figure 23 (which is a longer exposure of the autoradiogram represented in figure 22).

The position of these protected bands as mapped with both probes correspond to very similar positions on the rat CRH gene. The mapping pattern of these protected fragments is diagramed in figure 24. Here it can be seen that there appear to be as many as 10 potential points of transcriptional initiation, only some of which are 20 - 40 nucleotides downstream from a TATAA box (although there are other AT rich regions found in this 5' flanking DNA sequence).

In the data described here, there appear to be many more start sites for transcription than there are TATAA boxes. A possible explanation to account for these findings would suggest that these additional cap sites may be TATAA box independent. Alternatively, once one TATAA box is recognized by RNA Polymerase II then this entire region of the gene may be "open" for transcriptional activation. The ability to make available multiple areas for transcriptional

Figure 22. RNase Mapping of the Points of Transcriptional Initiation of the Rat CRH Gene.

The results of RNase protection assays performed with two genomic fragments (#1 and #2 shown in figure 21) are shown. Fragment #2 corresponds to an Hph I fragment derived from the 5' region of the rat CRH gene including a portion of Exon I, and would yield a protected RNA hybrid of approximately 130 bp if the putative cap site (defined in the text) was utilized. Fragment #1 corresponds to a Bgl II/Pst I fragment derived from the 5' region of the rat CRH gene including 92 bp of Exon I, and would yield a protected RNA hybrid of approximately 92 bp if the putative cap site was utilized. The following poly A RNA samples (amounts noted) were analyzed with fragment #2: P= whole pituitary, 2.0μg; T= testis (5.2μg), B= brainstem (medulla/pons) (2.0μg), C= cerebral cortex (2.0μg), H= hypothalmus (2.0μg), A= adrenal gland (5.0μg), Y= yeast total RNA (5.0μg). The following poly A RNA samples (amounts noted) were analyzed with fragment #1: T= testis (5.0μg), B= brainstem (medulla/pons) (2.0μg), C= cerebral cortex (2.0μg), Y= yeast total RNA (5.0μg). The autoradiogram was exposed for 24 hours at room temperature. DNA sequencing reactions were electrophoresed with the RNase protection samples generating the size markers (in nucleotides) on the left edge of the photograph.

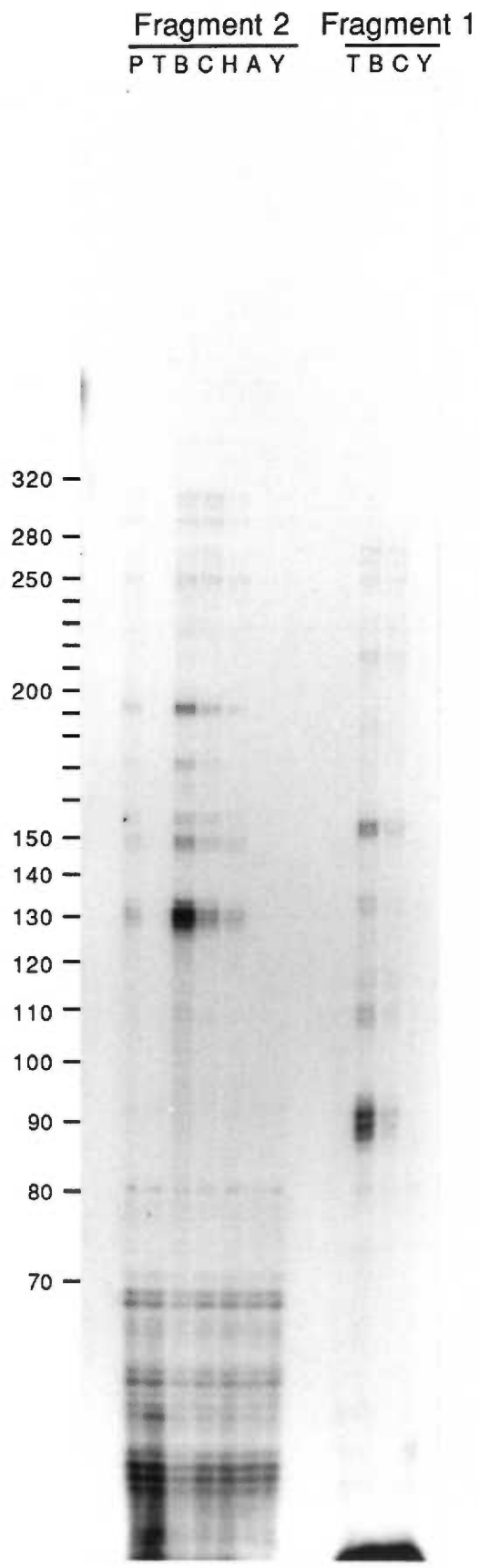

Fragment 2
P T B C H A Y
Fragment 1
T B C Y
320
280
250
200
150
140
130
120
110
100
90
80
70

Figure 23. RNase Mapping of Multiple Points of Transcriptional Initiation of the Rat CRH Gene.

The results of RNase protection assays performed with two genomic fragments (#1 and #2 shown in figure 21) are shown. Fragment #2 corresponds to an Hph I fragment derived from the 5' region of the rat CRH gene including a portion of Exon I, and would yield a protected RNA hybrid of approximately 130 bp if the putative cap site (defined in the text) was utilized. Fragment #1 corresponds to a Bgl II/Pst I fragment derived from the 5' region of the rat CRH gene including 92 bp of Exon I, and would thus yield a protected RNA hybrid of approximately 92 bp if the putative cap site was utilized. The following poly A RNA samples (amounts noted) were analyzed with fragment #2: P= whole pituitary, 2.0μg; T= testis (5.2μg), B= brainstem (medulla/pons) (2.0μg), C= cerebral cortex (2.0μg), H= hypothalmus (2.0μg), A= adrenal gland (5.0μg), Y= yeast total RNA (5.0μg). The following poly A RNA samples (amounts noted) were analyzed with fragment #1: T= testis (5.0μg), B= brainstem (medulla/pons) (2.0μg), C= cerebral cortex (2.0μg), Y= yeast total RNA (5.0μg). The autoradiogram was exposed for 90 hours at -70°C with an intensifying screen. DNA sequencing reactions were electrophoresed with the RNase protection samples generating the size markers (in nucleotides) on the left edge of the photograph. The lines drawn between the two photographs indicate cap sites mapped with the Hph I and Bgl II/Pst I probes which correspond to similar positions in the 5' flanking DNA of the rat CRH gene.

