REGULATION OF CART mRNA EXPRESSION

Ph.D THESIS

By

ANITA LAKATOS

Department of Medical Biology

Pécs University
Faculty of Medicine

Ph.D Program: Biochemistry and Molecular Biology
Ph.D Subprogram: Signal Transduction Pathways
Program Director: Sümegi Balázs Ph.D D.Sc.
Subprogram Director: József Szeberényi M.D. Ph.D D.Sc

Pécs
2003.

DEDICATED TO

My son, Soma Doherty,
my sister, Szilvia Lakatos and my
parents for their love and
strength

"Nem az a fontos, hogy honnan jössz, hanem az, hogy hova mész."

Illyés Gyula

TABLE OF CONTENTS

1. INTRODUCTION

1.1 Discovery of CART

Cocaine-induced molecular alterations are believed to be responsible for many of the drug's behavioral consequences. Attention has therefore been focused on determining the changes in gene expression after cocaine treatment. Changes in expression of a number of genes have been observed in response to acute and chronic cocaine administration. CART (i.e., cocaine- and amphetamine-regulated transcript) gene which was discovered in 1995 by Douglass et al. as an mRNA transcript by differential display method is included in this gene group. CART mRNA was increased in rat striatum and nucleus accumbens after acute cocaine and amphetamine treatment (Douglass and Daoud 1996).

To confirm that the cloned PCR product represents a striatal mRNA that is transcriptionally regulated by psychomotor stimulants, Northern blot analysis was conducted. It revealed the appearance of an RNA doublet approximately 700 and 900 bases in length after 1 hour cocaine administration. The profile of CART mRNA within the cerebellum, striatum and hippocampus was determined by Northern blot analysis and surprisingly showed transcriptionally silent brain regions in the hippocampus after acute cocaine and amphetamine treatment. Douglass et al. (1995) already pointed out that CART was a new, previously uncharacterized rat mRNA that was not significantly related to any known mRNA.

Lastly, regional expression of the CART mRNA throughout the adult rat brain was more precisely determined by *in situ* histochemical analysis (Couceyro et al. 1997) without cocaine administration. *In situ* hybridization revealed a wide distribution pattern of CART mRNA expressing cells in various brain regions of different species and a very discrete localization of the mRNA to specific neuronal and endocrine cell groups. This indicates a role within several neuronal circuits in the brain and periphery, especially in those areas involved in motivation, reward, and feeding. CART mRNA has also been found in the telencephalon. The signals were limited to a few areas. The labeling in the neostriatum was evident, consisting of the nucleus accumbens and olfactory tuberale. Within the neocortex, moderate labeling was seen

only in the primary somatosensory (layer 4) and the piriform areas. Intense labeling was observed in the induseum griseum. A moderate signal was observed in the dentate gyrus of the rostral hippocampus and the amygdaloid complex. In the diencephalon, the hypothalamus exhibited the most extensive labeling distribution of any brain region examined. Particularly, the hypothalamic supraoptic, paraventricular, and arcuate nuclei showed very intensive labeling signal. Subsequently, CART was found to be the third most abundant mRNA in the hypothalamus. In contrast to the hypothalamus, mesencephalon contained the fewest number of hybridization positive cells. However, within this region the Edinger-Westphal nucleus exhibited intense labeling. The rhombencephalon also contained regions of hybridization positive cells. The pituitary represented the major site of transcriptional expression. The other brain regions: the posterior horn of the spinal cord; sympathetic ganglions, adrenal medulla; and the intestine contained small detectable levels of CART mRNA (Couceyro et al. 1997).

1.2. Characteristic features of CART DNA and mRNA

CART mRNA expression patterns in the human brain were qualitatively similar to the rat brain CART mRNA distribution indicating that the protein product of the CART mRNA plays a functional role or roles across the mammalian species.

Since, CART mRNA has been described in many vertebrate species including rodents (mouse, rat), goldfish (Volkoff and Peter, 2001) and human. Comparing human CART cDNA to rat CART cDNA, the human CART cDNA sequence was about 80 % identical to the rat cDNA with about 92 % homology in the protein - coding region. Subsequently, the CART mouse gene was mapped to chromosome 13 encoding a 2.5 kb segment, and the human gene was mapped to chromosome 5 containing approximately 2 kb segment, while the rat CART gene has not been identified yet. The nucleotide sequence of human CART was determined after chromosomal mapping (Challis et al. 2000). The chromosomal mapping made possible to analyze the genetic variations of CART gene. Several polymorphisms have been described in the 3' UTR region and 5' flanking region. In the 3' UTR region, three polymorphisms have been identified (A1475G, 1457delA and among Pima Indians, C1442G) (Giudice et al., 2001)

and only one mutation found in the coding region, Ser39Thr. The prevalence of these variants was determined among obese and control groups. No difference was found between the obese patients heterozygous for one of these polymorphisms and those homozygous for the wild – type allele. Six polymorphic sites were identified in the upstream 5' flanking region; A-->G at -156, T-->C at -390, T-->G at -484, G-->T at -915, G-->C at -929 and C-->T at -962. The allele frequency of the -156 variant was significantly higher in the obese group than in the control group. The -156 polymorphism's linkage disequilibrium with the site may be associated with genetic predisposition to obesity. To confirm this association between the CART 5' flanking region polymorphic site and the inherited obesity, further investigations are necessary (Walder et al. 2000; Yamada et al. 2002, Challis et al., 2000).

Additional rat CART cDNA sequence analysis characterized the rat CART mRNA in helping to understand the appearance of an RNA doublet following Northern blot analysis. The characterization revealed that in the termination of synthesis of CART mRNA, alternate poly A site utilization plays a role in the 3' non-coding region which resulted in the appearance of an RNA doublet, either approximately 700 or 900 bases in length. Moreover, alternative splicing produced further diversity within the coding region of the transcripts in the rat, and resulted in two mRNA species, one with the absence of an in frame 39 base insert within the protein coding region, called short form of CART mRNA apart from the 700 or 900 bases in length (Douglass et al. 1995). In all probability, the observed CART mRNA doublet following Northern blot analysis corresponds to four mRNAs instead of two mRNAs (Kuhar et al. 2002). The shorter species was about twice as abundant as the longer one in rat. Interestingly, in human, the short form was the only one found.

1.3. Characteristic features of CART peptides

Rat CART cDNA sequence analysis also helped to predict the translation product of CART gene. The synthesized protein: either 129 or 116 amino acids in length depending on the alternative splicing of 39 bases in the coding region. Examination of the deduced amino acid sequence suggested that the CART product would be a neuropeptide that was secreted

and processed. This suggestion was based on the identification of a common hydrophobic leader sequence and the amino terminus indicating involvement in a secretion pathway, and several pairs of basic amino acids, which are commonly found in propeptides that are processed before subsequent utilization (Koylu et al. 1997; Smith et al. 1997; Couceyro et al. 1998; Koylu et al. 1998).

Thim et al. (1999) proved this hypothesis by purifying CART peptides from adrenal gland, hypothalamus, nucleus accumbens and pituitary gland of the rat and determined the peptide structures by using microsequencing and mass spectrometry. It demonstrated a tissue-specific processing of CART that may point to different biological functions of CART peptides in the peripheral and central nervous system. From the adrenal gland, the CART (1-89) and CART (10-89) peptides were isolated. In contrast, from the hypothalamus and nucleus accumbens, shorter form peptides CART (42-89) and CART (49-89) were purified. Surprisingly, the long splice variant was found in all of the tissues examined (Kristensen et al. 1998; Thim et al. 1998a; Thim et al. 1998b; Broberger 1999).

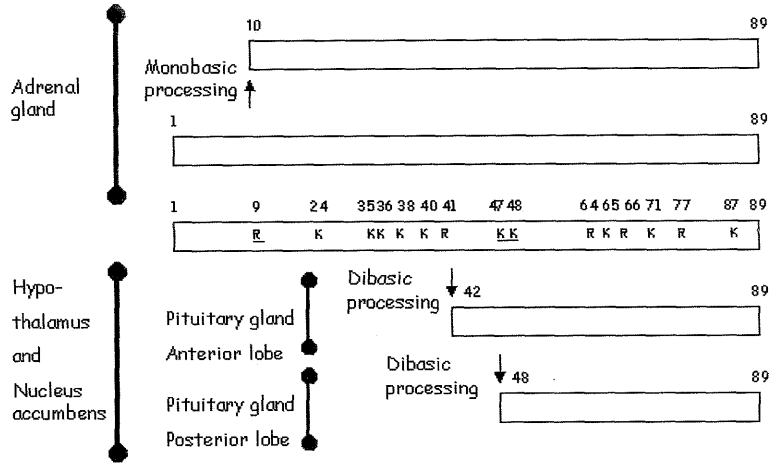


Figure 1. Schematic representation of the tissue-specific posttranslational processing of pro-peptide (116 amino acid lenght peptide, after cleavage of the first 27 amino acid leader sequence) CART protein in the rat. The lysine and arginine residues are indicated by K and R. The underlined residues represent the identified mono- and dibasic processing sites. The schema based on the CART peptides identified by Thim at al. (1999).

Kuhar et al. (1999) confirmed this processing by Western blot analysis. In order to examine the processing and localization of CART putative peptides in the brain, polyclonal antibodies were prepared against several CART peptide fragments (H1: 24-39; C1: 28-54; C2: 57-79; C3: 82-103; C4: 106-129). Using these antibodies, several different fragments of the CART protein were identified in the brain. It should be noted that after describing the cleavage forms of CART peptides, the literature used two nomenclatures. The peptides CART (42-89) and CART (49-89) are described above using nomenclature based on the 89 residue pro-peptide (Thim et al. 1999) have the same primary structure as CART (55-102) and CART (62-102) referred to the longer 102 residue peptide (Kristensen et al. 1998). These fragments were also identified by reverse-phase, high-pressure liquid chromatography (Murphy et al. 2000). It seemed that the two fragments were likely to be produced from the pre-proCART protein and presumably they had different processing modifications. These antibodies mentioned above were also used for immunohistochemical staining to determine the localization of CART peptides. The distribution of CART peptides corresponded to the expression pattern of CART mRNA (Kuhar and Dall Vechia 1999; Kuhar and Yoho 1999).

It has been shown that CART peptides are neurotransmitters (Smith et al. 1999; Thim et al. 1999). Electronmicroscopic analysis showed that the peptides were found exclusively in large dense vesicles in neurons in the brain, processed to smaller peptides and released by calcium-dependent mechanisms into synapses. CART peptides are co-localized with numerous well-known neurotransmitters like GABA, galanin, MCH, oxytocin, POMC, somatostatin, as well as nitric oxide synthase (Broberger 1999; Sergeyev et al. 2001).

These neuroanatomical findings suggested various possible physiological functions. CART peptides could play a role in the stress response, feeding behavior, immune function, autonomic regulation, fluid balance, metabolic processes, sexual function and endocrine control (Kuhar and Dall Vechia 1999). The putative functions are listed in Table 1.

Table 1. The possible physiological functions of CART peptides

Feeding behavior
• Paraventricular nucleus of the hypothalamus
• Arcuate nucleus
• Parabrachial nucleus
• Perifornical cells in the hypothalamus
Stress
• Hypothalamic nuclei
• Pituitary (anterior or posterior)
• Adrenal medulla
• Intermediolateral cell columns of the spinal cord
Sensory processing
• Mitral and tufted cells of the olfactory bulb
• Some retinal ganglion cells
• Layer IV of the cerebral cortex in barrels
• Thalamic relay nuclei
• Nucleus of the solitary tract
• Lamina 1 of the spinal cord
• Afferent vagal fibres
Reward and reinforcement
• Nucleus accumbens
• Ventral tegmental area of the midbrain
• Basolateral amygdala
Central autonomic network
• Nucleus of solitary tract
• Nucleus ambigous
• Central nucleus of amygdale
• Paraventricular nucleus of hypothalamus

In order to prove presumed physiological functions, CART peptides were made by using molecular biology. The preparation of CART peptides by peptide synthesis was very difficult. A yeast expression system was developed for the production of relatively large amounts of CART peptides including the naturally occurring CART (55-102) as well (Brunetti et al. 2000).

Injection of the peptides into the brain resulted in behavioral effects. Strong evidence implicates CART in the control of feeding behavior. As mentioned above, CART is highly expressed in the arcuate and paraventricular nuclei of the hypothalamus. These areas are known to be involved in the control of appetite. CART neurons show c-Fos-immunoreactivity after intravenous injections of leptin in rats. Hypothalamic CART mRNA levels are decreased in hypoleptinemic states, such as in the *ob/ob* mouse, and after prolonged fasting. In these conditions, CART expression is restored by leptin administration. Intracerebrovascular injection of recombinant CART peptide into rats and mice inhibits normal and starvation-induced feeding and blocks the feeding response induced by neuropeptide Y (Kuhar and Dall Vechia 1999; Trayhurn et al. 1999; Ahima et al. 2000; Elias et al. 2000; Johansen et al. 2000; Aja et al. 2001; Asakawa et al. 2001). Conversely, after central administration of anti-serum against CART in rats, feeding response was increased. These data strongly implicate CART as an endogenous satiety factor. However, a new finding suggests that CART peptides may stimulate feeding at least in the hypothalamus.The injection of multifunctional CART peptides into the VTA produces psychostimulant-like behavioral effects including increased locomotor activation and establishment of conditioned place preference.

There is also evidence that the CART protein has neurotrophic action *in vitro*. The short form of CART protein, which is found in humans, has been shown to have neurotrophic properties in several primary-cell-culture neurons. These include dopaminergic, hippocampal and retinal neurons, and motoneurons. The effects produced include increased dopamine uptake, increased cell survival and increased neurite length. In a recent study, Balkan et al. suggested the involvement of the CART peptide in the stress response and its modulation by glucocorticoids (Balkan et al. 2001). CART studies predict its role in development and neurotrophic activity, too.

Even with all of the information available, the CART receptor has not been found yet. Apart from the receptor missing, substantial evidence has been gathered that CART peptides are involved in psychostimulant - like effects, feeding, and stress. To understand and confirm the multifunctional effects of CART protein, the CART receptor needs to be identified. Furthermore, elucidating the detailed molecular mechanism involved in the regulation of CART gene may serve as a model to address the multifunctional behavior of CART peptides.

First , the CART was identified as an upregulated mRNA transcript after acute cocaine and amphetamine treatment. Secondly, the cyclic adenosine 3', 5'-monophosphate (cAMP)-protein kinase A (PKA) signal tranduction pathway (Figure 2) is known to be induced by cocaine administration in rat nucleus accumbens (Nestler et al. 1998). Thirdly, it has been reported that after cAMP activation CART mRNA was increased in GH3 cells (Barrett et al. 2001). Therefore, the possibility of PKA-mediated transcriptional regulation in the CART gene expression is more than likely.

In this study, based on the aforementioned evidence, the attention was focused on determining regulatory mechanisms driving the changes in the CART gene expression.

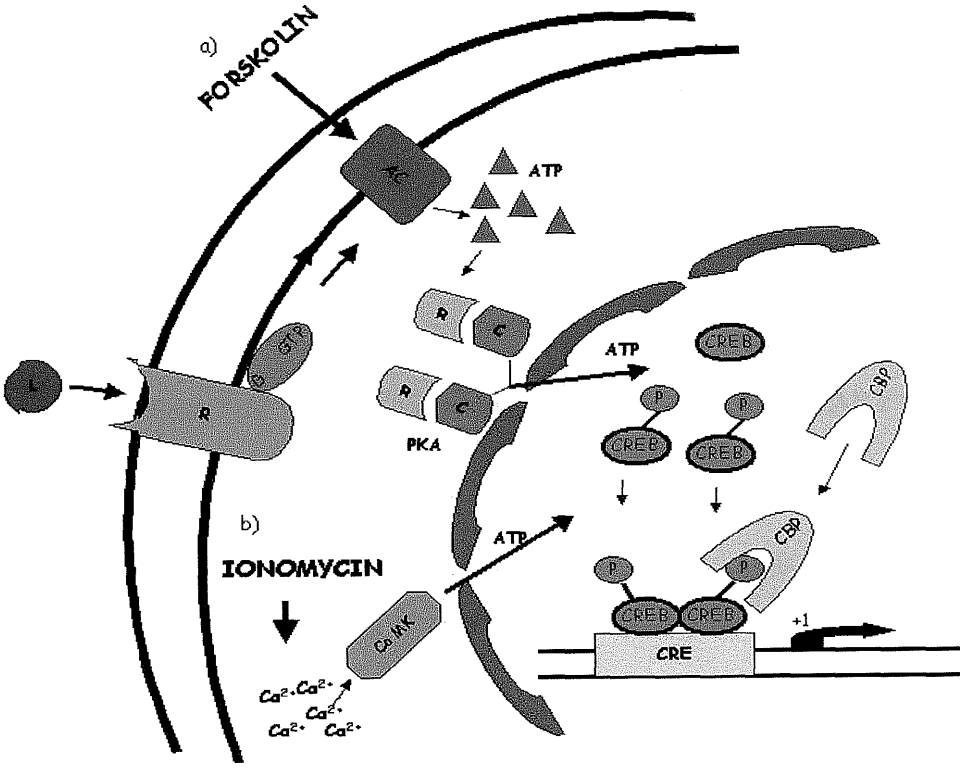


Figure 2 Scheme illustrating a) adenylate cyclase (AC) - cAMP-protein kinase A (PKA) transduction pathway. Forskolin increases the level of cAMP through membrane associated AC activation. The increased cAMP stimulates PKA activity and the phosphorylation of cytoplasmic as well as nuclear targets, including CREB. The cAMP-dependent transcription factor (CREB) interacts strongly with CREB-binding protein (CBP), in response to CREB phosphorylation at Ser-133. Then CREB and other cofactors are recruited to the cAMP response enhancer element (CRE) found in the promoters of several cAMP-responsive genes to activate transcription. b) Ionomycin elevates the level of intracellular Ca^{2+}. Ca^{2+} induced calmodulin kinase (CaMK) activates CREB phosphorylation that plays important role in CRE-mediated gene expression (P. Sassone 1998).