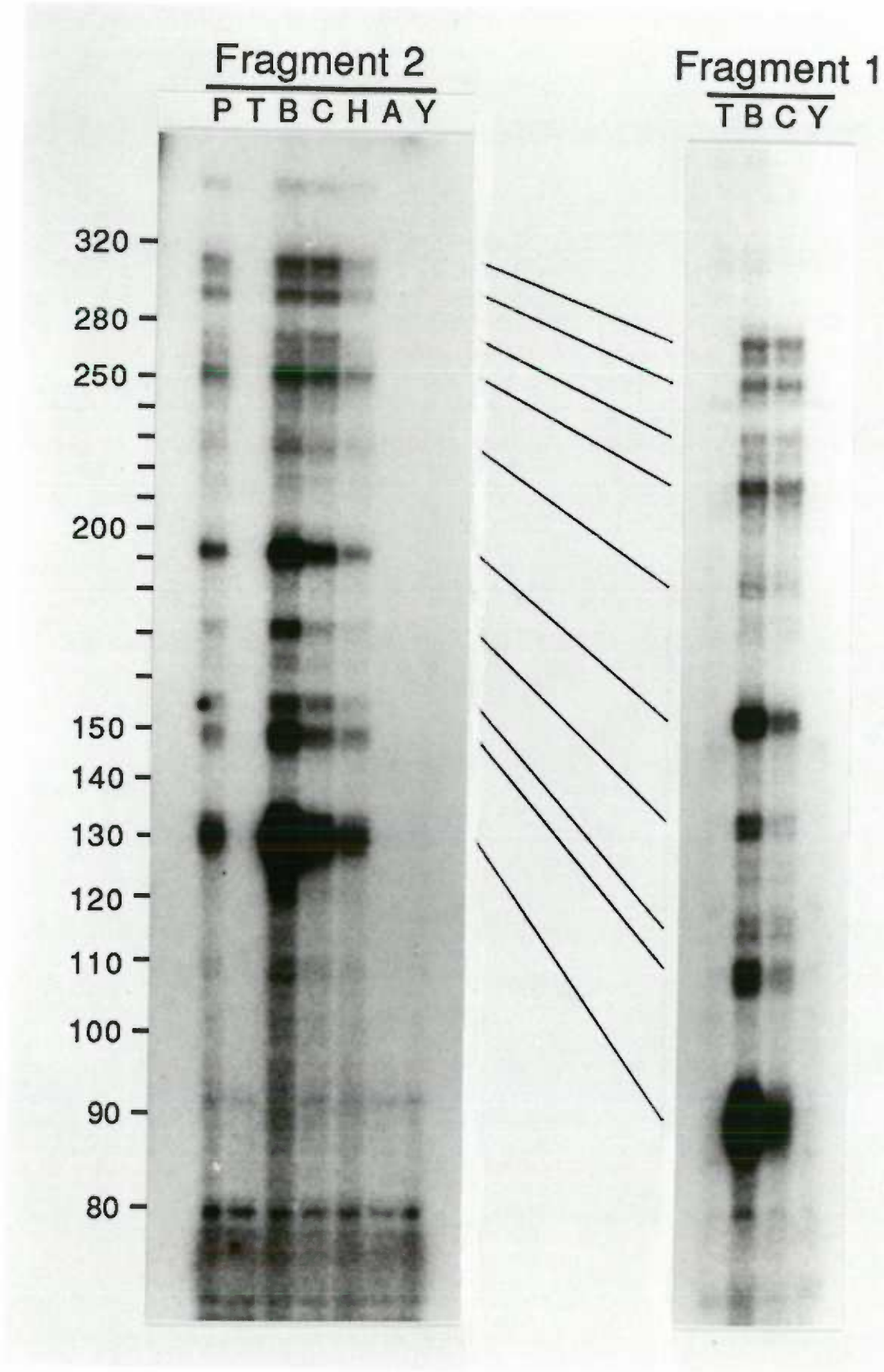
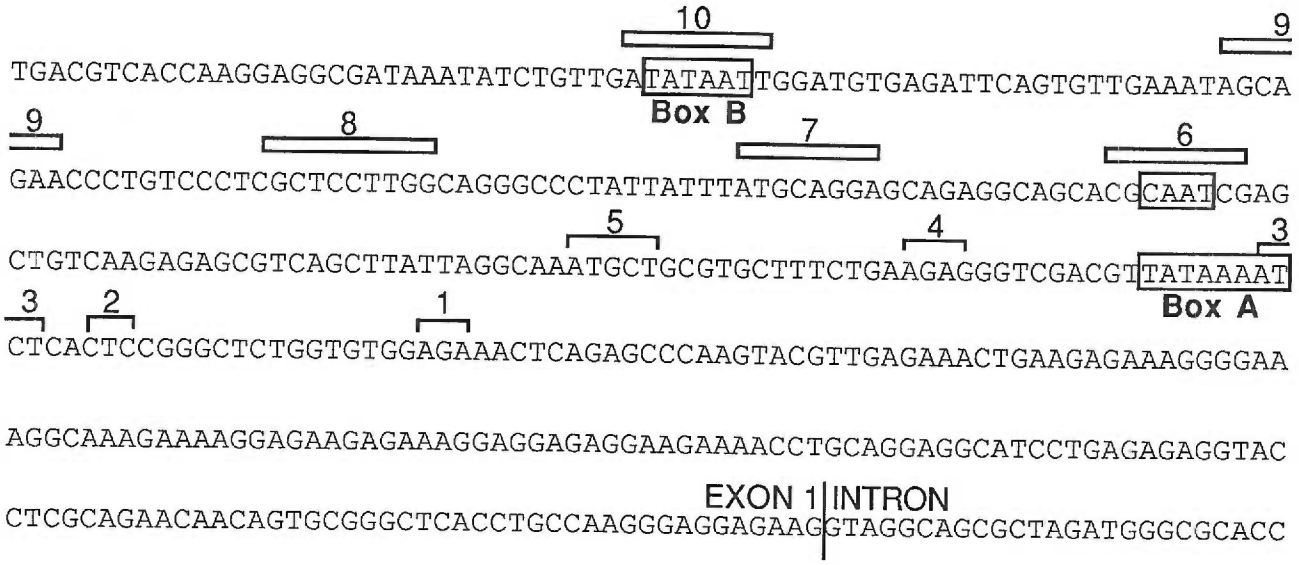

Fragment 2
P T B C H A Y
Fragment 1
T B C Y
320
280
250
200
150
140
130
120
110
100
90
80

Figure 24. Points of Transcriptional Initiation of the Rat CRH Gene.

The nucleotide sequence of the rat CRH 5' flanking region of the gene and 5' untranslated region of the CRH mRNA are shown along with a portion of the intron. TATAA sequences are boxed; Box A represents the major promoter and Box B may represent the minor promoter. The Exon I/Intron boundary is marked by a vertical line. Cap sites as determined by RNase protection assays are indicated by brackets (precise, within 1-2 bases) or open boxes (less precise, within 5 bases) above the nucleotide sequence. The cap sites are arbitrarily numbered sequencially from 3' to 5'.


10
9
TGACGTCACCAAGGAGGCGATAAATATCTGTTGATATAATTGGATGTGAGATTCAGTGTTGAAATAGCA
Box B
9
8
7
6
GAACCCTGTCCCTCGCTCCTTGGCAGGGCCCTATTATTTATGCAGGAGCAGAGGCAGCACGCAATCGAG
5
4
3
CTGTCAAGAGAGCGTCAGCTTATTAGGCAAATGCTGCGTGCTTTCTGAAGAGGGTCGACGTTATAAAAT
3
2
1
Box A
CTCACTCCGGGCTCTGGTGTGGAGAAACTCAGAGCCCAAGTACGTTGAGAAACTGAAGAGAAAGGGGAA
AGGCAAAGAAAAGGAGAAGAGAAAGGAGGAGAGGAAGAAAACCTGCAGGAGGCATCCTGAGAGAGGTAC
EXON 1
INTRON
CTCGCAGAACAACAGTGCGGGCTCACCTGCCAAGGGAGGAGAAGGTAGGCAGCGCTAGATGGGCGCACC

activation may be a form of amplification permitting a greater number of transcriptional complexes to position themselves in the 5' region of this gene to respond to increased cellular demands for more RNA from this gene locus. This mechanism seems possible due to the close proximity of the two TATAA box sequences (only 160 bp apart).

The major bands protected with both the Hph I and Bgl II/Pst cRNA probes map to a consensus sequence for the initiation of transcription which usually begin with an A residue found 20 - 30 nucleotides downstream from a TATAA box. Therefore, greater than 50 % of the transcription does appear to originate from this position on the rat CRH gene. The fact that multiple sites of transcriptional initiation are found in the rat CRH gene is not unusual. Multiple cap sites have been identified in other eukaryotic genes. For example, in the c-erbB-2 protooncogene, multiple cap sites, mapped by S1 mapping techniques, were identified and four of these were 5' to a TATAA box sequence (102). It was noted in this report that two DNA sequences, homologous with the consensus sequence for Sp 1 binding, were identified approximately 100 and 155 bp upstream (5') of the TATA box. This transcription factor was previously shown to bind to tandemly repeated GC boxes of the SV40 early promoter and stimulate transcription in vitro (103,104). The authors suggest that these Sp 1 binding sites may play a role in the utilization of cap sites found 5' to the TATAA box in the c-erbB-2 protooncogene. Interestingly, an Sp 1 binding site is found in the 5' flanking region of the rat CRH gene, located approximately 427 nucleotides 5' to the major cap site. It is

therefore possible that this Sp 1 binding sequence may also play a role in the transcriptional regulation of the rat CRH gene.

An additional possibility could be that some of these protected bands are due to artifacts inherent in the RNase protection assay. Although this possibility can not be completely discounted, RNA from several tissues which, by Northern blot analysis, do not contain CRH mRNA was assayed as negative controls. These RNAs did not demonstrate protected fragments which could result from some secondary structure or non-specific binding of the CRH cRNA probes. These results imply that only tissues which produce CRH mRNA demonstrate protected fragments. Primer extension experiments could address this question.

Also shown in figures 22 and 23 is the lack of detectable protected fragments in the testis lanes. For many months, I believed that this was due to the low abundance of CRH mRNA in testis, but Northern blot analysis does not support this notion. When equal quantities of poly (A) RNA from hypothalamus and testis are electrophoresed in adjacent lanes on a Northern blot and probed with a full length cRNA probe, the signal intensity in the testis lane is certainly equal to and is usually stonger than the hypothalamic signal. Therefore, this RNase protection data suggests that the testicular CRH mRNA initiates at a position on the rat CRH gene that is not included in either the Hph I or the Bgl II/Pst I probes.

The next potential point of variation tested by RNase protection assays was the position of the intron/exon II boundary. For these

studies, a Pvu II/ BamH I genomic fragment (fragment #5, figure 21) was isolated and subcloned into cRNA producing plasmids. This fragment spanned this intron/exon II boundary and produced a cRNA probe of 218 nucleotides which would yield a protected fragment of approximately 98 bp specific to exon II in hypothalamic samples. The autoradiogram of this assay analyzing brainstem, hypothalamus, cerebral cortex, heart and testis RNA is shown in figure 25.

Prominent protected bands approximately 98 bp in size were observed in all samples except heart and testis. This protected fragment size places the intron/exon II boundary approximately 14 nucleotides upstream from the point of translational initiation. A few high molecular weight bands were observed in the testis lanes, but these were judged to be nonspecific due to their presence in the heart RNA lanes. Again, testis CRH mRNA appeared undetectable using the intron/exon II probe in this assay.

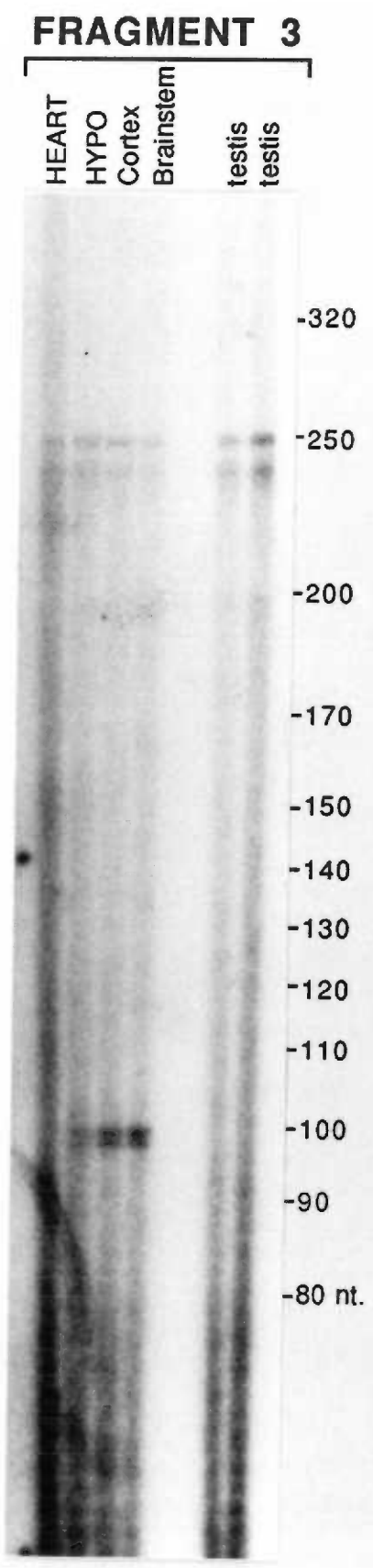
The results of both sets of RNase protection assays suggest that either the CRH mRNA in testis does not contain sequences found in exon I or the 5' end of exon II, or that the Northern blot data is somehow artifactual and the cRNA probes are detecting a similar but unrelated mRNA in testis which is RNase sensitive.

2. Northern Blot Analysis

From the RNase protection data it appears that the CRH mRNA in testis is significantly different in structure than the hypothalamic mRNA. The only data which suggested that CRH mRNA was found in this tissue was the original Northern blot analysis of CRH gene

Figure 25. RNase Mapping of the Intron/Exon II Boundary of the Rat CRH Gene.

The results of RNase protection assays performed with genomic fragment #3 (shown in figure 21) are shown. Fragment #3 corresponds to a Pvu II/BamH I fragment spanning 89 bp of the intron of the rat CRH gene and 98 bp of Exon II. The following poly A RNA samples (amounts noted) were analyzed with fragment #3: heart (2.0μg), hypothalamus (2.0μg), cerebral cortex (2.0μg), brainstem (medulla/pons) (2.0μg), testis (2.5μg), testis (5.0μg). The autoradiogram was exposed for 72 hours at -70°C with an intensifying screen. DNA sequencing reactions were electrophoresed with the RNase protection samples generating the size markers (nt= nucleotides) on the right edge of the photograph.


FRAGMENT 3
HEART
HYPO
Cortex
Brainstem
testis
testis
-320
-250
-200
-170
-150
-140
-130
-120
-110
-100
-90
-80 nt.

expression in peripheral tissues. The RNA samples on this blot were hybridized with the full length cRNA probe. One hypothesis to explain the size variation of the testicular CRH mRNA which is consistant with the RNase protection and Northern blot data is that this testicular CRH mRNA results from transcription initiated at a cap site more distant from those tested in the RNase protection assays and alternatively spliced with the protein portion of Exon II. Therefore, a promoter element either more 5' or 3' than those tested could be utilized.

In order to confirm the original Northern blot analysis and determine which portion of the full length CRH cDNA sequence was hybridizing to this testicular mRNA, Northern blot analysis was performed with various cRNA probes derived from different fragments of the rat CRH gene.

RNase protection assays have suggested that most or all of Exon I is absent from the testicular CRH mRNA. To confirm this result by Northern analysis, cRNA probes produced from a Sal I/Hph I fragment from the Exon I region (fragment # 3, figure 21) were used on blots containing both hypothalamic and testicular poly A RNA. The results from this analysis are shown on the left portion of figure 26. As can be seen in lane 2, the Sal I/Hph I cRNA probe hybridizes to a hypothalamic mRNA at approximately 1400 nucleotides but no detectable band is seen in the testis lane (lane 1). To confirm that testis mRNA is present in this lane, the same blot was rehybridized with the BamH I cRNA probe which is specific to Exon II (fragment #6,

Figure 26. Northern Blot Analysis of Testicular and Hypothalamic Poly A RNA Using Exon I- and Exon II-Specific cRNA Probes.

Poly A RNA isolated from rat testis (lane 1) and hypothalami (lane 2) were analyzed by Northern blots. A Sal I/ Hph I genomic fragment (corresponding to a portion of Exon I) and a BamH I cDNA fragment (corresponding to a portion of Exon II) were subcloned into plasmids to produce cRNA probes specific to these regions. The position of these fragments in relationship to the rat CRH gene is diagrammed in figure 21. The autoradiogram on the left, resulting from the hybridization with Exon I cRNA probe, was exposed for 48 hours at -70°C with an intensifying screen. This blot was then incubated in sterile dH_2O at 100°C for 10 minutes to remove the hybridized Exon I cRNA probe. The blot was then autoradiographed for 24 hours at -70°C with an intensifying screen. This blot did not demonstrate any detectable signal after this exposure. The autoradiogram on the right, resulting from the re-hybridization of the same Northern blot with Exon II cRNA probe, was exposed for 24 hours at -70°C with an intensifying screen. The positions of denatured RNA molecular weight markers form the RNA Ladder (BRL) are indicated between the two autoradiograms.