2. OBJECTIVES OF THE RESEARCH PROJECT

2.1. Identifying transcription binding sites for the CART promoter region using the Trasfact computer program.

2.2. To determine whether the putative transcription binding sites on the CART promoter participate in the activation of CART gene expression in GH3 cells.

2.3. Characterizing the relevant transcription factors and their functions which can be recruited into the CART promoter during CART gene activation.

2.4. To identify transcriptional regulatory networks that can play substantial role in the regulation of CART mRNA expression.

3. EXPERIMENTAL PROCEDURES

3.1. Cell lines

List of cell lines used:

GH3 rat pituitary adenoma cell line: The epithelia - like clone generates growth hormone at a greater rate than the GH1 cells and also produces prolactin. Studies on the control of the production of these protein hormones by these cells have shown that hydrocortisone stimulates the production of growth hormone and inhibits prolactin production.

AtT20 mouse pituitary adenoma cell line: This clone has been used successfully for several DNA mediated transfection studies relating to endocrine and exocrine secretory pathways.

PC12 rat phaeochromocytoma cell line: This cell line was derived from a transplantable rat pheochromocytoma. The cells respond reversibly to NGF by induction of the neuronal phenotype.

3.2. Cell culture

PC12 cells were grown in Dulbecco's modified eagle medium (DMEM), supplemented with 5% calf serum and 10% horse serum.
GH3 cells were maintained in F-12K Ham's (Kaighn's modification) media supplemented with 12% horse and 2.5 % fetal bovine serum.Mouse AtT20 cells were grown in Dulbecco's Minimal Essential Medium supplemented with 10 % horse serum. All tissue cultures were maintained in a humidified incubator at 37 ^{0}C under 5 % CO_2.

For the Northern blot and the mobility shift assay, $5x10^6$ GH3 cells were seeded to a poly-lysine-coated 10 cm cell culture dish to 70-80 % confluence. Thereafter, the cells were fasted in F-12K Ham's media supplemented with 0.5 % horse serum for 12-16 hours. Following serum starvation, the cells were treated with 20 μM forskolin for 0, 0.5, 1, 3, 6, 12 and 24 hours in F-12K Ham's media supplemented with 0.5 % horse serum. In some experiments, prior to the addition of the forskolin treatment, GH3 cells were treated with 30 μM N-[2-(p-Bromocinnamylamino) ethyl] 5- isoquinolinesulfonamide (H89), selective protein kinase A (PKA) inhibitor, for 2 hours.GH3 cells were treated with 10 μM for 0.5, 1, 3, and 6 hours under the same conditions described for forskolin treatment.

For the transfection experiments, $1x\ 10^6$ GH3 cells were counted and seeded to poly-L-lysine-coated 8 well plates.

3.3. Sequencing and analysis of the mouse CART gene promoter region

A *Bam*HI subclone, BAM5'C (approximately 125 kb in length), containing the entire CART gene was generated (Adams et al. 1999). The CART 5'-flanking region in BAM5'C (*Bgl*II/*Sac*I segment) was sequenced. All sequences were determined by cycle sequencing reactions.

The sequenced region was analyzed for putative transcription factor binding sites using the Transcription Factor Database (TRANSFAC) and MatInspector V2.2 (Wingender et al. 2000).

3.4. Generation of plasmid constructs

pGL3-Luciferase Reporter (Promega) and pSV-Galactosidase Control Vectors (Promega) provide a basis for the quantitative analysis of factors that potentially regulate mammalian gene expression. These factors may be *cis*-acting (promoters and enhancers) or *trans*-acting (various DNA-binding factors).

pGL3-Basic Luciferase Reporter Vector: This vector lacks eukaryotic promoter and enhancer sequences. Expression of luciferase activity in cells transfected with this plasmid depends on the promoter activity of the insertion in certain cells. Without insertions, this vector serves as a control for the background activity.

pGL3-Control Luciferase Reporter Vector: This vector contains SV40 promoter and enhancer sequences, resulting in strong expression of luciferase gene in many types of mammalian cells. The control vector serves as positive control to monitoring transfection efficiency.

pSV-Galactosidase C Control Vector: This vector is co-transfected with the DNA of interest and acts as an internal control for transient expression assays (Figure 3).

Cloning:

Various lengths of 5' upstream sequences were cloned into the promoter-less vector; pGL3-Basic itself and generated clones were tested for promoter activity. Constructs were generated by digesting the genomic clone, Bam5'C with *Bgl*II/*Nco*I, or *Kpn*I/*Sac*I, or *Sma*I/*Nco*I. pGL3-Basic was digested and dephosphorylated followed by ligation with the digested genomic fragment using T4 DNA ligase. The clones were transformed into *E. coli* TOP10 cells and plasmid DNA was isolated. Constructs were confirmed by dideoxy nucleotide sequencing.
The pGL3-luciferase expressing constructs made -3451 CART-LUC, spanning -3451 to +23, -641 CART-LUC, spanning -641 to +30, and -102 CART-LUC, spanning -102 to + 23 to +30 where +1 is the predicted site of transcription initiation. Furthermore, mutated 641CART-LUC with abrogated CRE site and CREB dominant negative mutant, (pCMV500-A-CREB) in which basic residues within the bZIP domain have been mutated to acidic residues, referred to as A-CREB were generated (Ahn et al. 1998)

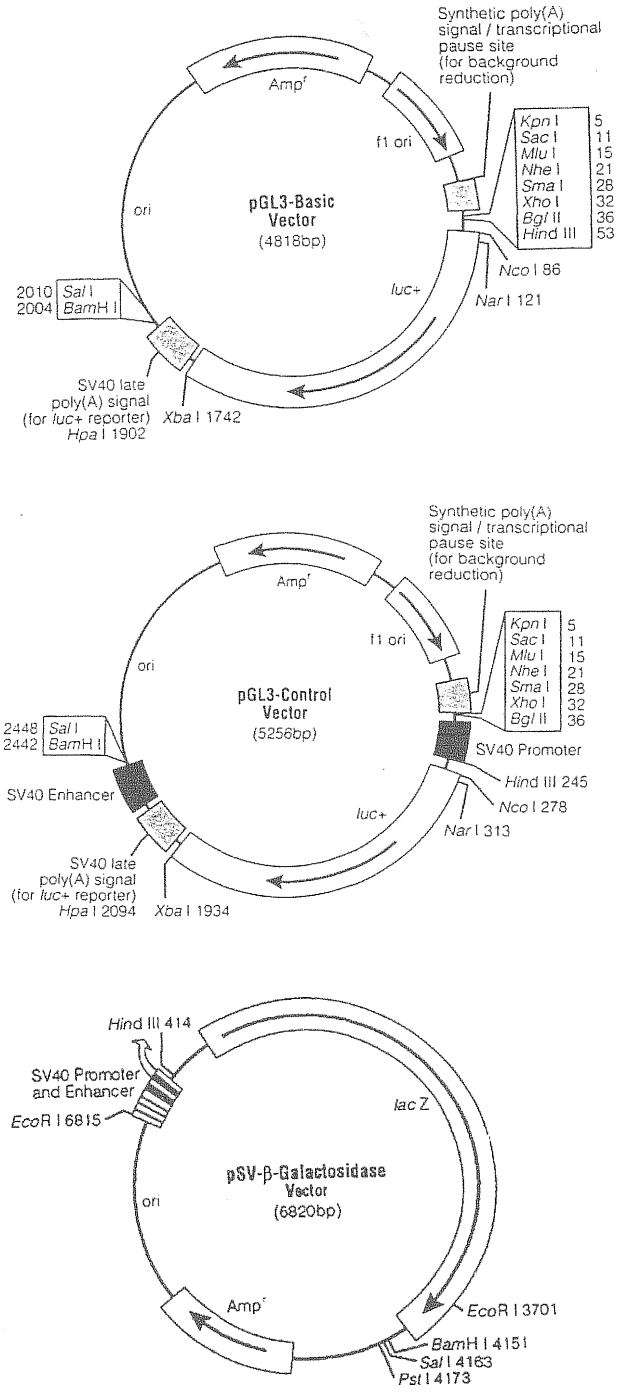


Figure 3. Schematic pictures of the vectors used for transfection experiments (see details in text).

3.5. Transfection

For each transfection, 2 μg of one of the pGL3-luciferase expressing constructs, 2μg of pSV-β-Galactosidase Vector and 6 μl of FuGENE 6 Transfection Reagent (Roche) were mixed in the appropriate serum-free media and incubated at 25 ^{0}C for 45 minutes following the manufacturer's instruction. 100 μl of the complexed DNA/FuGENE 6 mixture was added to each well and incubated for 18 hours at 37 ^{0}C under 5 % CO_2. In some cases, 2 ml of supplemented media containing 20 μM forskolin was added and incubated for additional amounts of time. Cells were lysed and luciferase expression measured using the Luciferase Assay System (Promega).

Luciferase activity was quantified using a luminometer. β-Galactosidase activity was measured using the β-Galactosidase Enzyme assay system (Promega) and the absorbance read at 420 nm. Protein concentration was determined using the Bradford method (Bio-Rad Protein Assay). In all experiments, luciferase expression was normalized to β-Galactosidase activity using equal protein concentration (Abbott et al. 2001). Statistical significance was determined using one-way ANOVA followed by a Tukey Test or a Student t-test (SigmaStat 2.0.).

3.6. RNase protection assay

10 μg of RNA from AtT20, GH3 and PC12 cells was dissolved in High-Speed Hybridization buffer (Ambion) and ^{32}P-CART RNA antisense probe was added to each sample. After 20 minutes hybridizing at 68 ^{0}C, the probe and RNA were digested with an RNase mixture at 37 ^{0}C for 30 minutes. After inactivation and precipitation of samples, the protected fragments were subjected to 8% denaturing polyacrylamide gel electrophoresis. Then, the gel was dried and exposed to X-ray film at minus 80 ^{0}C for 15 hours.

3.7. Northern blot assay

Total RNA was isolated from frozen rat cerebellum and hypothalamus or GH3 cells. Tissues (50-100 mg) were homogenized in 1 ml of Trizol Reagent (Life Tech.) using a power homogenizer. 10^7 GH3 cells were lysed in 1 ml of Trizol Reagent and RNA was isolated according to the manufacturer's recommendation. Total RNA was run in a 1.2 % formaldehyde agarose gel. RNA was transferred by capillary action in the presence of 10x SSC and fixed by UV cross-linking. Prehybridization was carried out at 50 ^{0}C in 6x SSC, 5x Denhardt's solution (Sigma), 1 % SDS, 20 % formamide, 50 μg/ml sonicated salmon sperm DNA (Amersham) for 6 hours. Hybridization buffer was changed and 2x 10^6 cpm of a ^{32}P-labeled cocktail consisting of 2 or 3 distinct oligonucleotides was incubated at 50 ^{0}C for 24 hours. Oligonucleotides were 5' end labeled by using γ-^{32}P-ATP (6000 Ci/mmol) and polynucleotide kinase (Stratagene). The CART cocktail consisted of three oligonucleotides with the following sequence: 5'TGAAAACAAGCACTTCAAGAGGAAAG3', 5'TGCAACGCTTCGATCTGCAACATAG3', 5'CTCATGCGCACTCTCTCCAGCG3'.
The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was made of two oligonucleotides having the following sequences:
5'AGTTGTCATTGAGAGCAATGCCAGC3', 5'AGTAGACTCACGACATACTCAGCA3'.
Nonspecific hybridization was removed by washing the blots with increasing stringency washes (5x SSC, 0.1% SDS up to 2x SSC, 0.1% SDS) at room temperature. Blots were analyzed by autoradiography. Blots were stripped and re-probed with the GAPDH probe. CART mRNA levels were normalized to GAPDH mRNA.

3.8. Western blot assay

Total protein was extracted in 100 μl of lysis buffer {1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 5 mM EDTA, and 0,02 % sodium azide} containing the following protease and phosphatase inhibitors: 5 nM ocadaic acid, 200 μM sodium othovanadate, 1x protease mixture (Maniatis) and 0.2 mM PMSF.

Equal amounts of 1x sample buffer (12.5 mM Tris-HCL (pH 7.2), 0. 4 % SDS, 0.2 M 2-mercaptoethanol, 0.5 mg bromphenol blue) were added to 25 µg of protein lysate, which was boiled for 5 minutes and loaded onto a 0.1 % SDS - 1x Tris-glycine gel.
The gel was run at 120V for 2 hours and then electrically transferred overnight (50V at 4 ^{0}C) onto Immobilon -P membrane (Millipore) using a transfer buffer consisting of 1x Tris-glycine. The blot was incubated in PBS-T blocking buffer (1.36 M NaCl, 2.7 mM KCl, 4.3 mM $Na_2 HPO_4$ x 7 H_2O, 1.4 mM KH_2PO_4, 0.01% Tween, pH:7.6) for 1 hour. After the initial blocking step, the blot was incubated in blocking buffer that contained anti-CREB antibody at a 1:500 dilution (Santa-Cruz) for 1 hour at room temperature. After incubating with the first antibody, the blot was washed at room temperature and then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody, anti-rabbit IgG (Santa Cruz), at 1:2000 dilutions in blocking buffer. CREB binding was detected using a chemiluminescent detection kit (ECL+Plus). The blot was washed with 0,2 M NaOH to remove previous protein complexes and re-incubated overnight at 4 °C with anti-phosphorylated - CREB antibody (P- CREB) (Cell Signaling) in blocking buffer. The blot was washed and incubated with a HRP - conjugated secondary antibody, goat-anti-mouse IgG, at a 1:2000 dilution for 1 hour at room temperature and developed using a chemiluminescent detection kit as described above.

3.9. Electrophoretic mobility shift assay (EMSA)

DNA-protein interactions were studied by EMSA. Nuclear protein extracts were prepared from forskolin-treated GH3 cells as described by Szeberényi et al. (1998) with the following modifications. These included the addition of phosphatase inhibitors: 5 nM ocadaic acid, 200 µM sodium orthovanadate, and protease inhibitors: 2 mM PMSF, 2.5 mM leupeptin, 1 µM pepstatin, and 1.5 µM aprotinin (Sigma). Total nuclear protein (15 µg) was incubated with 2 ng of ^{32}P-5'end-labeled oligonucleotide, containing the CART-CRE site (5'AGCATTGACGTCA3') in the binding buffer composed of 10 mM Tris-HCl (pH 7.5), 50mM NaCl, 1 mM EDTA, 5% glycerol, and 0,05 µg/µl Poly(d)I-C)) (Roche). Total nuclear protein extract and labeled oligonucleotide (approximately 1x10^5 cpm) were incubated for 10

minutes at room temperature. In some cases, a 20- or 100-fold molar excess of a specific competitor (nonlabeled CART oligo), or 100-fold molar excess of a nonspecific competitor Oct 1 (5' TGTTGAGGGGACTTTCCCAGC 3') was added to the mixture, to assess the specificity of the DNA-protein binding complex before the addition of the labeled oligonucleotide. After 10-minute incubation with unlabeled oligonucleotide, the ^{32}P-labeled CART oligonucleotide was added and incubation was continued for 20 minutes.

For the supershift assay, 2 μg of CREB antibody (Santa Cruz) was incubated with the nuclear extract and the ^{32}P-labeled CART oligonucleotide for 45 minutes. Supershift analysis was also done using 4 μg of P-CREB antibody (Santa Cruz). P-CREB antibody was incubated with the nuclear protein extract in binding buffer for 30 minutes at room temperature, followed by addition of the ^{32}P-labeled CART oligonucleotide and incubated for another 45 minutes. The DNA -protein complexes were separated by electrophoresis on a 6% non-denaturing polyacrylamide gel (1x TBE, 2.5% glycerol). Gels were run (120 V) in the presence of 0.5x TBE buffer for 1.5 hour at 4 ^{0}C. Dried gels were exposed for 24 hours and analyzed by using a PhosphoImager.