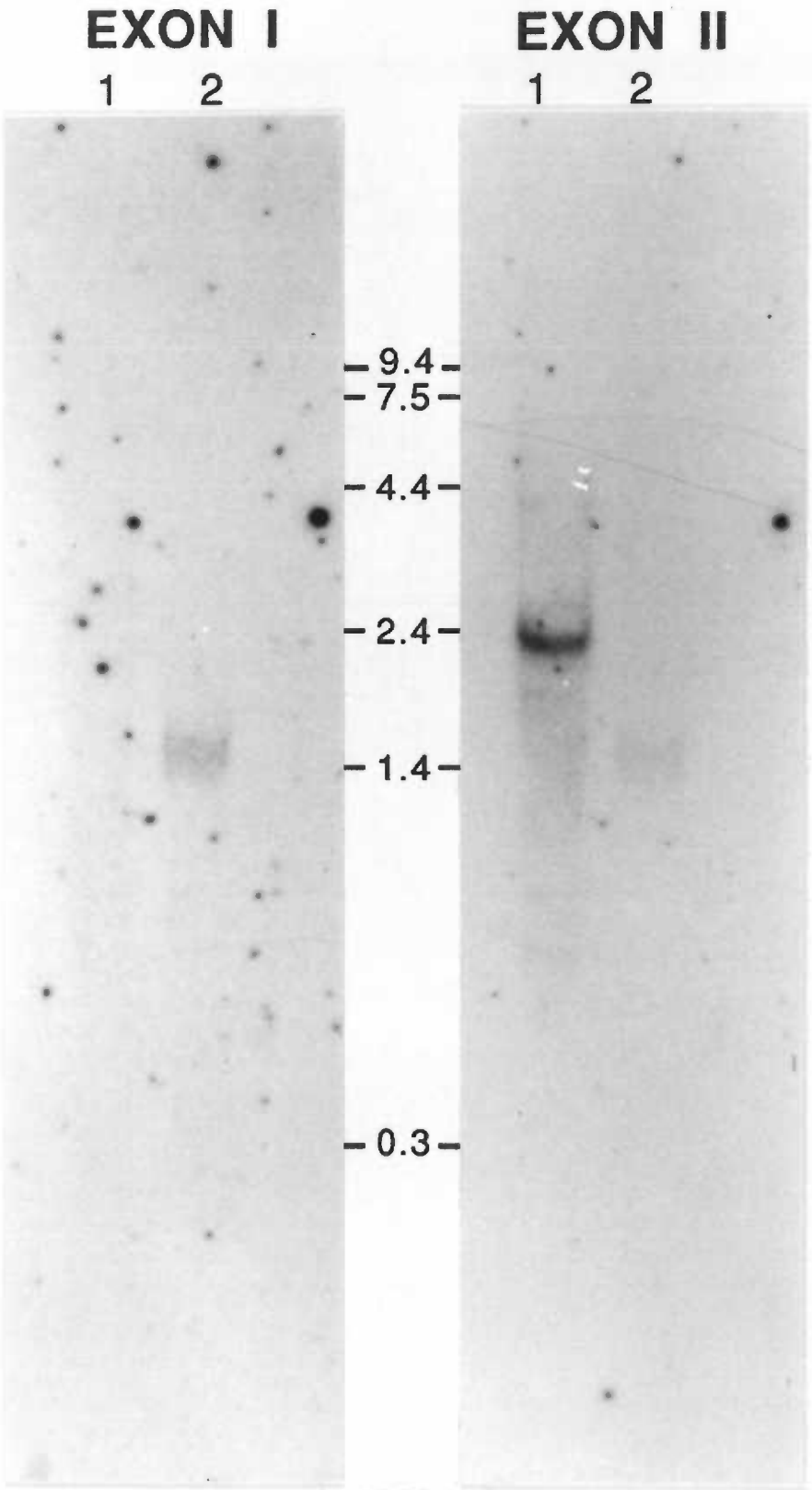

EXON I
1
2
EXON II
1
2
9.4
7.5
4.4
2.4
1.4
0.3
(x 1000 nt.)

figure 21) and is shown on the right in figure 26. Both hypothalamic and testicular CRH transcripts are apparent in this autoradiogram and supports the previous observation showing that the testis transcript is more abundant than the hypothalamic transcript. These Northern blot results also confirm the RNase protection results suggesting that a significant portion of Exon I is absent in the CRH mRNA in testis.

The CRH transcript in testis appears to contain sequences found in exon II. However, the 5' end of exon II was not completely present in this transcript as shown by RNase protections, suggesting that this RNA does not contain a hypothalamic-like CRH signal peptide region (which is encoded on this portion of the RNA in hypothalamus). It seems likely that if the testicular CRH RNA is to encode a CRH peptide an additional 5' untranslated and signal peptide encoded region would be required. These sequences would also have to be spliced in frame with the homologous portion of exon II (which spans the CRH peptide encoding region).

Interesting, several AT-rich sequences were identified within the intron of the rat CRH gene. It is possible that these sequences could serve as promoter-like elements and direct transcription from within this "intron". This possibility could account for the variation in the size of the testicular CRH mRNA, since, as was determined from Northern blots, the size difference between the CRH mRNA in testis and brain was approximately the size of the intron.

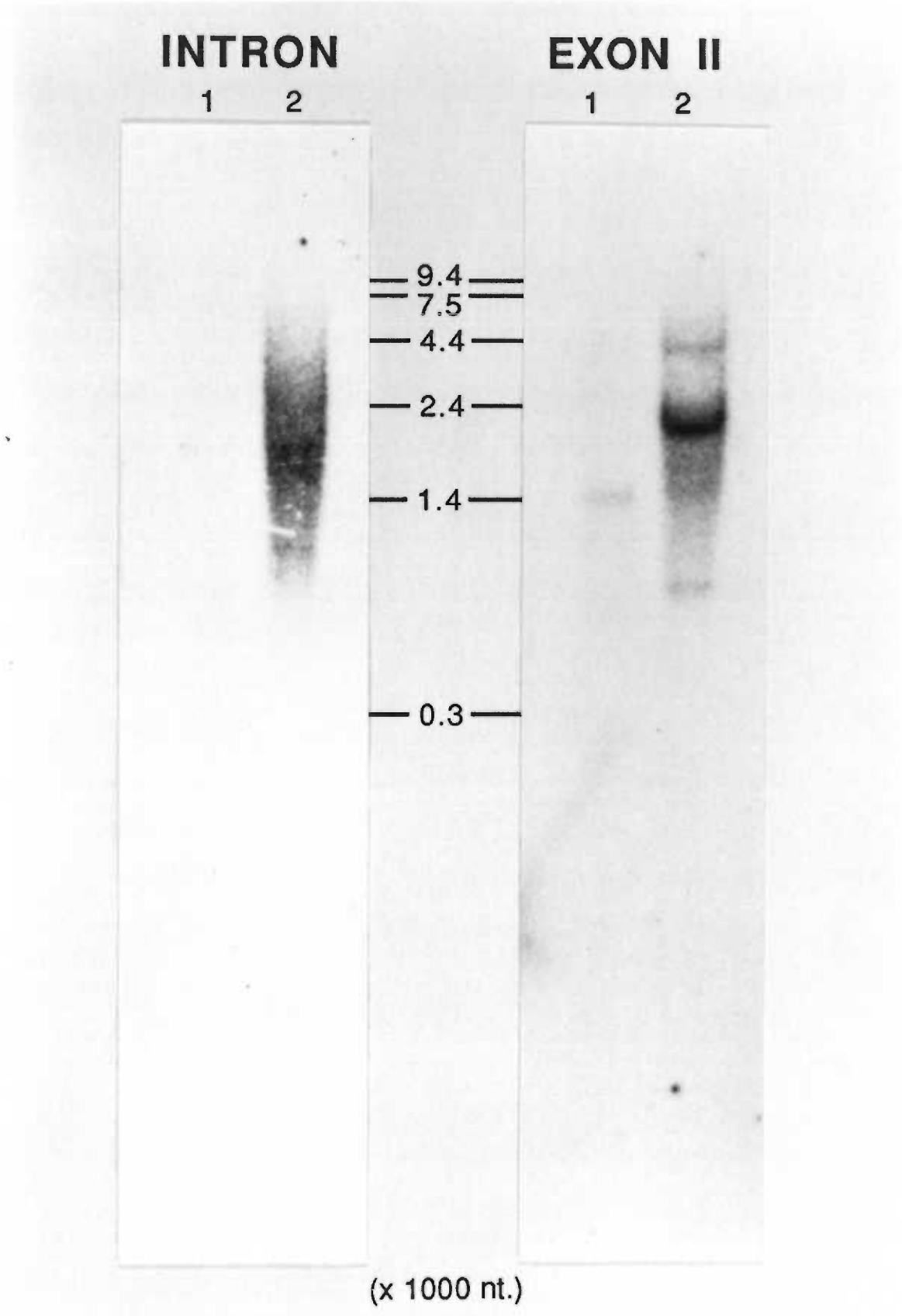
In order to determine if a portion of the intronic sequence was

found in the testicular CRH mRNA, a 534 bp Pvu II genomic fragment (fragment # 4, figure 21) was isolated from the intron and subcloned into cRNA producing plasmids. A cRNA probe produced from this cloned Pvu II fragment was used to probe Northern blots containing both hypothalamic and testicular poly A RNA. After a 48 hour exposure of the blot hybridized with the Pvu II cRNA, no specific bands were evident, however a diffuse signal was evident between 1400 and 4400 nucleotides and is shown on the left in figure 27. The origin of this diffuse signal in unknown but may relate to repetitive elements often found within intronic sequences. However, when this blot was washed in dH_2O at 100°C for 10 minutes and then re-hybridized with the BamH I fragment specific to Exon II (fragment 6, in figure 21), a specific band of approximately 2000 nucleotides was apparent on short exposures of this blot (24 hours) shown in the right in figure 27. From these results, it appears that the intron probe does not hybridize to the same testicular transcript detected with the BamH I cRNA probe (which is specific for Exon II). As expected, the Exon II-specific also hybridizes to a specific hypothalamic CRH mRNA. It therefore appears unlikely that the CRH mRNA in testis contains a signifcant portion of the intronic sequences resulting from transcriptional initiation within the intron.

RNase protection and Northern blot analysis suggest that the testicular CRH mRNA is quite different in structure from the brain transcript. It appears that Exon I, the intron and the 5' end of Exon II are absent from the testis transcript, but a significant portion of

Figure 27. Northern Blot Analysis of Testicular and Hypothalamic Poly A RNA Using Intron- and Exon II-Specific cRNA Probes.

The presence of intronic sequences, derived from the rat CRH gene, in poly A RNA samples (isolated from rat testis (lane 2) and hypothalami (lane 1)) was evaluated by Northern blot analysis. A Pvu II genomic fragment (corresponding to a portion of the intron) and a BamH I cDNA fragment (corresponding to a portion of Exon II) were subcloned into plasmids to produce cRNA probes specific to these regions. The position of these fragments in relationship to the rat CRH gene is diagrammed in figure 21. The autoradiogram on the left, resulting from the hybridization with the Pvu II-intron cRNA probe, was exposed for 48 hours at -70°C with an intensifying screen. This blot was then incubated in sterile dH_2O at 100°C for 10 minutes to remove the hybridized Pvu II-intron cRNA probe. The blot was then autoradiographed for 24 hours at -70°C with an intensifying screen. This blot did not demonstrate any detectable signal after this exposure. The autoradiogram on the right, resulting from the re-hybridization of the same Northern blot with BamH I-Exon II cRNA probe, was exposed for 24 hours at -70°C with an intensifying screen. The positions of denatured RNA molecular weight markers form the RNA Ladder (BRL) are indicated between the two autoradiograms.


INTRON
1
2
EXON II
1
2
9.4
7.5
4.4
2.4
1.4
0.3
(x 1000 nt.)

Exon II represented in the BamH I fragment is present in the testis CRH mRNA. This testicular RNA could still encode a CRH peptide, since the DNA sequence encoding the CRH peptide is found within the BamH I fragment sequence. However, this CRH mRNA appears to be alternately spliced due to the absence of the 5' end of Exon II as suggested from the RNase protection assays with fragment # 5 (figure 21). It also appears likely that this transcript arises from a promoter element not tested in the RNase protection assays which does not appear to lie within the intron and may be found further upstream (5'). Therefore, the testicular CRH mRNA as detected by Northern blot analysis appears to contain a region homologous to a region of Exon II and may represent an alternatively spliced CRH mRNA whose transcription initiates from a more 5' promoter element. In further sequencing the 5' flanking DNA of the rat CRH gene, an additional TATAA sequence has been identified which is found approximately 1.1 kb 5' to Exon I. Cell specific promoters have been identified in the human α1-antitrypsin gene which are approximately 2 kb apart (105). Precise information concerning the sequence of this testicular transcript will await its isolation from a rat testis cDNA library utilizing an Exon II specific probe.

RESULTS AND DISCUSSION

Chapter 5: Transcriptional Regulation of the rat CRH gene by cAMP

Summary

The mechanisms of transcriptional regulation of the rat CRH gene are poorly understood. The isolation of the CRH gene and the description of the promoter elements 5' to this gene will now permit investigations into the molecular mechanisms which activate or repress transcription of this gene. In experiments described in this chapter, gene transfer techniques were ultilized to identify DNA sequences involved in induction of expression of the rat CRH gene. These studies focus on the cAMP regulation of this gene, and suggest that this gene can be regulated by cAMP-mediated pathways in vivo.

Introduction

The regulatory mechanisms involved in control of CRH mRNA levels have not been well characterized. Jingami et al. demonstrated an increase in hypothalamic CRH mRNA levels to 153 % of control levels 14 days after adrenalectomy, and this effect could be prevented by the administration of dexamethasone (24). Young has used in situ hybridization to monitor CRH mRNA levels in rats under salt loading and dehydration stimuli (106). This study demonstrated that CRH mRNA is regulated differently within separate populations of hypothalamic neurons under various conditions. Secretion studies have suggested that catecholamines (28), opioid peptides (29-32), and

dexamethasone (33) inhibit secretion of CRH while acetylcholine (28), serotonin (29) and angiotensin II (33) stimulate CRH secretion. In many instances, compounds which serve to stimulate secretion of neuroendocrine peptides also serve to increase cellular levels of the corresponding mRNA, Thus, these factors may also act in vivo as modulators of CRH gene expression. However, it is not known whether these compounds affect only secretion of CRH, or whether they also alter the transcription of the CRH gene. Lastly, the molecular mechanisms responsible for regulation of CRH transcription by any of these compounds are unknown.

Advances in molecular biological techniques over the past five years have provided us with the tools to examine these questions. The availability of cloned genes and the development of new methods for the efficient transfer of DNA into eukaryotic cells have revolutionized the field of gene regulation. One can examine the molecular mechanisms involved in the regulation of expression at a variety of levels (i.e., transcription, RNA and protein processing, post-translational modifications, secretion) using gene transfer methods. Transcriptional regulation appears to be controlled by a variety of cis-acting and trans-acting regulatory elements. The DNA sequences representing cis-acting elements are quite diverse in function and structure, and may alter the general stimulation of transcription (promoters and enhancers), tissue-specific gene expression, or the induction (or repression) of transcription by the action of specific pharmacological compounds. The trans-acting

regulatory elements are often specific protein factors which interact with the cis-acting DNA sequences. "Activated" receptors (i.e. glucocortocoid receptor) or cellular phosphoproteins which translocate to the nucleus and interact with specific genomic DNA sequences to modulate trancription of specific genes are examples of trans-acting factors.

Gene transfer techniques can be divided into two classes: 1) stable transfections and 2) transient assay systems. To study stably transfected cell lines, one isolates a clone of eukaryotic cells that has integrated the foreign gene into its chromosomal DNA. The major limitation of this approach is an inability to control the region of the genome into which the introduced gene is integrated. Thus, different clones carrying the same gene may show significant variability in RNA and protein production depending upon differences in location of the foreign gene in the host chromosome. In transient expression systems, the foreign gene is introduced into eukaryotic cells and its expression is assayed within a few hours or days before it can integrate into the host DNA. These transient assay systems can be used to rapidly examine the regulation and expression of cloned genes in a variety of cellular environments.

A number of transient expression systems are presently available for studing transcriptional regulation. The fusion gene system will be utilized in these studies in which the control region of the gene of interest is fused to the coding sequence of a gene whose protein product is readily assayed by enzymatic means. The amount

of enzyme activity reflects the amount of protein produced under the control of the linked regulatory region and thus provides direct indication of the level of gene expression. The E. coli chloramphenicol acetyltransferase (CAT) reporter function developed by Gorman et al (107). was selected for these studies because CAT can be rapidly and sensitively assayed, and there is no interfering endogenous activity in mammalian cells. By fusing the 5' end of the rat CRH gene to the coding region of the CAT gene, we can analyze the efficiency of the CRH promoter, test for tissue-specific elements, and map DNA control elements responsible for alterations in transcription via regulatory compounds (i.e. steroid hormones, peptide hormones and neurotransmitters).

Many extracellular signals (peptide hormones, neurotransmitters) are recognized through specific membrane receptors, and binding of the signal molecule to the receptor initiates a rapid series of intracellular events that eventually translates this external signal into a specific cellular response such as RNA and/or protein synthesis, secretion, cell division, etc. It is now generally accepted that "second messenger" molecules are involved in this signal transduction. cAMP is a known second messenger molecule in cells and is thought to be involved in transcriptional regulation of eukaryotic genes. Activation of adenylate cyclase by a variety of different mechanisms causes increased levels of intracellular cAMP. Elevated cAMP concentrations cause increased cAMP-dependent protein kinase activity and the resultant changes in protein

phosphorylation have been postulated to modulate gene transcription. The regulation of CRH transcription by hormones which mediate their effects through activation or inhibition of adenylate cyclase had not previously been examined in vivo. However, the rat CRH 5' flanking sequence contains a DNA element homologous to a 12-15 bp cAMP-responsive "consensus sequence" derived from studies on the human proenkephalin (108), rat somatostatin (109) and rat phosphoenolpyruvate carboxykinase (110) genes. The fusion gene system provides us with a rapid method for testing the ability of the cAMP-responsive "consensus sequence" to confer regulation by cAMP mediated pathways on the rat CRH gene.