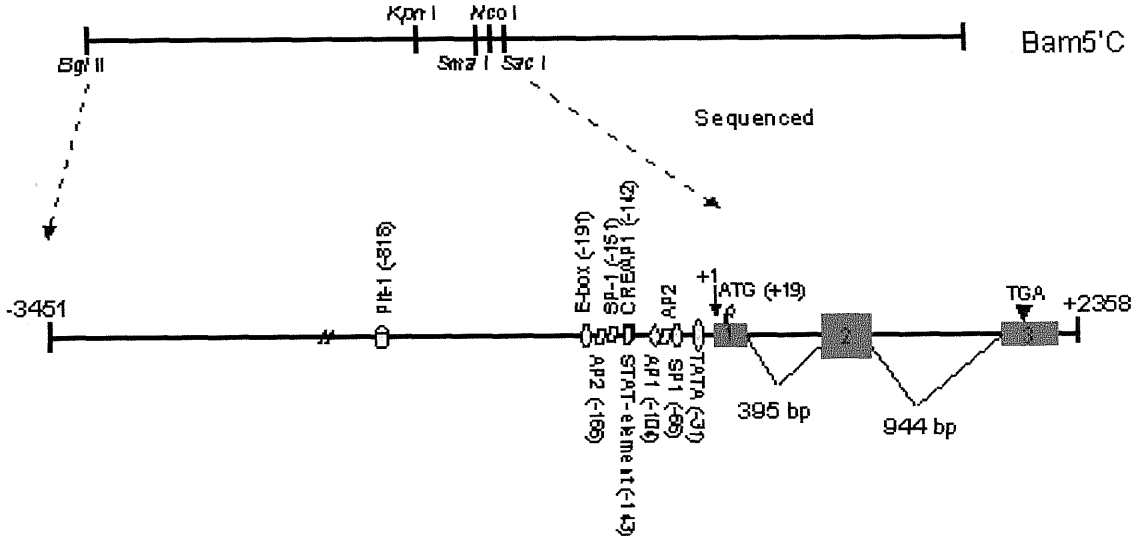
4. EXPERIMENTAL RESULTS

4.1. Computer search for enhancer elements in the CART promoter region

To understand the molecular mechanism involved in the transcriptional regulation of CART gene expression, a mouse BAC genomic library was screened using rat CART cDNA probe. One hybridization clone was identified (Adams et al.1999). A *Bam*HI subclone, BAM5'C, containing the entire CART gene was generated (Figure 4b). The CART 5' flanking region in BAM5'C (*Bgl*II / *SacI* segment) was sequenced. The sequenced region was analyzed for putative transcription factor binding sites. This analysis revealed a region in the mouse CART gene 5' upstream sequence containing a cluster of transcription factor binding sites, including a canonical cyclic AMP response element (CRE), two AP-1 sites, one SP1 and several AP2 sites, along with a TATA-like sequence and an E-box (Figure 4a). There is also a STAT-response element, $TT(N)_6AA$, in an overlapping STAT/CRE/AP1 site. Further upstream, there is a putative binding site for the pituitary-specific transcription factor Pit-1, AWWWTATNCAT, where W is either an A or T (Figure 4a).

The CART gene upstream sequence was further analyzed using a neural network promoter predicting algorithm (Ohler et al., 1999) in order to predict transcription initiation sites for the mouse CART gene. The transcription initiation for the mouse CART gene was predicted at the A located 19 nucleotides upstream of the initiating AUG codon (Figure 4b), in agreement with the previously published rat and human 5' ends (Douglass et al., 1995). The alignment of the mouse proximal promoter region with the corresponding human region (Accession Number NT019389) identified a 320 bp region immediately upstream from the CART coding sequence that shares 83.4% nucleotide identity. The region contains the binding site for the clustered set of transcription factors, including the overlapping CRE/AP1/STAT site (Figure 4a).

a)



b)

-320
Human GAAGGCATTT TCCATTtcct aggccctcc cgcttcccgT CCCTCACCTc TaGCCCCTCg
Mouse GAAGGCATTT TCCATT..... .....T CCCTCACCT t Tt GCCCCTCa

-260
Human GcTTCAGCTC aGGCCCCTcG GGgAGCaTCC CTtGCcGtGa GACtGACAGC cTTTgGGGGC
Mouse GtTTCAGCTC tGGCCCCTaG GGaAGCtTCC a

-200 E-box AP2
Human gCAGGGTCcT GTTcTCTGCG CTCTAGCC**CA TCTG**TGCGCA GAGCCTCGTT CCC**aGGCgCC**
Mouse aC gGGGTCtT GTTtTCTGCG CTCTAGCC**CA TCTG**TGCGCA GAGGCTCGTT CCC**gGGCtCC**

-160 STAT/CRE/AP-1 AP-1
Human tGGAaCCCGG **CGGGCATTGA CGTCAA**gCGc CgGCGGAGCG CTGCCTACAG ACGGt**TGACC**
Mouse cGGAgCCCGG **CGGGCATTGA CGTCAAa** CGgCaGCGGAGCG CTGCCTACAG ACGGcTGACC

-100
Human **CGGG**CcCTCC TCCACACCCC CtCCTTCTT CgCC**tCCTCC** CTCTTt CGtG CACggG ggcT
Mouse **CGGG**C tCTCC TCCACACCCC CgTCCTTCTT CtCC**cCCTCC** CTCTTcCCgG CACccGa t Tt

-40 TATA-box +1 +22
Human CgggCtcaC**TATAAa**AgGtg GGAGcGCGtg GTGCCCcAGC AaCGAcGAGt TtCAGAACgATG
Mouse C.aaCcggC**TATAAgA**aGcc GGAGaGCGca GTGCCCgAGC AgCGAgGAGg TcCAGAACcATG

Figure 4. Structure of the mouse CART gene and sequence of the CART promoter region. **(a)** A diagram of the region in Bam 5'C that was

sequenced. The cluster of the transcription factor binding sites is shown. The diagram is not drawn to scale. **(b)** Sequence alignment between human and mouse promoter region. Differences between human and mouse are in lower case. The transcription binding sites are indicated.

4.2. Assessing the transcription factor binding site for promoter activity

To examine the regulation of CART gene promoter, GH3 mouse pituitary adenoma cell line served as an *in vitro* model because these cells endogenously express CART mRNA measured by RNase protection assay. Furthermore, to compare the activities, predict tissue specificity, and determine the importance of certain CART promoter regions in the gene regulation, AtT20 and PC12 cell lines were used as well as an *in vitro* model.

The activity of various 5'-proximal regions of the CART gene was tested for the ability to drive gene expression when cloned into pGL3-Basic vector upstream of the luciferase gene. Three luciferase expressing constructs were made and tested, -3451CART-LUC, -641CART-LUC, and -102CART-LUC that contain 3451, 641, and 102 bp mouse CART 5' upstream sequence (+1 is the predicted site of transcription initiation), respectively (Figure 5).

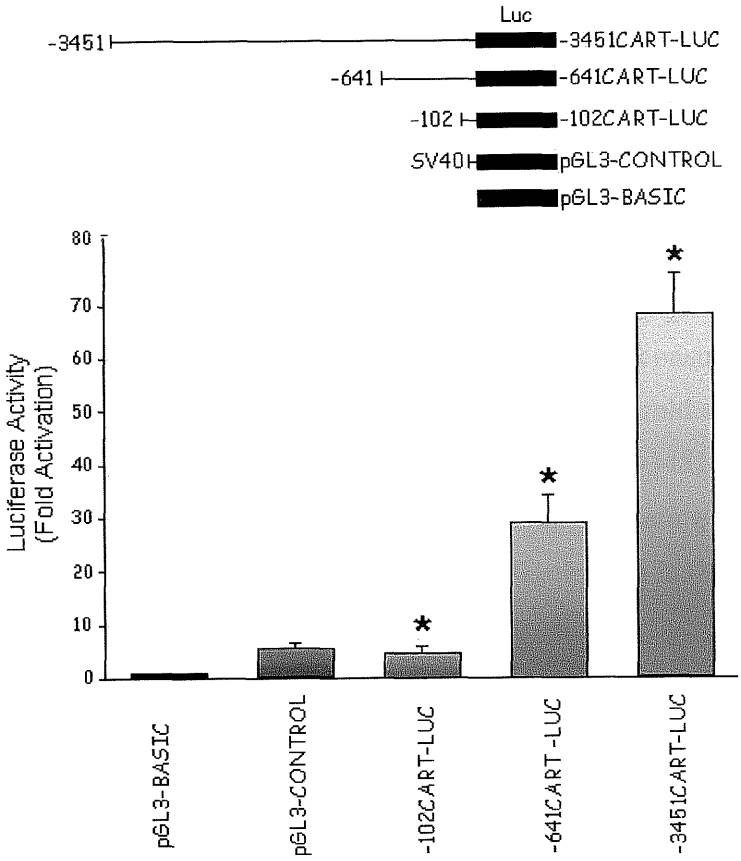


Figure 5. Luciferase expression constructs contain varying lengths of the CART promoter. Value of the activity of the three constructs is expressed as the folds increase relative to that found with pGL3-BASIC (see text for details). Statistical significance (*) was assessed using one-way ANOVA followed by a Tukey test (p=<0.001).

The -102CART-LUC construct contains the TATA-like box and an SP1 site. The -641CART-LUC construct contains the STAT/CRE/AP1 composite site and the -3451CART-LUC contains a putative Pit-1 binding site. Pit-1 is a transcription factor belonging to the POU

domain proteins that is expressed exclusively in the central nervous system and in the pituitary (Palmer et al. 2001).

Cells were transiently co-transfected with pSV-β-Galactosidase and one of the CART promoter containing constructs. Luciferase activity of each construct was compared to that produced by pGL3-BASIC, the parent vector that lacks a promoter for background activity. pGL3-CONTROL, which has SV40 promoter to drive luciferase expression, was used as a positive control. In GH3 cells, pGL3-CONTROL had a 6-fold increase in luciferase activity above pGL3-BASIC. This is similar to the 5.4-fold activation by -102CART-LUC, indicating that this segment of CART upstream sequence can function as a promoter and drive luciferase expression (Figure 4). Moreover, the -641CART-LUC construct containing a larger upstream segment of CART promoter produced much greater, 29-fold activity. A dramatic 59-fold increase in luciferase activity was observed after the -3451CART-LUC construct transfection, suggesting the importance of this region in mediating the promoter activity (Figure 5). Because the promoter analysis revealed a CRE element in the CART promoter, this element should respond to an elevated cAMP level in the cells.

To test this hypothesis, GH3 cells were treated with forskolin known to be an adenylate cyclase (AC) activator, an enzyme that converts adenosine triphosphate (ATP) to cAMP (Figure 2). It is well known that forskolin increases c-*fos* mRNA expression and as such the effect of forskolin on c-fos expression was tested (Treisman, 1996). The cells were treated with forskolin for 15, 30, 45 and 60 minutes. Northern blot analysis of the total mRNA showed a maximal expression after 30 minutes treatment (Data not shown).

Then, the endogenous CART gene was measured for responsiveness to forskolin treatment. GH3 cells were treated with 20 μM forskolin for 0, 1, 3, 6, 12 and 24 hours and 40 μg total RNA was analyzed by Northern blot analysis (Figure 6). Forskolin treatment increased CART mRNA levels compared to 0 hour, control exposure. The involvement of protein kinase A (PKA) was also tested. In the same experiment, some GH3 cells were treated with 30 μM H89, a selective inhibitor of PKA for 2 hours prior to and during forskolin treatment. The time course showed that the maximal increase in CART mRNA levels occurred after 6 hours of forskolin treatment. Treatment with H89 prior and during forskolin treatment reduced CART mRNA level up to 50% (Figure 6). This result confirms that cAMP is a second messenger for

positive regulation of CART gene expression and PKA as protein kinase is also involved in the hypothetical transcription pathways of CART gene.

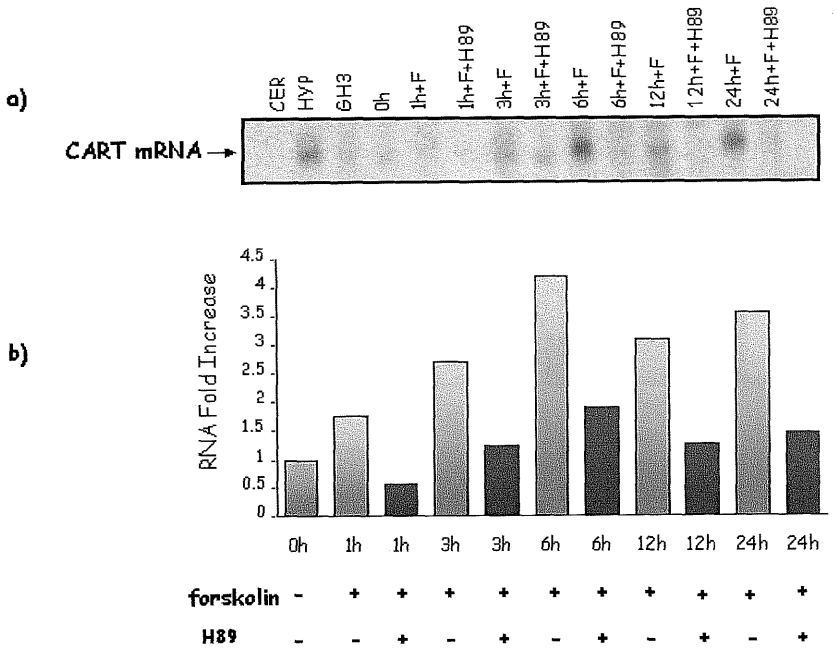


Figure 6. Changes in the endogenous CART mRNA level in response to 20 μMforskolin (F) and 30μM H89 treatments. a) Northern blot analysis of CART mRNA levels in GH3 cells at different times. b) CART mRNA levels were quantified and normalized to GAPDH levels.

To determine which cis-elements present in the CART promoter were responsible for the responsiveness to cyclic AMP induction by forskolin, two luciferase expressing constructs were tested. The -641CART-LUC and -102CART-LUC constructs were transfected into GH3 cells and 18 hours after transfection media was changed and cells were treated with 20μM forskolin for 7 hours. A 2-fold increase in luciferase activity after forskolin treatment was produced with -641CART-LUC, while no statistically significant increase in luciferase activity was observed with -102CART-LUC and pGL3-CONTROL (Figure 7). This suggests

that the region between -102 and -641 containing overlapping STAT/CRE/AP1 transcription factor binding sites that are responsive to cyclic AMP.

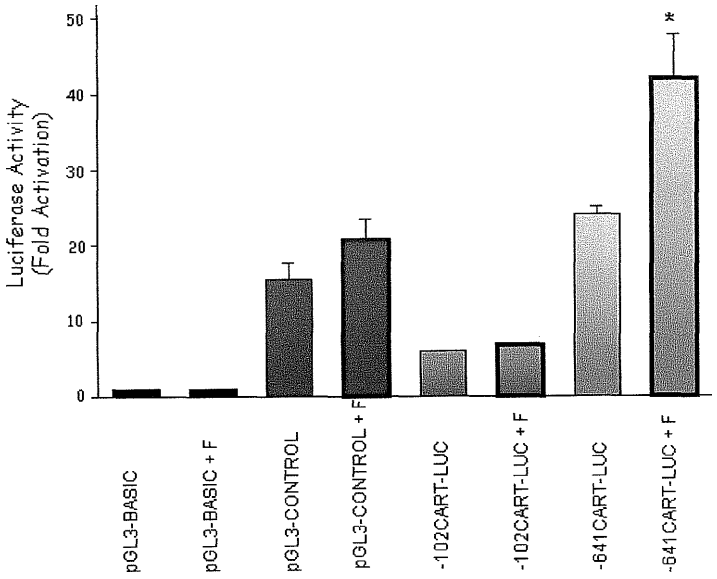


Figure 7. cAMP responsiveness of -102CART-LUC and -641CART-LUC in GH3 cells. GH3 cells were transiently transfected with each construct with or without 20 μM Forskolin (F) treatment. Luciferase activity was measured 7h after F treatment. Statistical significance was assessed using a student *t*-test ($p = 0.037$)

A comparison of luciferase activity between -641CART-LUC and pGL3-CONTROL after forskolin treatment showed that -641CART-LUC exhibits a continuous increase in expression beginning at 1 hour and continuing through 48 hours (Figure 8).

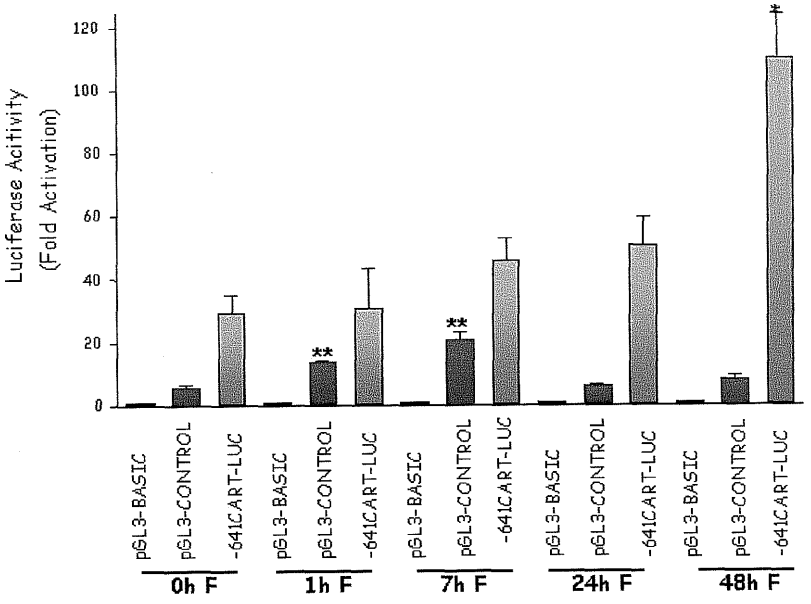


Figure 8. Time course of 20 μMforskolin (F) effect on -641-LUC in GH3 cells. Cells were cotransfected with pGL3-basic, pGL3-control and -641CART-LUC constructs. Luciferase activity were assessed at different time after F treatment. Each value is expressed as the fold increase relative to pGL3-BASIC. Statistical significance (*) was determined usin one-way ANOVA followed by a Tukey test. (**) indicates that the increase in the values of pGL3 -CONTROL at 1h and 7h are significantly different than the 0h time point (p=<0.05)

The promoter activity of -641CART-LUC was examined in AtT20 and PC12 cells as well. -641CART-LUC showed higher activity in PC12 cells than in AtT20 cells (Figure 9). However, promoter activity was the highest in GH3 cell (29-fold above background). Forskolin treatment did not have any significant effect on luciferase expression of -641CART-LUC construct in PC12 and AtT20 cells. The high activity of -641CART-LUC in GH3 cells,

and the lower activity in PC12 and AtT20 cells suggest that the STAT/CRE/AP1 overlapping transcription binding sites enhance expression in a cell type specific manner.