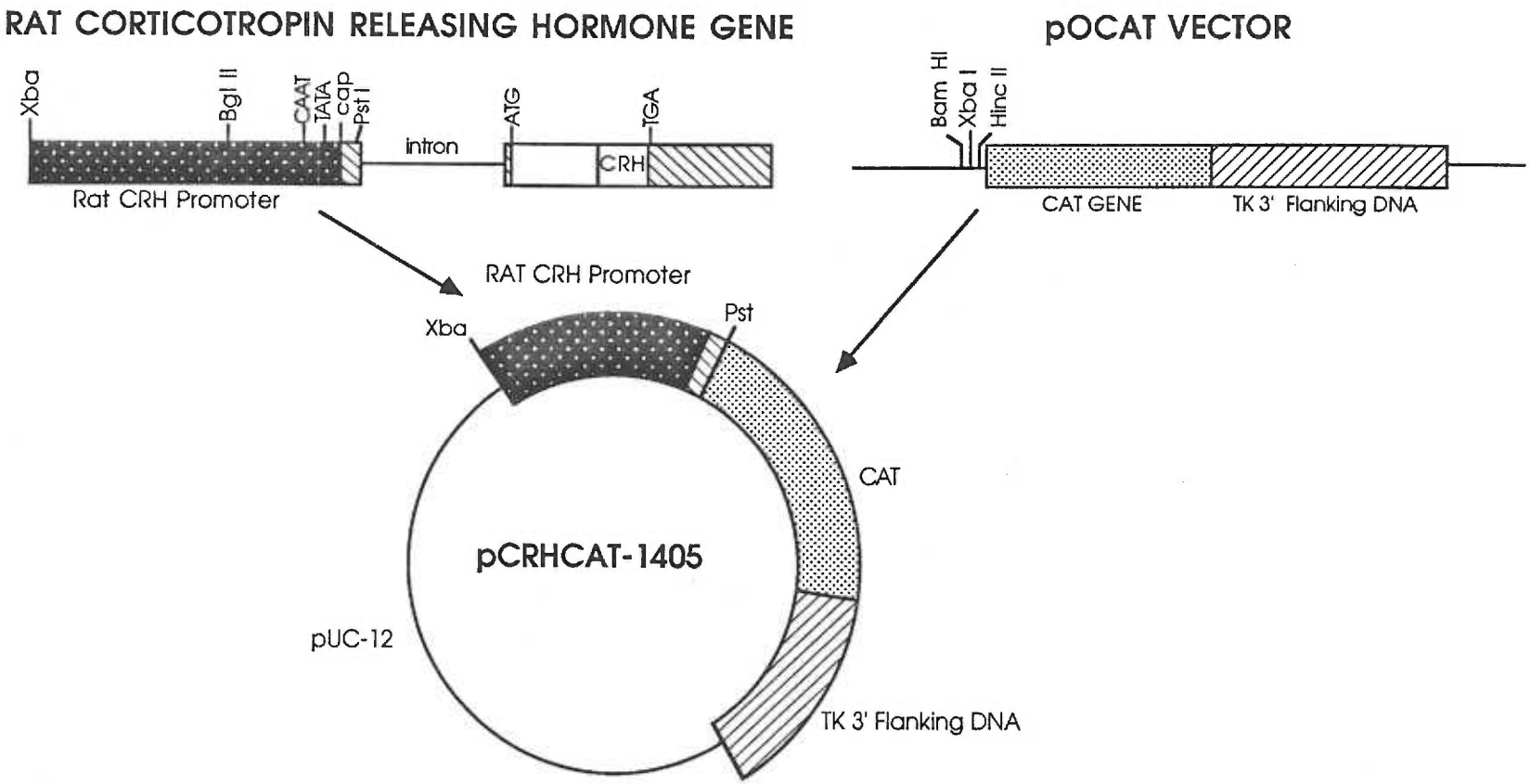
Results and Discussion

The fusion gene construct (pCRHCAT-1405) contains 1.4 kb of rat CRH 5' flanking sequence and 94 bp of the first exon fused to the CAT gene (see figure 28). The 3' untranslated sequence, termination signals, poly A addition sites, and 3' flanking sequence are from the herpes thymidine kinase (TK) gene. This plasmid DNA was transfected into a variety of transformed mammalian cell lines by the calcium phosphate precipitation method, including a glycerol shock after 4 hours to aid in DNA uptake. The cells were harvested after 48 hours, lysed with Triton X-100 (0.5 %), and assayed for CAT activity as described (in Methods).

The basal levels of CAT activity were examined after

Figure 28. Schematic Representation of CRHCAT-1405 Fusion Gene Construction.

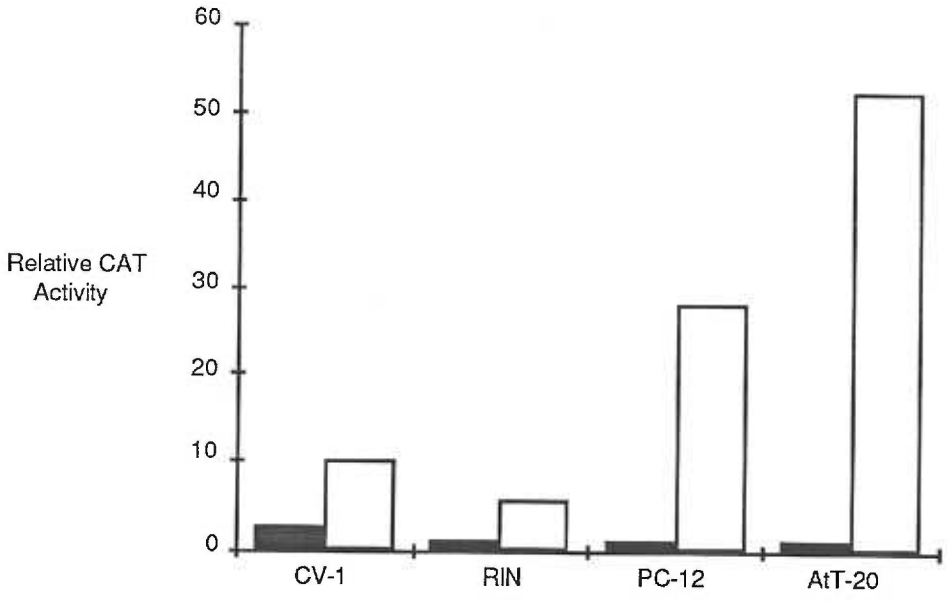
The rat CRH gene is diagrammed on the upper left with the 5' and 3' untranslated regions denoted by hatched boxes. The open region represents the protein coding region of the CRH mRNA, and CRH denotes the location of the 41 amino acid CRH peptide in the 187 amino acid precursor molecule. The pOCAT vector shown in the upper right contains the coding region of the E. coli chloramphenicol acetyltransferase (CAT) gene and the herpes thymidine kinase (TK) mRNA polyadenylation and termination signals in a pUC-12 derived vector. The 1499 bp Xba/Pst restriction fragment from the rat CRH gene, containing 1405 bp of 5' flanking sequence and 94 bp of 5' untranslated CRH mRNA, was inserted immediately 5' to the CAT gene in the pOCAT vector to form pCRHCAT-1405.


RAT CORTICOTROPIN RELEASING HORMONE GENE
Xba
Bgl II
CAAT
TATA
cap
Pst I
intron
ATG
TGA
CRH
Rat CRH Promoter
pOCAT VECTOR
Bam HI
Xba I
Hinc II
CAT GENE
TK 3' Flanking DNA
RAT CRH Promoter
Xba
Pst
CAT
pCRHCAT-1405
pUC-12
TK 3' Flanking DNA

transfection of pCRHCAT-1405 into a variety of mammalian cells. In all cases, the basal CAT expression was very low (approximately 2-fold over a promoterless construct), suggesting that either the CRH promoter is a relatively weak promoter, or a tissue-specific element may be present on the 1.4 kb of 5' CRH sequence preventing the expression of the fusion gene construct in these cell lines. (None of these cell lines tested have been shown to express the endogenous CRH gene) Although the basal levels of CAT activity were low, the levels of expression were increased dramatically (up to approximately 50-fold over a promoterless construct) in each cell line in the presence of 8BrcAMP (a cAMP analogue) and isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor which prevents the breakdown of intracellular cAMP. Both the RIN (rat pancreas) and CV-1 (monkey kidney) cells showed a 2.5 to 5 fold increase in CAT activity in the presence of 8BrcAMP and IBMX, while PC-12 (rat pheochromocytoma) and AtT-20 (mouse anterior pituitary) cells showed 30 and 50 fold inductions, respectively (see figure 29). Thus, the rat CRH gene is transcriptionally activated via a cAMP-mediated pathway with varied levels of induction in the different cell lines. Several possible explanations could account for these findings. These differences in induced activity may be due to variable intracellular cAMP levels under induced conditions reflecting different levels of adenylate cyclase or phosphodiesterase activities in these cell lines. Alternatively, different trans-acting proteins which bind to the cis-acting regulatory elements involved in the

Figure 29. Regulation of pCRHCAT-1405 Gene Expression by cAMP.

The plasmid CRHCAT-1405 containing 1.4 kb of the rat CRH 5' flanking DNA and 94 bp of the 5' untranslated region of the CRH mRNA linked to the CAT gene was transfected into four different cell lines. The CAT enzyme activity in CV-1(monkey kidney), RIN (rat pancreas), PC-12 (rat pheochromocytoma), and AtT-20 (mouse anterior pituitary) cells following transfection with CRHCAT-1405 is shown. Solid boxes represent basal activity (no regulators) and stippled boxes represent CAT activity in transfected cells treated with 8BrcAMP (1mM) and IBMX (0.5mM). Regulators were added 6 hours before the cells were harvested. In all experiments a plasmid containing the ß-galactosidase gene linked to the Rous sarcoma virus (RSV) promoter was co-transfected to provide an internal control for differences in transfection efficiency between different precipitates (71). CAT activity was assayed as described in Methods. Acetylated products were resolved by thin layer chromatography, excised and counted in a scintillation counter. The counts per minute (cpm) determined were then normalized to the ß-galactosidase activity in each extract. The basal activity in PC-12 cells was assigned a value of 1.0 and the CAT activity of the other cell lines was expressed relative to that value.


Relative CAT Activity
60
50
40
30
20
10
0
CV-1
RIN
PC-12
AtT-20

cAMP-mediated induction of the CRH gene may be present in these various cell lines.

The complete nucleotide sequence of 2 kb of the 5' flanking DNA of the rat CRH gene was obtained. Convenient restriction sites were found which dissected the 1.4 kb of 5' flanking DNA of the rat CRH gene into two smaller regions which when cloned into the CAT pasmid could be used to localize the cAMP-responsive element. Taking advantage of a Bgl II site at -497, a second CRHCAT plasmid was constructed (pCRHCAT-497) which contained 497 bp of CRH 5' flanking DNA and 94 bp of 5' untranslated CRH mRNA fused to the CAT gene. This contruct showed similar levels of induction with 8BrcAMP and IBMX (as pCRHCAT-1405) in all cell lines tested and indicated that the cAMP responsive element was between -497 and +94 bp on the rat CRH gene.

A third fusion gene (pCRHCAT-132) was constructed which contained only 132 bp of rat CRH 5' flanking DNA (and 94 bp of 5' untranslated CRH mRNA) fused to the CAT gene. When this plasmid was transfected into PC-12 cells, the basal level of CAT expression was similar to cells transfected with pCRHCAT-497, but treatment of the transfected cells with 8BrcAMP and IBMX or forskolin (an activator of adenylate cyclase) no longer caused an induction in CAT expression. A summary of the CAT activity results obtained with these various CRH promoter/CAT fusion plasmid constructs is shown in figure 30. Similar results were obtained using CV-1 cells transfected with CRHCAT-131. This finding was most interesting,

Figure 30. Localization of DNA Sequences Necessary for cAMP Regulation of CRHCAT Fusion Genes.

Fragments of the rat CRH 5' flanking DNA (-1405 to +94 bp, -497 to +94 bp, and -132 to +94 bp) were inserted upstream of the bacterial CAT gene. The open boxes represent CRH 5' flanking DNA and the hatched boxes represent the 94 bp of the 5' untranslated region of the CRH mRNA. The CRHCAT fusion gene constructs were tested for cAMP induction of CAT activity after transfection into PC-12 cells as described in Methods. The cpms of actylated chloramphenicol product were normalized to the ß-galactosidase activity in each extract. The basal activity of CRHCAT-1405 was assigned a value of 1.0. Fold induction was calculated by dividing the cpm of acetylated chloramphenicol products in forskolin-treated samples by the cpm of acetylated chloramphenicol products in untreated samples.

CRHCAT DELETION MUTANTS

CRHCAT CONSTRUCTS		Relative CAT Activity		
		Control	Forskolin	Fold Induction
CRHCAT-1405	-1405 	1.0	19.4	19.4
CRHCAT-497	-497 	1.6	24.3	15.2
CRHCAT-132	-132 	1.0	1.4	1.4

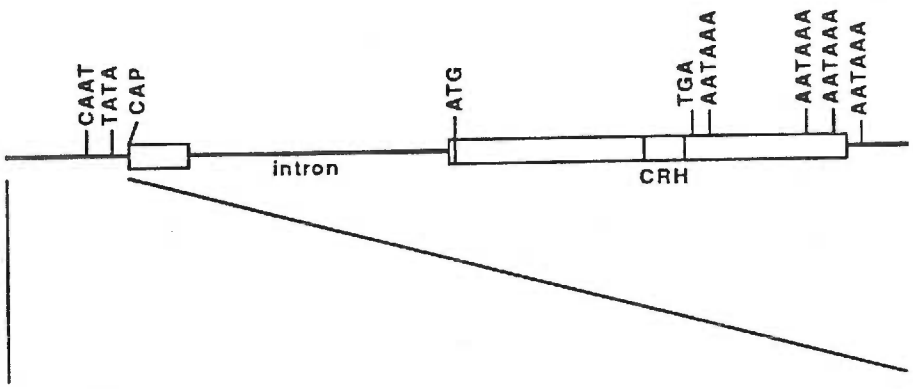
since the region of 5' flanking CRH DNA sequence homologous (at 11 of 14 bases) to a 15 bp region of the human proenkephalin cAMP responsive element (106) is located at position -104 to -118 bp upstream of the putative cap site. Thus, even though this CRHCAT construct contains a sequence similar to an identified cAMP regulatory element, it no longer confers cAMP induction.

Upon closer analysis of the 5' flanking sequences, an additional cAMP regulatory consensus sequence was found in the 5' flanking DNA of the rat CRH gene (-229 to -222). This sequence shares a lower degree of homology with the human proenkephalin cAMP responsive element over a 15 bp region. However, research in our laboratory, as well as others, on the DNA elements responsive to cAMP induction has suggested that these cAMP responsive DNA sequences share a smaller 8 nucleotide "core" sequence ($^{5'}$TGACGTCA$^{3'}$). It is interesting to note that the most 3' consensus sequence (-118 to -104) lacks this exact core sequence. It is therefore tempting to speculate that this 3' consensus sequence, which did not by itself confer cAMP responsiveness to the CRH promoter, is in fact not the cAMP responsive element in this gene, but rather the cAMP responsive element is located about 80-100 nucleotides further upstream. The position of these two consensus sequences within the 5' flanking region of the rat and human CRH genes is shown in figure 31.

Gene transfer experiments have suggested that the rat, and possibly the human, CRH gene is transcriptionally regulated by cAMP-mediated pathways in vitro. The region of the rat CRH gene

Figure 31. Nucleic Acid Homology between 5' Flanking Sequences of Rat and Human ProCRH Genes.

A schematic representation of the rat proCRH gene is shown at the top. The exons are shown as blocks and the intron by a line. The TATAA and CAAT sequences, putative cap site, translation initiation ATG, translation terminator TGA, and poly A addition signals (AATAAA) are indicated. The location of the CRH peptide is indicated by CRH. The nucleotide sequences of the 5' flanking DNA from the rat (upper sequence -336 bp) and human (lower sequence-333 bp) CRH genes are shown at the bottom of the figure. The vertical lines denote conserved nucleotides. The TATAA and CAAT boxes are shown in black. The location of two cAMP "consensus" sequences are indicated by striped boxes. Nucleotide -1 corresponds to the first nucleotide 5' to the putative CRH mRNA cap site.