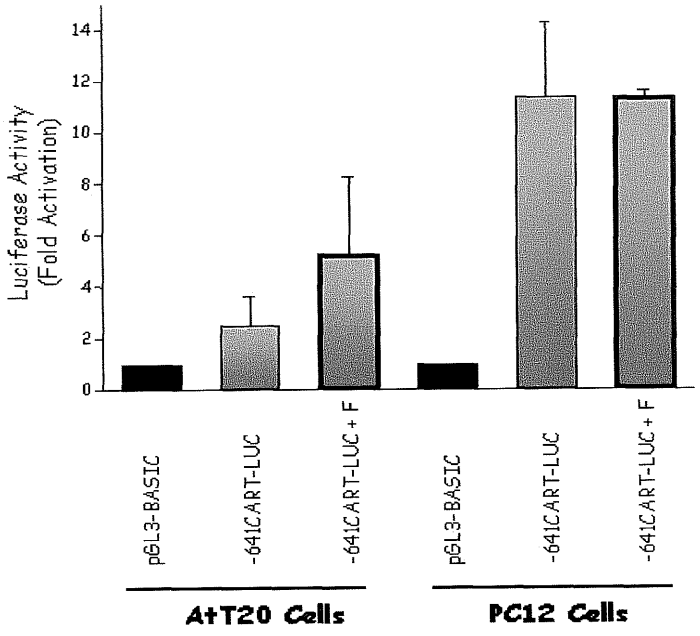


Figure 9. Comparison of the activity of -641CART-LUC in AtT20 and PC12 cells in the presence and absence of 20 μMforskolin (F) (for details see text).

Additionally, a mutation (see below, in Figure 11) that abrogated the CRE site in -641CART-LUC construct resulted in a 4.7-fold drop of luciferase expression in GH3 cells after forskolin treatment. (Figure 10). All these results together delineate the relevance of the CRE transcription binding site on the CART promoter in GH3 cells. Also, these findings propose that this overlapping STAT/CRE/AP1 site plays an important role in the determination of tissue specificity of CART gene expression.

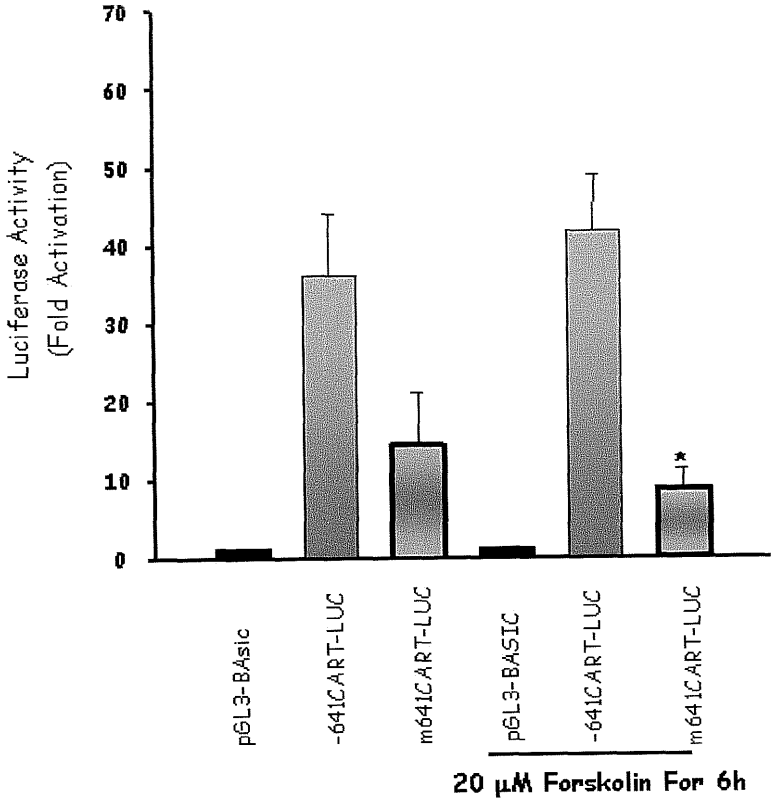


Figure 10. Mutation of 6 of the 8 bases of the CRE consensus (TGACGTCA) results in a 4.8 fold reduction in luciferase expression after forskolin treatment (statistical significance was determined by a Two-Sample student's t-test ($p < 0.05$ vs -641CART-LUC).

4.3. Characterizing the putative transcription factors and their involvement in the regulation of the CART gene

Subsequently, electromobility shift assays were carried out to identify in particular the transcription binding sites that are responsive to elevated cAMP level and responsible for the promoter activity in transfected GH3 cells and the induced endogenous CART mRNA expression after forskolin treatment. A 27-bp double-stranded oligonucleotide containing the CRE site, corresponding to nucleotides -153 to -127 of the CART promoter, was synthesized and 5' end labeled with ^{32}P, referred to as CART oligo (Figure 11).

In order to test if this CRE site binds protein factors from GH3 cells, nuclear extracts from cells treated with 20 μM forskolin for varying periods of time (0.5, 1 and 3 hours) were incubated with ^{32}P-labeled CART oligo. Treatment with forskolin under the same conditions has been shown to enhance endogenous CART gene expression in GH3 cells (Barrett et al 2001).

Figure 11. The structure of the CART promoter of the mouse gene showing several transcription factors binding sites including the overlapping STAT/CRE/AP1 site. The sequence of CART CRE oligo used in EMSA and the mutant oligo used to generate m641CART-LUC are given.

The ^{32}P-labeled CART promoter oligonucleotide clearly binds to nuclear factors in gels and results in the detection of a shifted DNA/protein complex (Figure 12). The binding increased with forskolin treatment time, and addition of 20x excess unlabeled CART oligo served to

assess the specificity of the DNA-protein binding complex. This experiment indicates that the GH3 cells, in response to forskolin treatment, produced increased binding to the CART promoter oligonucleotide.

Western blotting study was conducted to examine whether the treatment with forskolin under the same conditions produced changes in CRE binding protein (CREB) and/or phosphorylated-CREB (P-CREB) levels. CREB is a well-characterized transcription factor known to be activated by cAMP and binds to the CRE site of promoters (Figure 2) (Guitart et al. 1992; Bonni et al. 1995; Coven et al. 1998)

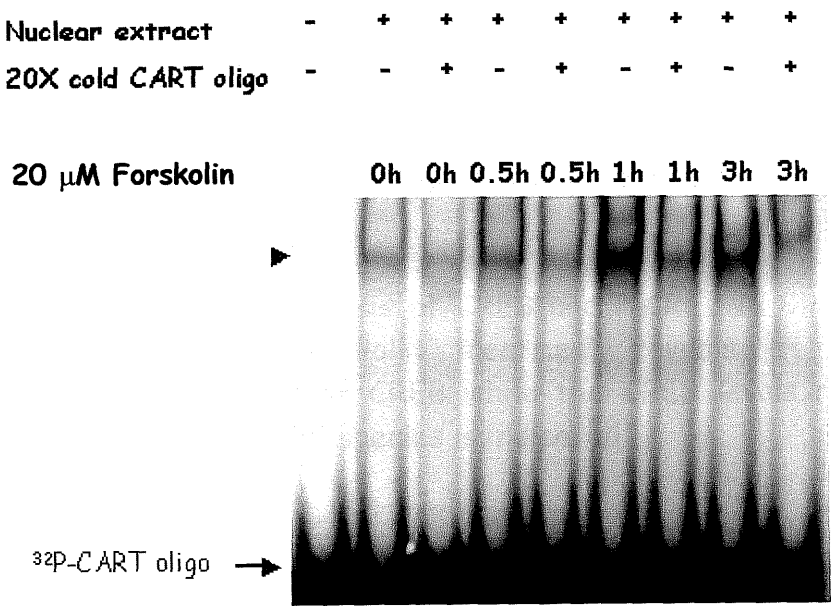


Figure 12. Autoradiogram illustrating the binding activity in nuclear extracts from GH3 cells with a ^{32}P-labeled CART CRE oligo. Forskolin treatment for varying amounts of time results in an increase in binding activity. The arrowhead indicates the CART oligo/protein complex (see the text for details).

Consequently, CREB became the first candidate for the transcription factor that can bind to the CRE site of CART proximal promoter and be accountable for the increased CART mRNA expression. It revealed an increased P-CREB level with duration of treatment with forskolin, while CREB levels remained unchanged (Figure 13). These results suggested that the nuclear protein binding to the CART promoter oligonucleotide could be P-CREB.

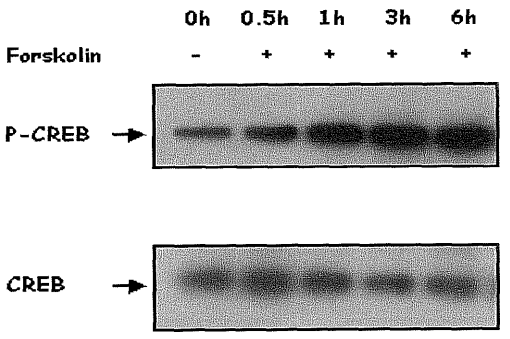


Figure 13. Western blot analysis of CREB and P-CREB in GH3 cells at various times after forskolin treatment reveals an increase in the phosphorylation of CREB while the CREB levels remain unchanged. The 43 kDa CREB species is indicated.

Accordingly, mobility supershift assay was used to detect if the protein binding to the CART promoter site would interact with CREB and P-CREB antibodies since an increase of P-CREB was observed by Western blot. It was found that CREB and P-CREB antibodies supershifted the CART oligo/protein complex (Figure 14). In addition, molar excess of unlabeled cold CART oligo competed with radiolabeled CART oligo, whereas an unrelated oligo (Oct1) did not, indicating specificity in the CART oligo/protein complex. The CART oligo/protein/CREB antibody complex was found in both untreated and treated cells as expected (Shaywitz and Greenberg 1999). Similar data were found after 0.5, 1 and 6 hours of forskolin treatment as well.

To confirm the role of CREB transcription factor in CART gene regulation, a CREB dominant negative mutant, A-CREB (Ahn et al., 1998) was co-transfected with the -641CART-LUC construct into GH3 cells treated with forskolin. A-CREB functions by heterodimerizing with endogenous CREB transcription factor and preventing its interaction with the CRE element. GH3 cells, co-transfected with the A-CREB mutant had a 3.4-fold reduction in luciferase expression as compared to cells co-transfected with the empty vector (Figure 15).

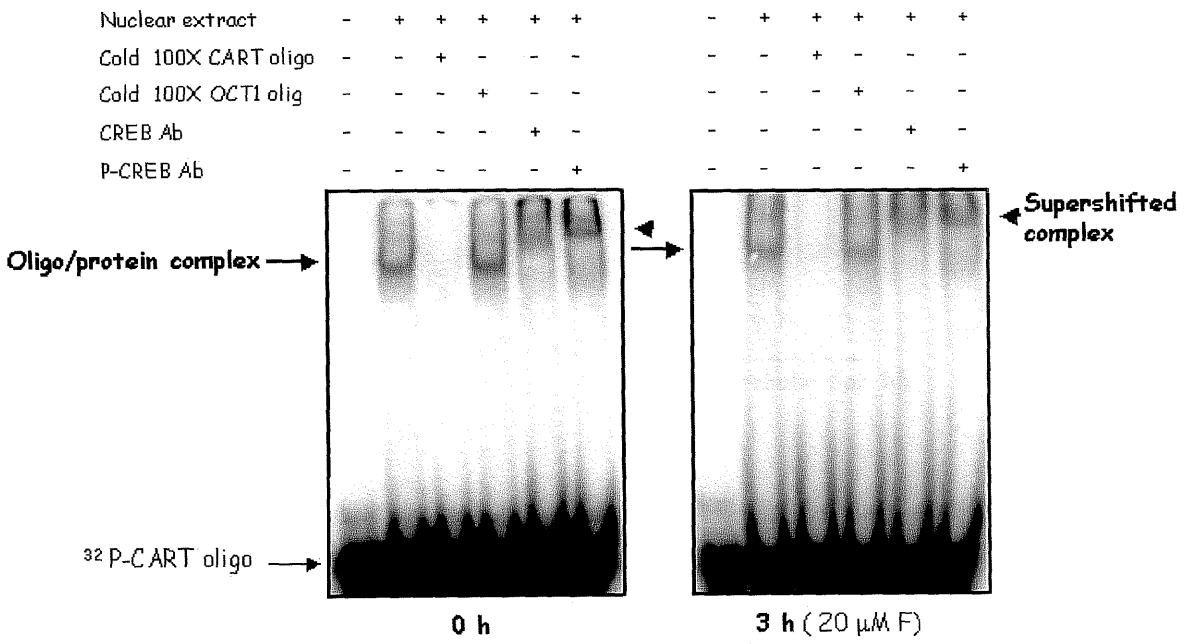


Figure 14. Supershift assay identifies CREB and P-CREB as components of the CART oligo/protein complex after forskolin treatmnet. Arrows indicate the CART oligo/protein complex while arrowheads indicate supershifted CART oligo/CREB and CART oligo/P-CREB complexes.

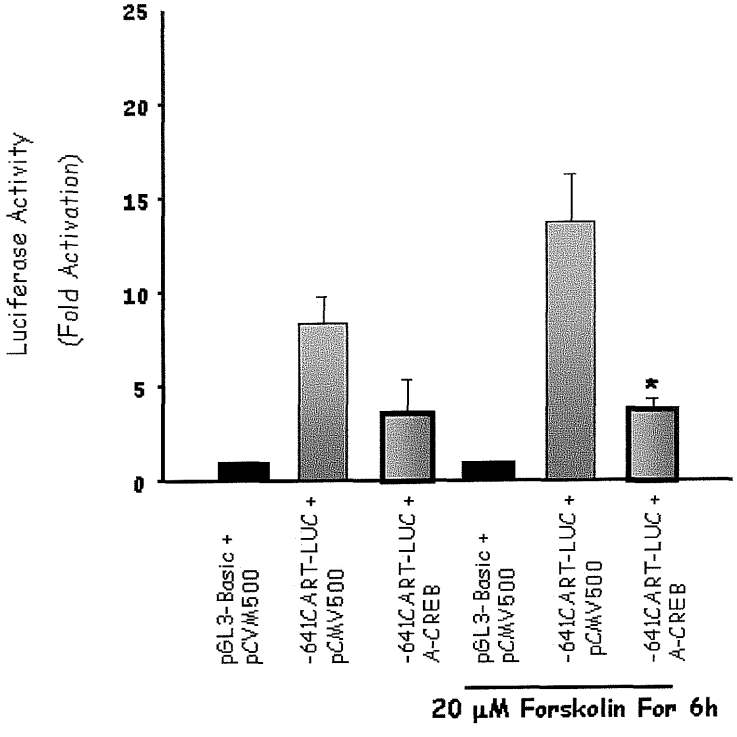


Figure 15. Luciferase expression from -641CART-LUC is decreased by 3.7 fold after forskolin treatment in the presence of a dominant negative mutant of CREB, A-CREB, which inhibits wild-type CREB from binding to DNA (Ahn et al., 1998). Statistical significance was determined by a Two-Sample student's t-test ($p < 0.05$ vs -641CART-LUC +pCMV500).

As a result of the experiments, in GH3 cells, the transcriptional regulation of the CART gene after activation of the cAMP pathway is likely to be mediated by the CREB transcription factor.

4.4. Investigating the importance of different signal transduction pathways in the regulation of CART gene

Ionomycin, an antibiotic has been reported to trigger the release of Ca^{2+} ions from the intracellular storage resulting in an increase of P-CREB (Yamaguchi, 2000.) (Figure 2). GH3 cells were treated with 10. μM ionomycin under the same condition as forskolin treatment. Northern blot analysis demonstrated a significantly changed expression pattern after 1 hour ionomycin treatment (Figure 16). It seems that Ca^{2+} ions are stronger activators for the CART gene than AC activation.

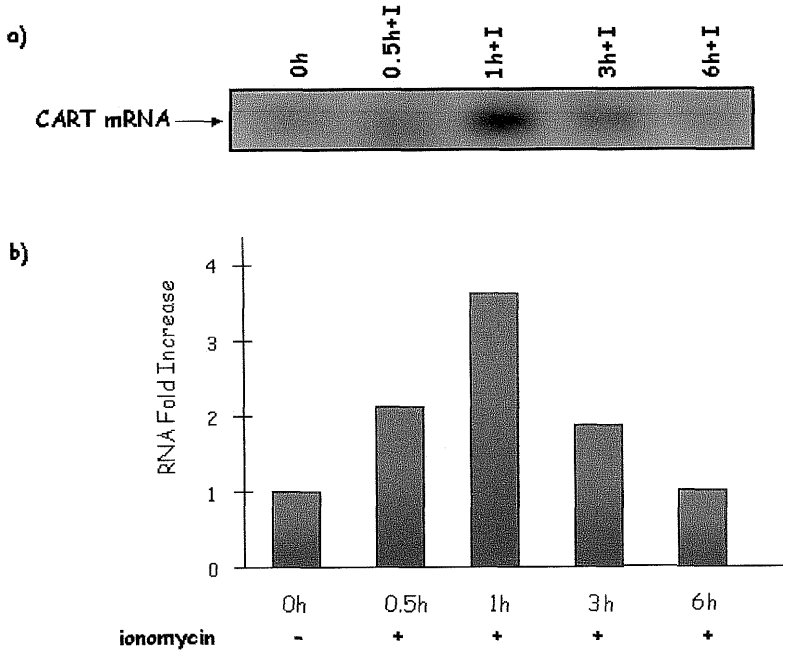


Figure 16. Changes in CART mRNA level in response to ionomycin (I) treatment.
a) Northern blot analysis of CART mRNA in GH3 cells at different time after ionomycin treatment.
b) CART mRNA levels were quantified and normalized to GAPDH. RNA fold increase was compared to 0h time point.