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-336  GGCCTATCATAGTAAGAGGTCAGTATGTTTTCCACACTTGGATAATCTCA  -287
-333  GGCCTTTCATAGTAAGAGGTCAATATGTTTT.CACACTTGGGAAATCTCA  -285

-286  TTCAAGAATTTTTGTCAATGGACAAGTCATAAGAAGCCCTTCCATTTTAG  -237
-284  TTCAAGAATTTTTGTCAATGGACAAGTCATAAGAAGCCCTTCCATTTTAG  -235

-236  GGCTCGTTGACGTCACCAAGGAGGCGATAAATATCTGTTGATATAATTGG  -187
-234  GGCTCGTTGACGTCACCAA.GAGGCGATAAATATCTGTTGATATAATTGG  -186

-186  ATGTGAGATTCAGTGTTGAAATAGCAGAACCCTGTCCCTCGCTCCTTGGC  -137
-185  ATGTGAGATTCAGTGTTGAGATAGCAAAATTCTGCCCCTCGTTCCTTGGC  -136

-136  AGGGCCCTATTATTTATGCAGGAGCAGAGGCAGCACGCAATCGAGCTGTC  -87
-135  AGGGCCCTATGATTTATGCAGGAGCAGAGGCAGCACGCAATCGAGCTGTC  -86

-86   AAGAGAGCGTCAGCTTATTAGGCAAATGCTGCGTGCTTTCTGAAGAGGGT  -37
-85   AAGAGAGCGTCAGC.TATTAGGCAAATGCTGCGTGGTTTTTGAAGAGGGT  -37

-36   CGACGTTATAAAATCTCACTCCGGGCTCTGGTGTGG                 -1
-36   CGACACTATAAAATCCCACTCCAGGCTCTGGAGTGG                 -1
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responsible for confering this cAMP regulation has been localized within the 5' flanking region of this gene between bases -497 and +94 relative to the defined cap site. DNA sequences homologous to a cAMP "consensus" core sequence are found within this mapped portion of the 5' flanking region of the CRH gene.

SUMMARY AND CONCLUSIONS

In this thesis, the isolation and characterization of the rat corticotropin releasing hormone cDNA and gene were described. DNA complementary to the rat hypothalamic mRNA coding for the corticotropin-releasing hormone (CRH) precursor was cloned from a rat hypothalamic lambda gt10 cDNA library using a synthetic deoxyoligonucleotide complementary to 20 nucleotides from the CRH peptide-encoded region of the human CRH gene (66). Several cDNA clones were isolated and characterized, the longest of which was shown to encode the entire rat CRH protein precursor. The elucidation of the structure of the rat CRH precursor provided many interesting findings. First, the amino acid sequence of the rat CRH peptide, as deduced from the cDNA, was shown to correspond precisely to the amino acid sequence published by Rivier et al. (72). Additionally, this work demonstrated that the rat CRH precursor is highly homologous at both the amino acid and nucleotide levels to the previously published ovine and human CRH precursors particularly in the CRH peptide and putative signal peptide regions. The high degree of homology between the CRH sequences in these species seems to emphasize the physiological importance of this peptide. The most significant contribution of this work was the isolation of the rat CRH cDNA itself, providing a molecular biological tool to investigate the CNS and peripheral sites of synthesis and the regulation of synthesis of the CRH prohormone. The regulatory mechanisms and

factors which act as modulators of cellular CRH mRNA levels remain largely uncharacterized. Several neurotransmitters and neuromodulators have been suggested to regulate the secretion of CRH from the hypothalamus but it is not known whether these secretory effects alter the synthesis of CRH. The rat CRH cDNA could be used to address these questions.

The central nervous system and peripheral tissue distribution of CRH mRNA were examined in an attempt to define the sites of synthesis of CRH using the newly acquired rat CRH cDNA as a hybridization probe. Prior to this effort, immunocytochemical techniques suggested that CRH was found in many regions of the brain as well as in peripheral endocrine tissues. However, Northern blotting techiques employed to identify the ovine CRH mRNA were unsuccessful, presumably due to the low abundance of this transcript in the hypothalamus. Two significant advances in Northern blot technology (Nylon membranes and cRNA probes) have greatly improved the sensitivity of Northern blots. These new advances in Northern blot analysis permitted the identification of CRH mRNA as a 1400 nucleotide transcript in hypothalamic tissue and, as immunohistochemical techniques had previously suggested, demonstrated that the CRH mRNA is produced in many regions of the CNS. Additionally, several peripheral sites of CRH synthesis were identified. This vast distribution of CRH may provide a mechanism for CRH containing neurons and endocrine cells to coordinate many different biological functions during an organisms response to

complex stimuli such as those experienced during stress.

Immunocytochemical techniques suggested that the rat testis produces CRH-like material and that this material is produced in Leydig cells and a population of germ cells. In an attempt to determine whether this CRH-like material was synthesized in the testis, Northern blot analysis was performed on mRNA extracted from adult rat testis. In this analysis, an RNA transcript was detected following high stringency hybridization conditions. However, the transcript was much larger than the CRH mRNA detected in brain regions. The nature of this size variation was analyzed through the use of RNase protection and Northern blotting techniques. The testicular CRH mRNA was shown to lack significant portions of Exon I, the intron and the 5' end of Exon II. The testicular CRH mRNA did, however, appear to contain a region which demonstrated a high degree of homology to a portion of Exon II, as demonstrated by Northern blot results using Exon II-specific probes. This CRH RNA transcript in testis may represent an alternatively spliced mRNA possibly encoding an additional protein sequence. This CRH mRNA detected in testis may still encode a CRH peptide due to the consistant hybridization of the portion of exon II which encodes the amino acids in the CRH peptide. The complete structure of the CRH transcript in testis will be determined by isolating this sequence from a testis cDNA library.

The molecular mechanisms underlying the expression of the CRH precursor and its gene are essential to an understanding of the

regulation within the hypothalamic-pituitary-adrenal axis. As mentioned above, many neurotransmitters and neuromodulators have been suggested to regulate CRH secretion from the hypothalamus but whether any of these compounds directly or indirectly alter the synthesis of the CRH precursor is unknown. In order to determine whether these compounds could alter the transcription of the rat CRH gene, this gene was isolated and characterized.

The structure of the rat CRH gene was shown to be very similar to the human CRH gene, containing two exons and a similarly sized intron. The nucleotide homology between these two genes was expected to be high in the region of the CRH peptide, due to the identical amino acid sequence in this region. However, the 94% homology in the promoter region of these genes was unexpected. This level of homology suggests that these two genes may share common transcriptional control elements and that the DNA binding proteins which mediate these transcriptional effects may also be similar.

The isolation of the CRH gene and the description of the promoter elements 5' to this gene will permit investigations into the molecular mechanisms which activate or repress transciption of this gene. In experiments described in this thesis, gene transfer techniques have been utilized to identify DNA sequences involved in induction of expression of the rat CRH gene. These studies focused on the cAMP regulation of this gene, and suggest that this gene can be regulated by cAMP-mediated pathways in vivo. Analysis of the 5'

flanking DNA of the human gene suggests that this gene is also regulated by cAMP-mediated pathways.

The elucidation of the structure and nucleotide sequence of the rat CRH cDNA has provided the information and molecular biological tools necessary to address difficult questions in a complex tissue (the brain). It is now possible to determine the effects of acute or chronic pharmacological treatment, psychological manipulation, or disease states on the biosynthesis of CRH. Experiments along these lines prior to the availability of cloned genes and molecular biological tools were very difficult if not impossible due to the minute concentrations of neuropeptides (particularly releasing factors) in the central nervous system.

The ability to place genes into various cellular environments has permitted us with the opportunity to address questions concerning the mechanisms of transcription. In this thesis, a cAMP-responsive region in the 5' flanking region of the rat CRH gene was described. Additional DNA elements are presently being pursued in our laboratory, including tissue-specific and glucocorticoid elements as well as the protein factors which bind to these DNA sequences. Several techniques are now available to investigate these trans-acting factors and isolate them. The isolation and characterization of these trans-acting factors will be a step forward in determining how the CRH gene is activated during period of acute and chronic stress. A more complete understanding of how the CRH gene is activated and suppressed could provide insights into

the pathophysiology of disease states in which the hypersecretion of CRH from the hypothalamus has been suggested to play a role.

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