In order to test if the CRE site binds protein factors from ionomycin-treated extracts, GH3 cells were treated with 10 μM ionomycin for 0, 0.5,1 and 3 hours and were incubated with ^{32}P-labeled CART oligo. Nuclear proteins bound to ^{32}P-labeled CART oligo and these DNA/protein complexes were supershifted with CREB and P-CREB antibody (Figure 17). In the same gel, the 100x excess of unlabeled AP1 oligo (Promega) competed for the labeled CART oligo (Figure 17) indicating that the same nuclear factor or factors have binding affinity to the CART and AP-1 oligo.

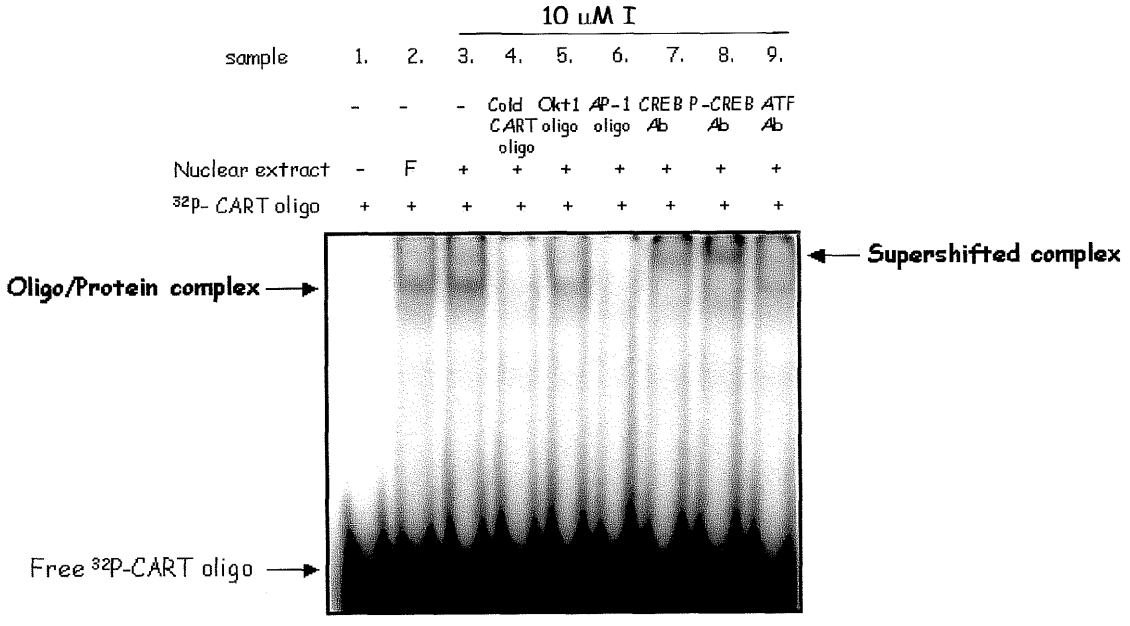


Figure 17. The binding activity of ^{32}P-CART oligo after 1h ionomycin treatment by EMSA (see text for details).

The binding activity of ^{32}P-CART after 1 h ionomycin treatment demonstrated increased binding to the probe. Since the sample 1 has no nuclear extract serves as negative control.

Sample 2 contains forskolin (F) treated cell extract that previously showed binding to the CART oligo (positive control). Sample 3 displayed complex formation in ionomycin treated cell extract. In the 4 and 5, the specificity of the CART oligo was detected. In sample 6, a competition between AP-1 and CART oligo is observed. In sample 7-8, the CART oligo/protein complex supershifted by CREB and P-CREB, respectively. ATF antibody failed to supershift CART oligo/protein complex in the sample 9.

.

5. DISCUSSION

CART mRNA expression has been reported to be altered in response to various stimuli including fasting, leptin, and psychostimulant drugs (Kuhar and Dall Vechia 1999; Johansen et al. 2000; Aja et al. 2001; Ludvigsen et al. 2001) but the signal transduction pathways involved in the CART mRNA regulation have not been studied yet.

The aim of this work was to characterize the CART promoter regulation by identifying putative transcription factor binding sites in order to describe signal transduction pathways involved in the regulation of CART gene expression. Sequence analysis of a 3.4 kb mouse genomic DNA fragment revealed a 320 bp region located upstream from the transcription start site, which is highly conserved between human and mouse CART gene. This region contains several transcription factor binding sites including an overlapping STAT/CRE/AP1 site located 148 nucleotides upstream from the predicted transcriptional initiation site. This binding may play an important role in the regulation of the CART gene. (Greenberg et al. 1999).

The transient transfection system is the primary tool for identifying and characterizing the interaction of *cis*-acting elements with their corresponding *trans*-acting factors and associated cofactors in mammalian cells (Qiu et al. 2002). In order to test the presumed promoter activity of the individual DNA fragment, different lengths of the mouse CART promoter were transfected for transcriptional activity into the GH3 rat adenoma line. This comparative transfection analysis revealed diverse transcriptional expression patterns after forskolin treatment in accordance with the different lengths of the CART promoter. These studies indicate that this individual DNA fragment displays significant promoter activity, moreover a major role for the overlapping STAT/CRE/AP1 transcription binding site in the regulation of the CART gene. This suggestion is supported by the mutation of the CRE site (Figure 11) in the -641CART-LUC construct that showed significant decrease (Figure 10) in the promoter activity. In addition, -3451CART-LUC displayed the highest promoter activity suggesting the importance of other transcription factor binding sites in CART gene expression

(Figure 5). Additionally, the transfection studies on AtT20 and PC12 cells showed promoter activity considerably reduced indicating a highly tissue – specific regulation of this promoter (Figure 9). Likewise, the Pit-1 binding site at position -818 on the mouse CART promoter suggest the Pit-1 transcription factor involvement in a cell-type specific transcriptional activation of the CART gene. Pit-1 is a POU-homeobox transcription factor that is responsible for either the commitment or maintenance of somatotrop, lactotrop, and thyreotrop cell lineages in the pituitary (Andersen et al. 1997; Rhodes et al. 1996).

To identify the possible regulation pathway/pathways, endogenous CART gene responsiveness to forskolin treatment was tested. Transcription activation of endogenous CART gene in GH3 cells showed a continuous increase after cyclic AMP activation. In accordance with the recent scientific viewpoint this elevation was probably due to PKA activation since forskolin turns on the cAMP-PKA pathway. PKA stimulates different transcriptions factors including CREB. CREB is known to bind to CRE elements in the promoter of number of genes (e.g. somatostatin and tyrosine aminotransferase) (Greenberg et al. 1999). Consequently, treatment with H89, an inhibitor of PKA, with simultaneous forskolin treatment, reduced the increase in the expression of endogenous CART mRNA. In summary, these studies together suggest that the CRE site in the CART proximal promoter could mediate the increased expression in CART mRNA levels in response to forskolin treatment via the PKA pathway.

Following this train of thoughts, it has been well described that the binding of cAMP to two PKA regulatory subunits, releases the catalytic subunits and enables them to phosphorylate target proteins. ATF/CREB family is one of the target proteins including several members, of which the CREB, CREM and ATF1 gene products are directly phosphorylated by the cAMP-dependent PKA. ATF/CREB family belongs to a larger leucine zipper family. This leucine zipper is a conserved structural motif at the C-terminus of these proteins formed by a heptad repeat of leucine residues (bZIP). This domain allows CREB, ATF1 and CREM to be able to bind to CRE sites as a dimer. The presence of highly conserved and related dimerization domains suggests that the different members of the CREB/ATF family might be able to form heterodimers and homodimers as well (Greeberg et al. 1999).

Furthermore, composite CRE/AP1 sites have been found in a number of genes in playing very important roles in neurotransmitter synthesis, including dopamine hydroxylase (Shaskus et al; 1992), prodynorphin (Messersmith et al; 1996), proenkephalin (Comb et al., 1986), and cholecystokinin (Hansen et al., 1999).

CREB is one of the main transcription factors involved in many genes' regulation in the brain; therefore, CREB became the first candidate, which may moderate the expression of CART gene via the PKA pathway by binding to the CRE site on the CART promoter. The results of mobility shift assays pointed out that treatment of GH3 cells with forskolin produced an increased level of P-CREB via the PKA pathway that binds to the CART promoter oligonucleotide containing CRE/AP1 site. These findings are very important because phosphorylation of CREB affects the ability of dimerization with different bZIP partners or the phosphorylation at Ser133 might trigger CREB binding to CRE (Greenberg et al. 1999). Transfection experiments with the dominant negative mutant CREB-A also supported a crucial role for CREB in the regulation of CART gene expression.

On the other hand, it is a well-known fact , that increase in the intracellular Ca^{2+} level causes an elevation of P-CREB (Shaywitz et al. 2000). Many kinases, some of whose activities are enhanced by Ca^{2} are capable of phosphorylating CREB *in vitro*, including Ca^{2+}/calmodulin-dependent kinases I, II, and IV (CaMKI,II, and IV) and PKC. The best characterized of the Ca^{2+}-activated CREB kinases are CaMKs. Phosphopeptide mapping indicates that all CaMKs phosphorylate CREB at Ser 133 *in vitro* (Finkbeiner et al. 1997; Shaywitz and Greenberg 1999; Kang et al. 2001; Wu et al. 2001; Arnould et al. 2002), the same residue that is also phosphorylated by PKA. In this study, it was also demonstrated that the increase of intracellular Ca^{2+} level by ionomycin treatment induced the gene expression of CART (Figure 16) through CREB recruitment to the CRE site of the promoter in GH3 cells (Figure 17). These observations indicate that the CART gene may be regulated by other signal transduction pathways activating CREB, not exclusively by cAMP-PKA. Additional studies are required to determine the precise role of intracellular Ca^{2+} in the regulation of the CART gene. Moreover, the mobility shift assay of ionomycin-treated cells (Figure 17) showed a competition between labeled CART probe and unlabeled AP-1 oligonucleotide indicating the possible role of other transcription factors (e.g. AP-1) in the regulation of CART gene expression.

The fact that other transcription factors can form complex with CRE can explain this phenomenon. After cAMP and Ca^{2+} stimuli, CREB makes different complexes involving several transcription factors. The molecular mechanism that accounts for the CRE/AP1 specificities of bZIP proteins has been a focus of research since this DNA recognition motif was first identified in 1987 (McKnight et al. 1991, Kerppola et al. 1991). The CRE (TGACGTCA) and AP1 (TGACTCA) target sites share the same consensus half-site and differ by only a single, central G-C base pair. It has been reported that the CRE/AP1 specificity of CREB is controlled by charged residues has interesting implications about how transcription factors seek and selectively bind to precise sequences within genomic DNA (Montclare et al. 2001) emphasizing the ease with which specificity can be altered through the formation of different heterodimers or through combinational interactions with cellular factors (McCabe and Burrell 2001).

Recently, *in vitro* binding studies have indicated that phosphorylation of CREB on Ser 133 does not alter the affinity of CREB for a palindromic CRE site (TGACGTCA; CART promoter has the same palindromic sequence), but it can be crucial at different levels of the transcription activation pathway for promoting target gene activation through recruitment of the 265-kDa, 2442 amino acid co-activator protein, CREB binding protein (CBP) (Jin et al. 2001). CBP co-activator is a member of acetyltransferase family (AT) including p300/CBP-associated factor (P/CAF), TAF_{II} 250, steroid receptor coactivator-1 (NCo-1/SRC-1), p300/CBP-interacting protein (P/CIF), and p160. These proteins are able to acetylate transcription factors (e.g. p53), histone proteins and other nuclear proteins (Perissi et al. 1999).

Based on studies, in accordance with our results, the Ser-133 phosphorylation of CREB is both necessary and sufficient for complex formation promoting the interaction between KIX domains. This interaction domain of CBP is required for binding to the P-CREB. CBP can serve as a molecular bridge that allows CREB to recruit and stabilize the RNA polymerase II (Pol II) transcription complex at the TATA box. Some studies have shown that the AT containing domain of CBP can only stimulate transcription from certain promoters and that the PKA activation of CREB required CBP, P/CAF and P/CIF. The diversity amongst the AT domains in different co-activators may account for their observed promoter selectivity (Fronsdal et al. 1998; Andrisani 1999; Aratani et al. 2001; Bhattacharjee et al. 2001; Dal

Peraro et al. 2001; Harton et al. 2001; Jin et al. 2001; McManus and Hendzel 2001; Yuan and Gambee 2001).

As mentioned above, CREB forms heterodimers with its family members (ATF1, CREM, ICER) (Cruzalegui et al. 1999; Shaywitz and Greenberg 1999; Bailey et al. 2000) and seems to make complexes with other factors binding to the CRE site, for example c- Jun and NF-κB through CBP (Shenkar et al. 2001). In this regard, our findings postulate a hypothesis in which the direct and indirect interaction of different transcription factors, co-activators with CREB involving the alternation of AT activity are also involved in the CART promoter regulation. Additional mutational analysis and mobility shift assay of the STAT/CRE/AP1 composite site with different transcription factors including CBP will be important in identifying which transcriptional binding sites and factors beside CREB are involved in the Ca^{2+}/cAMP responsiveness of CART promoter.

The present results may be of physiological significance since cocaine administration increases P-CREB levels in the brain (Blendy et .al. 1998) and increases CART mRNA levels in the striatum as well. Since the CART peptide appears to play a role in the action of diverse neurotransmitters and has itself multiple functions in the central and peripheral nervous system (Table 1), the further investigation of the regulation of the CART gene can elucidate the background of its multiple functions. On the other hand, using CART gene promoter studies as a model for investigating gene regulation, can provide a new approach helping to understand the complex regulation of genes and their expression in central nervous system.

6. SUMMARY OF NEW FINDINGS

1. Stimulation of the cAMP/PKA signal transduction pathway induces endogenous CART gene expression and activation of a 641 promoter fragment of the CART gene.

2. The CRE/AP1 site on the CART promoter binds proteins present in nuclear extracts from rat pituitary adenoma cells. CREB and P-CREB are demonstrated to have affinity to the CRE/AP1 site on the CART promoter.

3. Elevated intracellular Ca^{2+} level induces endogenous CART mRNA expression in rat pituitary adenoma cells.

7. REFERENCES

Abbott C. R., Rossi M., Wren A. M., Murphy K. G., Kennedy A. R., Stanley S. A., Zollner A. N., Morgan D. G., Morgan I., Ghatei M. A., Small C. J. and Bloom S. R. (2001) Evidence of an orexigenic role for cocaine- and amphetamine-regulated transcript after administration into discrete hypothalamic nuclei. *Endocrinology* **142,** 3457-3463.

Adams L. D., Gong W., Dall Vechia S., Hunter R.G., Kuhar M. J. (1999) CART: from gene to function *Brain Research* **848,** 137-140

Ahima R. S., Saper C. B., Flier J. S. and Elmquist J. K. (2000) Leptin regulation of neuroendocrine systems. *Front Neuroendocrinol* **21,** 263-307.

Ahn S, Olive M, Aggarwal S, Krylov D, Ginty DD, Vinson C. (1998) A dominant-negative inhibitor of CREB reveals that it is a general mediator of stimulus-dependent transcription of c-fos. *Mol Cell Biol*;**18**, 967-77

Aja S., Sahandy S., Ladenheim E. E., Schwartz G. J. and Moran T. H. (2001) Intracerebroventricular CART peptide reduces food intake and alters motor behavior at a hindbrain site. *Am J Physiol Regul Integr Comp Physiol* **281,** R1862-1867.

Andrisani O. M. (1999) CREB-mediated transcriptional control. *Crit Rev Eukaryot Gene Expr* **9,** 19-32.

Aratani S., Fujii R., Oishi T., Fujita H., Amano T., Ohshima T., Hagiwara M., Fukamizu A. and Nakajima T. (2001) Dual roles of RNA helicase A in CREB-dependent transcription. *Mol Cell Biol* **21,** 4460-4469.

Arnould T., Vankoningsloo S., Renard P., Houbion A., Ninane N., Demazy C., Remacle J. and Raes M. (2002) CREB activation induced by mitochondrial dysfunction is a new signaling pathway that impairs cell proliferation. *Embo J* **21,** 53-63.

Asakawa A., Inui A., Yuzuriha H., Nagata T., Kaga T., Ueno N., Fujino M. A. and Kasuga M. (2001) Cocaine-amphetamine-regulated transcript influences energy metabolism, anxiety and gastric emptying in mice. *Horm Metab Res* **33,** 554-558.

Asnicar MA, Smith DP, Yang DD, Heiman ML, Fox N, Chen YF, Hsiung HM, Koster A. (2001) Absence of cocaine- and amphetamine-regulated transcript results in obesity in mice fed a high caloric diet. *Endocrinology* **142**, 4394-400

Bailey J., Sparey C., Phillips R. J., Gilmore K., Robson S. C., Dunlop W. and Europe-Finner G. N. (2000) Expression of the cyclic AMP-dependent transcription factors, CREB, CREM

ınd ATF2, in the human myometrium during pregnancy and labour. *Mol Hum Reprod* **6,** 648-560.

Balkan B., Koylu E. O., Kuhar M. J. and Pogun S. (2001) The effect of adrenalectomy on cocaine and amphetamine-regulated transcript (CART) expression in the hypothalamic nuclei of the rat. *Brain Res* **917,** 15-20.

Bhattacharjee R. N., Underhill C. and Torchia J. (2001) Isolation of a p300/CBP cointegrator-associated protein coactivator complex. *Methods Mol Biol* **176,** 249-260.

Bonni A., Ginty D. D., Dudek H. and Greenberg M. E. (1995) Serine 133-phosphorylated CREB induces transcription via a cooperative mechanism that may confer specificity to neurotrophin signals. *Mol Cell Neurosci* **6,** 168-183.

Broberger C. (1999) Hypothalamic cocaine- and amphetamine-regulated transcript (CART) neurons: histochemical relationship to thyrotropin-releasing hormone, melanin-concentrating hormone, orexin/hypocretin and neuropeptide Y. *Brain Res* **848,** 101-113.

Brunetti L., Orlando G., Michelotto B., Recinella L. and Vacca M. (2000) Cocaine- and amphetamine-regulated transcript peptide-(55-102) and thyrotropin releasing hormone inhibit hypothalamic dopamine release. *Eur J Pharmacol* **409,** 103-107.

Carlezon Jr. W. A., Thome J., Olson V. G., Lane –Ladd S. B., Brodkin E. S., Hiroi N., Duman R.S.,Neve R. L., Nestler E. J. (1998) Regulation of cocaine reward by CREB *Science* **282**, 2272-75

Challis BG, Yeo GS, Farooqi IS, Luan J, Aminian S, Halsall DJ, Keogh JM, Wareham NJ, O'Rahilly S. (2000) The CART gene and human obesity: mutational analysis and population genetics. *Diabetes* **49**, 872-5

Couceyro P., Paquet M., Koylu E., Kuhar M. J. and Smith Y. (1998) Cocaine- and amphetamine-regulated transcript (CART) peptide immunoreactivity in myenteric plexus neurons of the rat ileum and co-localization with choline acetyltransferase. *Synapse* **30,** 1-8.

Couceyro P. R., Koylu E. O. and Kuhar M. J. (1997) Further studies on the anatomical distribution of CART by in situ hybridization. *J Chem Neuroanat* **12,** 229-241.

Coven E., Ni Y., Widnell K. L., Chen J., Walker W. H., Habener J. F. and Nestler E. J. (1998) Cell type-specific regulation of CREB gene expression: mutational analysis of CREB promoter activity. *J Neurochem* **71,** 1865-1874.

Cruzalegui F. H., Hardingham G. E. and Bading H. (1999) c-Jun functions as a calcium-regulated transcriptional activator in the absence of JNK/SAPK1 activation. *Embo J* **18,** 1335-1344.

Dal Peraro M., Alber F. and Carloni P. (2001) Ser133 phosphate-KIX interactions in the CREB-CBP complex: an ab initio molecular dynamics study. *Eur Biophys J* **30,** 75-81.

del Giudice EM, Santoro N, Cirillo G, D'Urso L, Di Toro R, Perrone L.(2001) Mutational screening of the CART gene in obese children: identifying a mutation (Leu34Phe) associated with reduced resting energy expenditure and cosegregating with obesity phenotype in a large family. *Diabetes* **50** 2157-60

Douglass J. and Daoud S. (1996) Characterization of the human cDNA and genomic DNA encoding CART: a cocaine- and amphetamine-regulated transcript. *Gene* **169,** 241-245.

Elias C. F., Kelly J. F., Lee C. E., Ahima R. S., Drucker D. J., Saper C. B. and Elmquist J. K. (2000) Chemical characterization of leptin-activated neurons in the rat brain. *J Comp Neurol* **423,** 261-281.

Finkbeiner S., Tavazoie S. F., Maloratsky A., Jacobs K. M., Harris K. M. and Greenberg M. E. (1997) CREB: a major mediator of neuronal neurotrophin responses. *Neuron* **19,** 1031-1047.

Fronsdal K., Engedal N., Slagsvold T. and Saatcioglu F. (1998) CREB binding protein is a coactivator for the androgen receptor and mediates cross-talk with AP-1. *J Biol Chem* **273,** 31853-31859.

Guitart X., Thompson M. A., Mirante C. K., Greenberg M. E. and Nestler E. J. (1992) Regulation of cyclic AMP response element-binding protein (CREB) phosphorylation by acute and chronic morphine in the rat locus coeruleus. *J Neurochem* **58,** 1168-1171.

Harton J. A., Zika E. and Ting J. P. (2001) The histone acetyltransferase domains of CREB-binding protein (CBP) and p300/CBP-associated factor are not necessary for cooperativity with the class II transactivator. *J Biol Chem* **276,** 38715-38720.

Jin K., Mao X. O., Simon R. P. and Greenberg D. A. (2001) Cyclic AMP response element binding protein (CREB) and CREB binding protein (CBP) in global cerebral ischemia. *J Mol Neurosci* **16,** 49-56.

Johansen J. E., Broberger C., Lavebratt C., Johansson C., Kuhar M. J., Hokfelt T. and Schalling M. (2000) Hypothalamic CART and serum leptin levels are reduced in the anorectic (anx/anx) mouse. *Brain Res Mol Brain Res* **84,** 97-105.

Kang H., Sun L. D., Atkins C. M., Soderling T. R., Wilson M. A. and Tonegawa S. (2001) An important role of neural activity-dependent CaMKIV signaling in the consolidation of long-term memory. *Cell* **106,** 771-783.

Koylu E. O., Couceyro P. R., Lambert P. D. and Kuhar M. J. (1998) Cocaine- and amphetamine-regulated transcript peptide immunohistochemical localization in the rat brain. *J Comp Neurol* **391,** 115-132.

Koylu E. O., Couceyro P. R., Lambert P. D., Ling N. C., DeSouza E. B. and Kuhar M. J. (1997) Immunohistochemical localization of novel CART peptides in rat hypothalamus, pituitary and adrenal gland. *J Neuroendocrinol* **9,** 823-833.

Kristensen P., Judge M. E., Thim L., Ribel U., Christjansen K. N., Wulff B. S., Clausen J. T., Jensen P. B., Madsen O. D., Vrang N., Larsen P. J. and Hastrup S. (1998) Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature* **393,** 72-76.

Kuhar M. J. and Yoho L. L. (1999) CART peptide analysis by Western blotting. *Synapse* **33,** 163-171.

Kuhar MJ, Adams S, Dominguez G, Jaworski J, Balkan B. (2002) CART peptides. *Neuropeptides* **36,** 1-8

Kuhar M. J. and Dall Vechia S. E. (1999) CART peptides: novel addiction- and feeding-related neuropeptides. *Trends Neurosci* **22,** 316-320.

Kuhar M. J, Adams L. D., Gong W., Hunter R.G., Dall Vechia S., Yoland Smith (2000) CART peptides. *Regulatory Peptides* **89,** 1-6

Ludvigsen S., Thim L., Blom A. M. and Wulff B. S. (2001) Solution structure of the satiety factor, CART, reveals new functionality of a well-known fold. *Biochemistry* **40,** p9082-9088.

McCabe J. T. and Burrell A. S. (2001) Alterations of AP-1 and CREB protein DNA binding in rat supraoptic and paraventricular nuclei by acute and repeated hyperosmotic stress. *Brain Res Bull* **55,** 347-358.

McManus K. J. and Hendzel M. J. (2001) CBP, a transcriptional coactivator and acetyltransferase. *Biochem Cell Biol* **79,** 253-266.

Nankova B, Kvetnansky R, McMahon A, Viskupic E, Hiremagalur B, Frankle G, Fukuhara K, Kopin IJ, Sabban EL.(1996) Induction of tyrosine hydroxylase gene expression by a nonneuronal nonpituitary-mediated mechanism in immobilization stress.*Mol. Brain Research* **35**, 164*172

Palmer G., Manen D., Bonjour J. P. and Caverzasio J. (2001) Species-specific mechanisms control the activity of the Pit1/PIT1 phosphate transporter gene promoter in mouse and human. *Gene* **279,** 49-62.

Qui Ping and Li Li (2002) Histone acetylation and recruitment of serum responsive factor and CREB-binding protein onto SM22 promoter during SM22 gene expression *Circ Research* **90**,858-865

Peraro Dal M, Alber F, Carloni P. (2001) Ser133 phosphate-KIX interactions in the CREB-CBP complex: an ab initio molecular dynamics study *Eur Biophys J*; **30**(1):75-81

Perissi V, Dasen JS, Kurokawa R, Wang Z, Korzus E, Rose DW, Glass CK, Rosenfeld MG (1999) Factor-specific modulation of CREB-binding protein acetyltransferase activity *Proc Natl Acad Sci* U S A ;**96**(7):3652-7

Sergeyev V., Broberger C. and Hokfelt T. (2001) Effect of LPS administration on the expression of POMC, NPY, galanin, CART and MCH mRNAs in the rat hypothalamus. *Brain Res Mol Brain Res* **90,** 93-100.

Shaywitz A. J. and Greenberg M. E. (1999) CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu Rev Biochem* **68,** 821-861.

Shaywitz A. J., Dove S. L., Kornhauser J. M., Hochschild A., and Greenberg M. E (2000) *Mol and Cell Biology* **20,** 9409-9422

Shenkar R., Yum H. K., Arcaroli J., Kupfner J. and Abraham E. (2001) Interactions between CBP, NF-kappaB, and CREB in the lungs after hemorrhage and endotoxemia. *Am J Physiol Lung Cell Mol Physiol* **281,** L418-426.

Smith Y., Koylu E. O., Couceyro P. and Kuhar M. J. (1997) Ultrastructural localization of CART (cocaine- and amphetamine-regulated transcript) peptides in the nucleus accumbens of monkeys. *Synapse* **27,** 90-94.

Smith Y., Kieval J., Couceyro P. R. and Kuhar M. J. (1999) CART peptide-immunoreactive neurones in the nucleus accumbens in monkeys: ultrastructural analysis, colocalization studies, and synaptic interactions with dopaminergic afferents. *J Comp Neurol* **407,** 491-511.

Thim L., Kristensen P., Larsen P. J. and Wulff B. S. (1998a) CART, a new anorectic peptide. *Int J Biochem Cell Biol* **30,** 1281-1284.

Thim L., Kristensen P., Nielsen P. F., Wulff B. S. and Clausen J. T. (1999) Tissue-specific processing of cocaine- and amphetamine-regulated transcript peptides in the rat. *Proc Natl Acad Sci U S A* **96,** 2722-2727.

Thim L., Nielsen P. F., Judge M. E., Andersen A. S., Diers I., Egel-Mitani M. and Hastrup S. (1998b) Purification and characterisation of a new hypothalamic satiety peptide, cocaine and amphetamine regulated transcript (CART), produced in yeast. *FEBS Lett* **428,** 263-268.

Trayhurn P., Hoggard N., Mercer J. G. and Rayner D. V. (1999) Leptin: fundamental aspects. *Int J Obes Relat Metab Disord* **23 Suppl 1,** 22-28.

Volkoff H, Peter RE. (2001) Characterization of two forms of cocaine- and amphetamine-regulated transcript (CART) peptide precursors in goldfish: molecular cloning and distribution, modulation of expression by nutritional status, and interactions with leptin. *Endocrinology* **142,** 5076-88

Walder K., Morris C. and Ravussin E. (2000) A polymorphism in the gene encoding CART is not associated with obesity in Pima Indians. *Int J Obes Relat Metab Disord* **24,** 520-521.

Widnell KL, Self DW, Lane SB, Russell DS, Vaidya VA, Miserendino MJ, Rubin CS, Duman RS, Nestler EJ. (1996) Regulation of CREB expression: in vivo evidence for a functional role in morphine action in the nucleus accumbens. *J Pharmacol Exp Ther* **276**, 306-15

Wu G. Y., Deisseroth K. and Tsien R. W. (2001) Activity-dependent CREB phosphorylation: convergence of a fast, sensitive calmodulin kinase pathway and a slow, less sensitive mitogen-activated protein kinase pathway. *Proc Natl Acad Sci U S A* **98,** 2808-2813.

Yamada K., Yuan X., Otabe S., Koyanagi A., Koyama W. and Makita Z. (2002) Sequencing of the putative promoter region of the cocaine- and amphetamine-regulated-transcript gene and identification of polymorphic sites associated with obesity. *Int J Obes Relat Metab Disord* **26,** 132-136.

Yermolaieva O, Chen J, Couceyro PR, Hoshi T. (2001) Cocaine- and amphetamine-regulated transcript peptide modulation of voltage-gated Ca2+ signaling in hippocampal neurons. *J Neurosci* **21**, 7474-80

Yuan L. W. and Gambee J. E. (2001) Histone acetylation by p300 is involved in CREB-mediated transcription on chromatin. *Biochim Biophys Acta* **1541,** 161-169.

3. ABBREVIATIONS

AC, adenylate cyclase

AP-1, activator protein 1

ATP, adenosine triphosphate

CaMK, Ca^{2+} induced calmodulin kinase

cAMP, cyclic adenosine 3', 5'-monophosphate

CART, cocaine.- and amphetamine-regulated transcript

CBP, CREB binding protein

CRE, cAMP response enhancer element

CREB, CRE binding protein

DMEM, Dulbecco's modified eagle Medium

EMSA, Electrophoretic mobility shift assay

F-12K Ham's, Kaighn's modification media

GABA, γ-amino-butyric acid

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

KIX, kinase interaction domain of CBP

MHC, melanin-concentrating hormone

mRNA, messenger RNA

NFκB, nuclear factor κB

NGF, nerve growth factor

PKA, protein kinase A, cAMP-activated protein kinase

PKB, protein kinase B

PKC, protein kinase C

POMC, pro-opiomelanocortin

STAT, signal transducer and activators of transcription

VTA, ventral tegmental area

9. ACKNOWLEDGEMENTS

I am greatly indebted to my advisor Dr. József Szeberényi for his enormous help, understanding, guidance, and for being a great teacher.

I would like to thank Dr. Michael J. Kuhar for the resources he provided and the opportunity to join his wonderful team. I extend my sincere gratitude to Dr. Geraldina Dominguez whose help was instrumental to me in acquiring certain but critical experimental skills.

Many thanks to Drs Larry J. Young, Beáta Töröcsik, István Lengvári, Alexandra Vincentic, Jeremy M. Boss, Andrea Lubics, Dóra Reglődi, Gábor Pethő, Andrea Nagy, Norbert Miskolczi for the productive discussions/support and their freindship.

My appreciation goes to the faculty, staff and students of the Department of Medical Biology, School of Medicine, Pécs University and Yerkes National Primate Research Center at Emory University for their support and assistance during my work.

I am devoted to my family, my Godparents Mrs. and Mr. András Cservenyák and my husband Dosu Doherty for their help and support toward achieving my goals.

10. PUBLICATIONS USED AS A BASIS FOR THE PRESENT THESIS

Geraldina Dominguez, Anita Lakatos, and Michael J. Kuhar (2002)
Characterizarion of the CART peptide gene promoter and its activation by a cyclic AMP-dependent signaling pathway in GH3 cells *J Neurochemistry* **80,** 880-893

Anita Lakatos, Geraldina Dominguez, Michael J.Kuhar (2002)
CART promoter CRE site binds phophoCREB *Brain Res Mol Brain Res.* **104**, 81-5

ırnal of Neurochemistry, 2002, **80**, 885–893

'haracterization of the cocaine- and amphetamine-regulated 'anscript (CART) peptide gene promoter and its activation y a cyclic AMP-dependent signaling pathway in GH3 cells

ɛraldina Dominguez,* Anita Lakatos† and Michael J. Kuhar*

'ivision of Neuroscience, Yerkes Research Center, Atlanta, Georgia, USA
'epartment of Biology, University Medical School of Pécs, Pécs, Hungary

ıstract

caine- and amphetamine-regulated transcript (CART) ɔtides are regulated neuropeptides that play a role in a 'iety of physiological processes. CART mRNA is also highly ɹulated as its levels change in response to psychostimulant ıgs and leptin. To understand the mechanisms involved in ɹulating CART mRNA levels, the mouse CART 5′-flanking ɹulatory region was studied. The sequence of 3.4 kb of the ıuse CART 5′-flanking region revealed a proximal promoter t contains a cluster of transcription factor binding sites, luding an overlapping STAT/CRE/AP1 site. In addition, the nost 320 bp of the CART promoter shares 83% nucleotide ntity between mouse and human. Three luciferase ɔressing constructs containing varying amounts of CART 5′ ɜtream sequence were generated and tested for promoter activity. Transient transfection of GH3 cells with constructs containing 641 and 3451 bp of upstream sequence displayed strong promoter activity, producing 29-fold and 51-fold stimulation, respectively, while, a construct containing 102 bp of upstream sequence displayed a 5.4-fold increase in activity. A construct containing the composite STAT/CRE/AP1 site was responsive to cyclic AMP induction by forskolin in GH3 cells. Forskolin treatment also resulted in a 4.5-fold increase in CART mRNA levels after 6 h and the addition of H89, an inhibitor of protein kinase A, reduced the levels by 50%. These studies indicate that the CART proximal promoter lies within the 5′-most 641 bp and that in GH3 cells the CART gene is regulated via a cyclic AMP-dependent pathway.

Keywords: AP1, CART, CRE, cyclic AMP, PKA, promoter.
J. Neurochem. (2002) **80,** 885–893.

.RT (cocaine- and amphetamine-regulated transcript) is a hly regulated mRNA, and CART peptides are regulatory ɔtides that are expressed only in neurons and not other ls in the nervous system. CART mRNA expression is ulated by psychostimulant drugs (Douglass *et al.* 1995; ɛergren and Hurd 1999; Brenz Verca *et al.* 2001) as well by leptin (Kristensen *et al.* 1998; Wang *et al.* 1999; ima and Hileman 2000; Dhillon *et al.* 2000) and sumably by other stimuli. CART mRNA and its cleaved ıtides are widely distributed in the brain and other locrine tissues, including the pituitary and adrenals ɔuglass *et al.* 1995; Koylu *et al.* 1997). CART peptides thought to have a role in multiple physiological functions, luding reward and reinforcement, feeding, stress, autonic and endocrine control, and sensory processing (Kuhar Dall Vechia 1999; Thim *et al.* 1999; Kuhar *et al.* 2000). ıaracterization of the mechanisms involved in CART e regulation will aid in understanding CART peptides' neostasis.

The rat, human, and mouse CART genes have been identified and sequenced (Douglass and Daoud 1996; Douglass *et al.* 1995; Adams *et al.* 1999). The CART gene is composed of three exons and two introns, with rat and mouse having alternatively spliced variants (Douglass *et al.* 1995; Adams *et al.* 1999). Splicing occurs within exon 2 of the long form, which results in a transcript that is missing 39 nucleotides (termed the short form). Also, in rats, two major CART mRNA species have been described which are the results of differential usage of polyadenylation sites.

Received September 28, 2001; revised manuscript received December 5, 2001; accepted December 10, 2001.

Address correspondence and reprint requests to Dr Geraldina Dominguez, Division of Neuroscience, Yerkes Research Center, Atlanta, GA 30329, USA. E-mail: gdoming@rmy.emory.edu

Abbreviations used: CART, cocaine- and amphetamine-regulated transcript; MCH, melanin-concentrating hormone; PKA, protein kinase A; POMC, proopiomelanocortin; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate buffer; TRH, thyrotropin-releasing hormone.

Our laboratory has started to address the molecular mechanisms involved in the regulation of CART mRNA expression. The data presented identifies putative *cis*-regulatory elements, and functionally tests various segments containing these *cis*-regulatory elements for promoter activity. The role of the cyclic AMP signal transduction pathway was also examined.

Experimental procedures

Tissue culture and stimulation

Rat pituitary GH3 and rat pheochromocytoma PC12 cells were maintained in Ham's F-12 media supplemented with 15% horse serum and 5% fetal bovine serum (Life Technologies, Rockville, MD, USA). Mouse pituitary AtT20 cells were maintained in Dulbecco's minimal essential media supplemented with 10% horse serum (Life Technologies). All tissue cultures were maintained in a humidified incubator at 37°C under 5% CO_2.

GH3 cells were grown in poly-L-lysine-coated 10 cm cell culture plates to 70–80% confluency. Media was then changed to Ham's F-12 supplemented with 0.5% horse serum for 16–24 h. In the some experiments, following serum deprivation, new 0.5% horse serum supplemented media was added containing 30 μM *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89) (Sigma, St Louis, MO, USA) for 2 h followed by addition of 20 μM forskolin (Sigma) to the media for 1, 3, 6, 12, and 24 h. Similarly, samples were treated with forskolin in the absence of H89.

RNA isolation and northern blot

Total RNA was isolated from frozen rat cerebellum and hypothalamus or GH3 cells. Tissues (50–100 mg) were homogenized in 1 mL of Trizol Reagent (Life Technologies, Grand Island, NY, USA) using a power homogenizer. GH3 cells were lysed in 1 mL of Trizol Reagent per 1 cm^2 of culture dish surface area. Samples were incubated in Trizol Reagent and RNA isolated according to the manufacturer's recommendation.

Total RNA was run on a 1.2% 3-[*N*-morpholino]propanesulfonic acid (MOPS, Sigma)/6% formaldehyde agarose gel. RNA was transferred by capillary action in the presence of 10 × saline sodium citrate buffer (SSC; 1.5 M NaCl 0.15 M sodium citrate, pH 7.0) and fixed by UV cross-linking. Prehybridization was carried out at 50°C in 6 SSC, 5 × Denhardt's solution (Sigma), 1% sodium dodecyl sulfate (SDS), 20% formamide, 50 μg/mL sonicated salmon sperm DNA (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) for 6 h. Hybridization buffer was changed and 2×10^6 cpm of a ^{32}P-labeled cocktail consisting of two or three oligonucleotides was added and incubated at 50°C for 24 h. Oligonucleotides were 5′ end labeled using γ-^{32}P (6000 Ci/mmol) and polynucleotide kinase (Stratagene, La Jolla, CA, USA). The CART cocktail consisted of three oligos with the following sequences: 5′-TGAAAACAAGCACTTCAAGAGGAAAG-3′, 5′-TGCAACGCTTCGATCTGCAACATAG-3′, and 5′-CTCATGCGCACTCTCTCCAGCG-3′. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was made up of two oligos having the following sequences: 5′-AGTTGTCATTGAGAGCAATGCCAGC-3′ and 5′-AGTAGACTCACGACATACTCAGCA-3′.

Nonspecific hybridization was removed by washing the blots with increasing stringency washes (5 × SSC, 1% SDS up to 2 × SSC, 0.1% SDS) at room temperature. Blots were analyzed by autoradiography or using a PhosphoImager (Storm, Molecular Dynamics, Sunnyvale, CA, USA). Blots were stripped and re-probed with the GAPDH probe. CART mRNA level was normalized to GAPDH RNA.

Sequencing and analysis of the mouse CART gene promoter region

A previously described mouse BAC genomic library was screened using a rat CART cDNA probe. One hybridization positive clone (approximately 125 kb in length) was identified (Adams *et al.* 1999). A *Bam*HI subclone, Bam5′C (approximately 9.5 kb in length), containing the entire CART gene was generated (Fig. 1). The CART 5′-flanking region in Bam5′C (*Bgl*II/*Sac*I segment) was sequenced. All sequences were determined by cycle-sequencing reactions using a dye-labeled chain terminator sequencing kit. Cloned fragment termini were sequenced using vector-derived primers; custom designed primers were used to walk across the region until double-stranded coverage across the region was obtained with a four-fold redundancy or greater. The sequence has been deposited with GenBank under accession number AF148071.

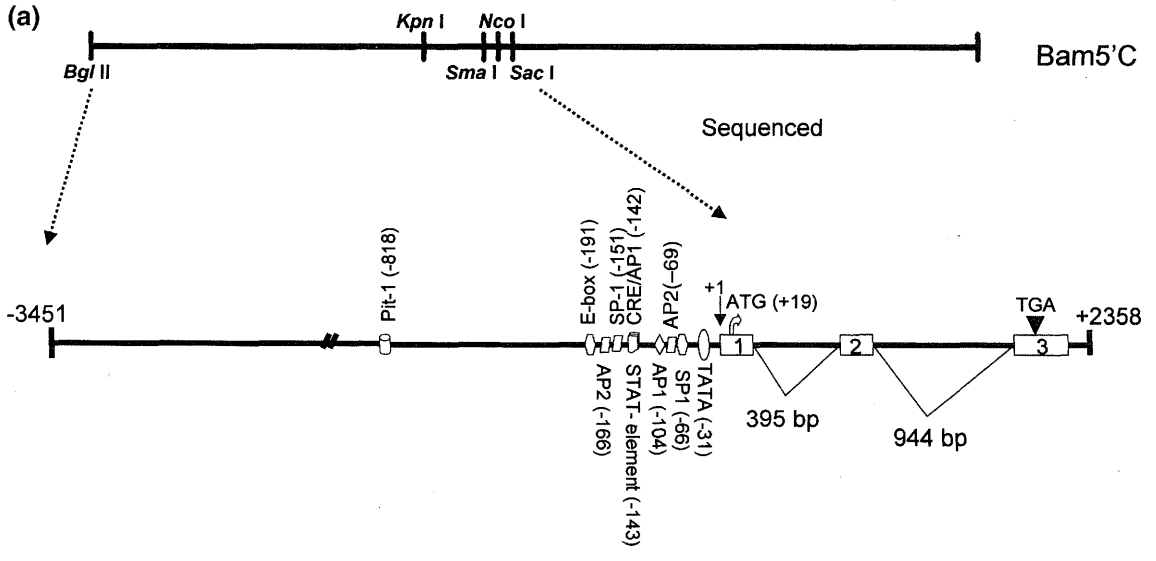
The sequenced region was analyzed for putative transcription factor binding sites using the Transcription Factor Database (TRANSFAC) and MatInspector V2.2 (Wingender *et al.* 2000). The sequence was also analyzed for specific patterns previously shown to be involved in restricting gene expression to a particular cell type using FindPatterns in the Wisconsin Package (Genetics Computer Group, Madison, WI, USA).

Cloning

Various lengths of 5′ upstream sequences were cloned into the promoter-less vector, pGL3-BASIC (Promega, Madison, WI, USA), and these clones were tested for promoter activity. Constructs were generated by digesting the genomic clone, Bam5′C, with *Bgl*II/*Nco*I, or *Kpn*I/*Sac*I, or *Sma*I/*Nco*I. pGL3-BASIC vector was digested and dephosphorylated followed by ligation to the appropriately digested genomic fragment using T4 DNA Ligase (Promega). Reactions were incubated overnight at 4°C and used to transform competent *Escherichia coli* TOP10 cells (Invitrogen, Carlsbad, CA, USA). Representative colonies were picked and plasmid DNA isolated. Constructs were confirmed by dideoxy nucleotide sequencing (Emory DNA Sequencing Core Facility). The three pGL3-luciferase expressing constructs made were: −3451CART-LUC, spanning −3451 to + 23; −641CART-LUC, spanning −641 to + 30; and −102CART-LUC, spanning −102 to + 23 where + 1 is the predicted site of transcription initiation.

Transfection and luciferase assay

GH3, AtT20, and PC12 cells were plated on 35-mm poly L-lysine-coated six-well plates at a density of 1×10^6 cells/well in 2 mL of fully supplemented media as described. For each transfection, 2 μg of pSV-β-Galactosidase Vector (Promega), 2 μg of one of the pGL3-luciferase expressing constructs, and 6 μL of FuGENE 6 Transfection Reagent (Roche Diagnostics Corporation, Indianapolis, IN, USA) were mixed in the appropriate serum free media and



(b)

```
          -320
Human     GAAGGCATTT TCCATTtcct aggcccctcc cgcttcccgT CCCTCACCTc TaGCCCCTCg
Mouse     GAAGGCATTT TCCATT.... .......... ..........T CCCTCACCTt TtGCCCCTCa

          -260
Human     GcTTCAGCTC aGGCCCCTcG GGgAGCaTCC CTtGCcGtGa GACtGACAGC cTTTgGGGGC
Mouse     GtTTCAGCTC tGGCCCCTaG GGaAGCtTCC CTgGCtGcGg GACgGACAGC gTTTcGGGGC

          -200                          E-box                        AP2
Human     gCaGGGTCcT GTTcTCTGCG CTCTAGCCCA TCTGTGCGCA GAGCCTCGTT CCCaGGCgCC
Mouse     aCgGGGTCtT GTTtTCTGCG CTCTAGCCCA TCTGTGCGCA GAGCCTCGTT CCCgGGCtCC

          -160  SP1   STAT/CRE/AP1                                    AP1
Human     tGGAaCCCGG CGGGCATTGA CGTCAAgCGc CgGCGGAGCG CTGCCTACAG ACGGtTGACC
Mouse     cGGAgCCCGG CGGGCATTGA CGTCAAaCGg CaGCGGAGCG CTGCCTACAG ACGGcTGACC

          -100                                SP1
Human     CGGGCcCTCC TCCACACCCC CtTCCTTCTT CgCCtCCTCC CTCTTtCCtG CACggGggcT
Mouse     CGGGCtCTCC TCCACACCCC CgTCCTTCTT CtCCcCCTCC CTCTTcCCgG CACccGattT

          -40     TATA-box                    +1                      22
Human     CgggCtcaCT ATAAaAgGtg GGAGcGCGtg GTGCCCcAGC AaCGAcGAGt TtCAGAACgATG
Mouse     C.aaCcggCT ATAAgAaGcc GGAGaGCGca GTGCCCgAGC AgCGAgGAGg TcCAGAACcATG
```

Fig. 1 Structure of the mouse CART gene and sequence of the proximal promoter region. (a) A diagram of the region in Bam 5′C (see Experimental procedures) that was sequenced. The genomic structure of the CART gene as found in the clone is shown. The three exons and two introns of the CART gene are indicated. Exons are drawn as clear numbered boxes and the length of the introns is given. The cluster of transcription factor binding sites is shown and each is represented by a unique symbol. The diagram is not drawn to scale. The location of each transcription binding site relative to the site of transcription initiation (+ 1) is given in parenthesis. (b) Sequence alignment between the human and mouse proximal promoter region. Differences between human and mouse are in lower case. The transcription binding sites are underlined. The predicted site of transcription initiation is indicated as + 1 and the initiating ATG is given. See text for additional details.

incubated at 25°C for 45 min as described by the manufacturer. One hundred microliters of the complexed DNA/FuGENE 6 mixture was added to each well and incubated for 18 h at 37°C under 5% CO_2. In some cases, media was replaced with 2 mL of supplemented media containing 25 μM forskolin and incubated for additional amounts of time. Cells were lysed and luciferase expression measured using the Luciferase Assay System as recommended by the vendor (Promega).

Luciferase activity quantified using a luminometer (Turner Designs Model TD-20/20 luminometer, Sunnyvale, CA). β-Galactosidase

activity was measured using the β-Galactosidase Enzyme Assay System (Promega) and the absorbance read at 420 nm. Protein concentration was determined using the Bradford method (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA, USA). In all experiments, luciferase expression was normalized to β-galactosidase activity using equal protein concentration.

Measurements were carried out on three or more independent transfected cultures that were done in duplicate. All results were expressed as mean ± SEM. Statistical significance was determined using one-way ANOVA followed by a Tukey test or a Student's *t*-test (SigmaStat 2.0, Jandel Corporation).

Results

CART's proximal promoter region contains transcription factor binding sites that are conserved between mice and humans. Analysis of the mouse CART gene 5′ upstream sequence identified a region containing a cluster of transcription factor binding sites, including a canonical cyclic AMP response element (CRE), two AP1 and SP1sites, and several AP2 sites, along with a TATA-like sequence and an E-box (Fig. 1). There is also a STAT-response element, TT $(N)_6$AA, in an overlapping STAT/CRE/AP1 site. Further upstream there is a putative binding site for the pituitary-specific transcription factor Pit-1, AWWTATNCAT, where W is either an A or a T (Fig. 1a).

The CART gene upstream sequence was further analyzed using a neural network promoter-predicting algorithm (Ohler *et al.* 1999) in order to predict transcription initiation sites for the mouse CART gene. Transcription initiation for the mouse CART gene was predicted at the A located 19 nucleotides upstream of the initiating AUG codon (Fig. 1b). This is in agreement with the previously published rat and human 5′ ends (Douglass *et al.* 1995; Douglass and Daoud 1996).

The CART peptide coding sequence is highly conserved between rodents and man, with greater than 90% nucleotide identity (Douglass and Daoud 1996). To determine if the 5′-flanking region of the mouse CART gene was similarly conserved across species, the human CART 5′-flanking sequence was identified in the databases. A contig (Accession Number NT019389) located on human chromosome 5 and spanning 877,249 bp was found. It contained the CART gene plus 5′ upstream sequence. A pairwise comparison between the mouse and human sequences was performed using GAP (GCG WISCONSIN PACKAGE; Accetys Inc., San Diego, CA, USA) with a gap weight of 50 and length weight of 3. Figure 1(b) is an alignment of the mouse proximal promoter region with the corresponding human region. The comparison identified a 320-bp region immediately upstream from the CART coding sequence that shares 83.4% nucleotide identity. The region contains the binding sites for the clustered set of transcription factors, including the overlapping CRE/AP1/STAT site.

Functional studies

To assess the importance of the regulatory elements within the CART upstream sequence, the activity of various 5′-proximal regions of the CART gene were tested for their ability to drive gene expression when cloned upstream of the luciferase gene. Three luciferase expressing constructs were made and tested; −3451CART-LUC, −641CART-LUC, and −102CART-LUC that contain 3451, 641, and 102 bp of mouse CART 5′ upstream sequence (+ 1 is the predicted site of transcription initiation), respectively (Fig. 2). We chose GH3 cells as our *in vitro* system because CART mRNA is expressed in the pituitary (Couceyro *et al.* 1997) and these cells had previously been shown to express high levels of CART mRNA (Barrett *et al.* 2001). GH3 cells were cotransfected with pSV-β-galactosidase (which serves as an internal control for normalization of transfection efficiency)

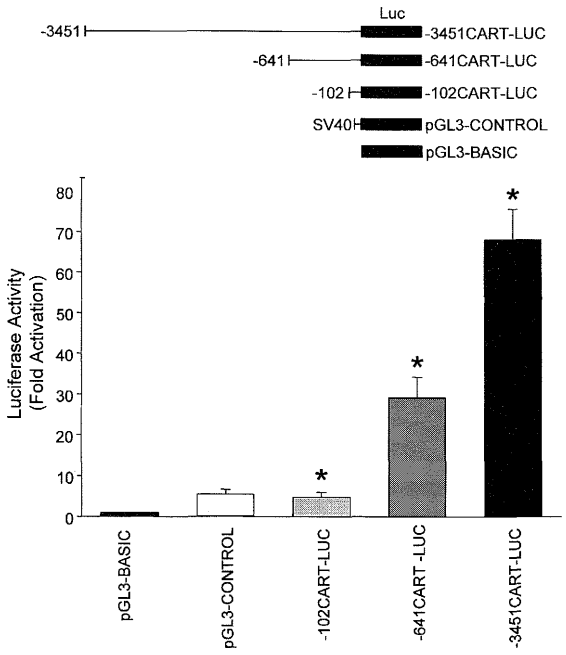


Fig. 2 Luciferase (LUC) expression of constructs carrying varying lengths of the CART promoter. The constructs were cloned into pGL3-BASIC vector which lacks a promoter, and the luciferase-expressing clones were transiently cotransfected with β-galactosidase vector, which serves as an internal control for normalization of transfection efficiency, into GH3 cells. Boundaries for the CART-LUC constructs are: −3451CART-LUC, −3451 to + 23; −641CART-LUC, −641 to + 30; −102CART-LUC, −102 to + 23 where + 1 is the site of transcription initiation (see Fig. 1b). pGL3-CONTROL contains the SV40 promoter to drive LUC expression and serves as a positive control. Each value is expressed as the folds increase relative to that found with pGL3-BASIC. Values are mean ± SEM from at least three independent experiments each done in duplicate. Statistical significance (*) was assessed using one-way ANOVA followed by a Tukey test. The differences in the values among the different constructs are greater than would be expected by chance ($p = < 0.001$).

and one of the CART containing constructs, −3451CART-LUC, −641CART-LUC, and −102CART-LUC. Luciferase activity of each construct was compared to that produced by pGL3-BASIC, the parent vector that lacks a promoter (background activity).

pGL3-CONTROL, which has a SV40 promoter to drive luciferase expression, was used as a positive control since it exhibits strong promoter activity in a variety of mammalian cells. In GH3 cells, pGL3-CONTROL had a six-fold increase in luciferase activity above pGL3-BASIC. This is similar to the 5.4-fold activation by −102CART-LUC, indicating that this segment of upstream sequence can function as a promoter and drive luciferase expression. However, constructs containing larger upstream segments produced much greater activity. −641CART-LUC produced a 29-fold activation and −3451CART-LUC had a 51-fold activation. The −102CART-LUC construct does not contain the E-box, AP2, SP1, or STAT/CRE/AP1 sites highlighted in Fig. 1(b) but it does contain the TATA-like box and an SP1 site. The −641CART-LUC construct contains the STAT/CRE/AP1 composite site, and −3451CART-LUC also contains a putative Pit-1 binding site. Pit-1 is a transcription factor belonging to the POU domain proteins that is expressed exclusively in the central nervous system and in the pituitary (reviewed by McEvilly and Rosenfeld 1999).

Cyclic AMP-dependent activation of the CART gene in GH3 cells

Because these promoter studies were carried out in GH3 cells, it is important to show that these cells normally express CART mRNA. Barrett *et al.* (2001) have shown that CART mRNA levels were up-regulated by activators of cyclic AMP, including forskolin, dibutryl-cyclic AMP, and PACAP-38 in these cells. To confirm and extend their results, a time course of forskolin induced activation of CART mRNA was performed. Forskolin is an activator of adenylate cyclase, which is the enzyme that converts adenosine triphosphate to cyclic adenosine monophosphate. GH3 cells were treated with 20 μM forskolin for 0, 1, 3, 6, 12, and 24 h as described in Experimental procedures and 40 μg of total RNA was analyzed by northern blot analysis (Fig. 3). Continuous exposure to forskolin increased CART mRNA levels compared to 0 h of exposure. The involvement of protein kinase A (PKA) was also tested (Fig. 3). In the same experiment, some GH3 cells were treated with 30 μM H89, an inhibitor of PKA, for 2 h prior to and during forskolin treatment. Pretreatment of PC12 cells with the same concentration of H89 results in a significant reduction of forskolin-induced protein phosphorylation (Chijiwa *et al.* 1990). The time course showed that the maximal increase in CART mRNA levels occurred after 6 h of forskolin treatment. Because it is well established that forskolin increases cFos mRNA (reviewed by Herdegen and Leah 1998), the effect of forskolin on cFos expression in GH3

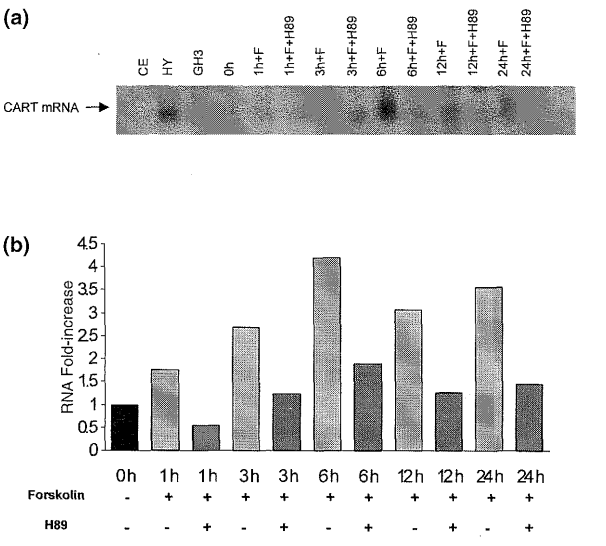


Fig. 3 Changes in CART mRNA levels in response to forskolin (F) and H89 treatments. (a) Northern blot analysis of CART mRNA levels in brain regions and GH3 cells treated with forskolin (F) alone or F plus H89 as described in Experimental procedures. The 0 h time point shows CART mRNA in the absence of any treatment. Total RNA (40 μg) was transferred by capillary action and probed with a CART oligonucleotide cocktail. Brain regions analyzed: CE, cerebellum as a negative control (20 μg); and HY, hypothalamus as a positive control (20 μg). (b) CART mRNA levels were quantified and normalized to GAPDH levels. RNA fold increase was determined by setting the 0 h time point to 1 and comparing the other time points to the 0 h time point. Data is from a single experiment but is representative of data from three independent experiments.

cells under the same experimental conditions was tested; maximal expression was seen after 30 min, after which time levels decreased (data not shown). Treatment with H89 prior to and during forskolin treatment reduced the CART mRNA levels by approximately two-fold.

To determine which *cis*-elements present in the CART promoter were responsible for the responsiveness to cyclic AMP induction by forskolin, two luciferase expressing constructs, −641CART-LUC which contains the clustered transcription factor binding sites including the overlapping STAT/CRE/AP1 site, and −102CART-LUC which lacks the STAT/CRE/AP1 site were tested. The −641CART-LUC and −102CART-LUC constructs were transfected into GH3 cells and 18 h after transfection, media was changed and cells were treated with 25 μM forskolin for 7 h. As illustrated in Fig. 4, a two-fold increase in luciferase activity after forskolin treatment was produced with −641CART-LUC, while no statistically significant increase in luciferase activity was observed with −102CART-LUC and pGL3-CONTROL. This suggests that the region between −102 and −641 contains *cis*-elements that are responsive to changes in cyclic AMP levels.

A comparison of luciferase activity between −641CART-LUC and pGL3-CONTROL after forskolin treatment showed

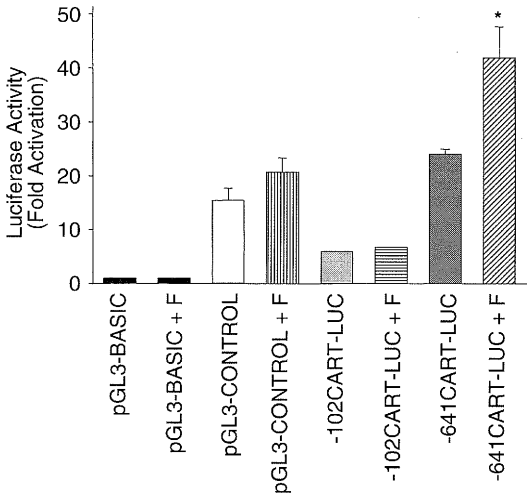


Fig. 4 Cyclic AMP responsiveness of −102CART-LUC and −641 CART-LUC in GH3 cells. GH3 cells were transiently transfected with each construct as described in Experimental procedures and luciferase activity assayed 7 h after forskolin (F) treatment. Samples are compared to the corresponding nontreated control. Each value is expressed as the folds increase relative to pGL3-BASIC. Values are mean ± SEM from at least three independent experiments each done in duplicate. Statistical significance (*) was assessed using a student *t*-test. The difference between the values of −641CART-LUC and −641CART-LUC + F is significant ($p = 0.037$).

that −641CART-LUC exhibits a continuous increase in expression beginning at 1 h and continuing through 48 h (Fig. 5). In comparison, pGL3-CONTROL exhibited greatest luciferase activity after 7 h of forskolin treatment, with a three-fold decrease after 24 h.

Cyclic AMP responsiveness of −641CART-LUC in cells of different origins. Promoter activity of −641CART-LUC in AtT20 and PC12 cells was examined. AtT20 and PC12 cells were transfected as described in Experimental procedures.

−641CART-LUC was more active in PC12 cells than in AtT20 cells (Fig. 6) however, promoter activity was highest in GH3 cells (29-fold above background, Fig. 2). Forskolin treatment of AtT20 and PC12 cells did not have a significant effect on luciferase expression. In addition, in AtT20 cells, increasing the amount of 5′ upstream sequence by using −3451CART-LUC did not significantly affect the luciferase expression level in the presence or absence of forskolin treatment, which is contrary to what we observed in GH3 cells (data not shown). CART mRNA is expressed at much lower levels in AtT20 cells than in GH3 cells (Dominguez G., unpublished result and Barrett *et al.* 2001).

Discussion

CART mRNA levels change in response to various stimuli including fasting, leptin, and psychostimulant drugs (Douglass *et al.* 1995; Kristensen *et al.* 1998; Fagergren and Hurd 1999; Hurd *et al.* 1999; Wang *et al.* 1999; Ahima *et al.* 2000; Dhillon *et al.* 2000), but the transcription factors involved in CART mRNA regulation have not been studied. The aim of this work was to characterize the CART promoter by identifying putative transcription factor binding sites and to test segments containing these elements for functional promoter activity.

Sequence analysis of a 3.4-kb mouse genomic DNA identified a 320-bp region, located immediately upstream from the transcriptional start site, that is highly conserved

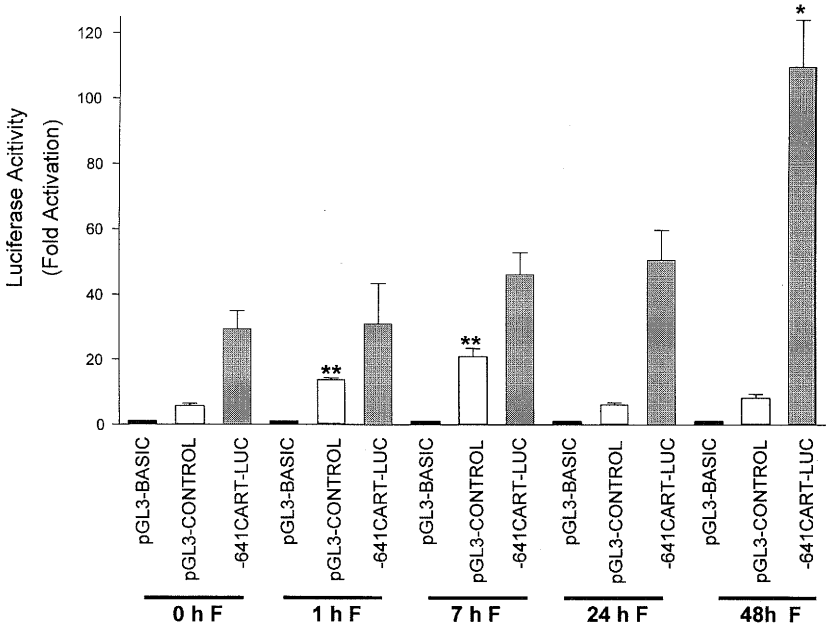


Fig. 5 Time course of forskolin (F) effect on −641CART-LUC in GH3 cells. Cells were treated with forskolin (25 μM) for 1, 7, 24, and 48 h. Each value is expressed as the folds increase relative to pGL3-BASIC. Values for pGL3-CONTROL are included for comparison. Values are mean ± SEM from at least three independent experiments each done in duplicate. Statistical significance (*) was assessed using one-way ANOVA followed by a Tukey test. The differences between the values of −641CART-LUC at 48 h F and the other time points are greater than would be expected by chance ($p = < 0.001$). **Indicates that the increase in the values of pGL3-CONTROL at 1 h and 7 h are significantly different than the 0 h time point ($p = < 0.05$).

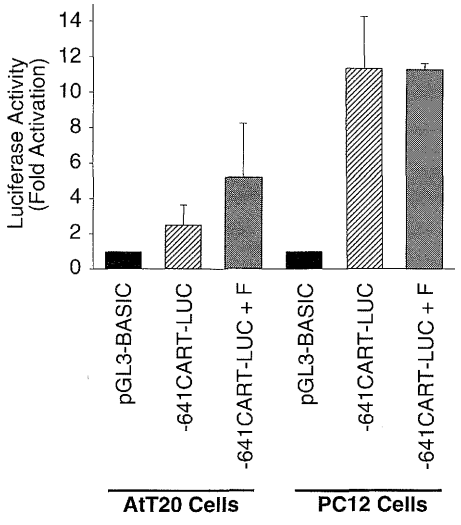


Fig. 6 Comparison of the activity of −641CART-LUC in AtT20 and PC12 cells in the presence and absence of forskolin (F). AtT20 and PC12 cells were transiently transfected with −641CART-LUC as previously described. Luciferase activity is expressed as the folds increase relative to pGL3-BASIC.

between the mouse and human CART gene (Fig. 1b). The region contains several transcription factor binding sites and thus suggests that the mouse and human CART gene could be transcriptionally regulated by the same transcription factors. For example, the overlapping STAT/CRE/AP1 site located 148 nucleotide upstream from the predicted site of transcription initiation is completely conserved between mouse and human. Additionally, the conservation of these binding sites suggests that these transcription factors have an important role in the regulation of the CART gene.

Transcriptional activation of cellular genes by a cyclic AMP-responsive element (CRE)-binding protein (CREB)-mediated response usually peaks after 30 min of stimulation and decreases after 2–4 h (reviewed by Mayr and Montminy 2001), the continuous increase that we observed in GH3 cells (Fig. 5) suggests that the cyclic AMP responsiveness of the CART promoter may not be due only to CREB-mediated activation but to activation by other transcription factors. Theoretically, the STAT/CRE/AP1 element could allow complex transcriptional regulation via CREB and cJun proteins as well as by signal transducer and activator of transcription (STAT) proteins (Shaywitz and Greenberg 1999). Composite CRE/AP1 sites have been found in a number of genes involved in neurotransmitter synthesis, including dopamine β-hydroxylase (Shaskus *et al.* 1992), prodynorphin (Messersmith *et al.* 1996), proenkephalin (Comb *et al.* 1986), and cholecystokinin (Hansen *et al.* 1999). For example, the inflammation-induced model proposed by Messersmith *et al.* (1998) for prodynorphin gene transcription describes the interaction between Fos/Fra, phosphorylated-CREB, and phosphorylated-cJun at the composite DYNCRE3 site. Mutational analysis of the STAT/CRE/AP1 composite site will be very important in identifying which transcription factors are involved in the cyclic AMP responsiveness of the CART promoter.

The presence of a STAT-binding motif suggests that the CART gene could be regulated directly via cytokine signaling. This could be a mechanism by which leptin stimulates CART mRNA transcription, since the leptin receptor signals through the Janus kinases (JAKs)/STAT pathway (reviewed by Good 2000). The presence of the SP1 adjacent to the STAT-response element (Fig. 1b) in the CART gene promoter sequence adds additional support for this, since SP1 has been shown to play a role in mediating the STAT response (Look *et al.* 1995). Interestingly, a model of leptin action in a thyrotropin-releasing hormone (TRH) neuron includes direct regulation of the TRH promoter by leptin via the phosphorylation of STAT3 (Harris *et al.* 2001). Recently, Elias *et al.* (2001) showed that leptin directly acts on hypothalamic CART neurons that coexpress TRH mRNA.

The Pit-1 binding site at position −818 (Fig. 1a) in the mouse CART promoter suggests the involvement of Pit-1 in cell-type specific transcriptional activation of the CART gene. Pit-1 is a POU-homeobox transcription factor that is responsible for either the commitment or maintenance of somatotroph, lactotroph, and thyrotroph cell lineages (Lin *et al.* 1994; Rhodes *et al.* 1996; Andersen *et al.* 1997). This transcription factor has been shown to be required for the tissue specific expression of several genes including growth hormone, prolactin, and thyrotropin (Haugen *et al.* 1996; Karin *et al.* 1990; Voss and Rosenfeld 1992). The Pit-1 protein has also been shown to participate in synergistic interactions with other transcription factors, including cJun (Farrow *et al.* 1996), thyroid hormone receptor (Chang *et al.* 1996), and estrogen receptor (Ying and Lin 2000). The 1.8-fold increase in luciferase activity exhibited by −3451CART-LUC as compared with −641CART-LUC in GH3 cells (Fig. 2) suggests that Pit-1 may play a role in CART gene expression. In AtT20 cells, −3451CART-LUC resulted in no significant increase in luciferase activity in the presence or absence of forskolin (data not shown). In AtT20 cells, prolactin mRNA is not induced by the Pit-1 pathway (Girardin *et al.* 1998). Additionally, the luciferase activity of −641CART-LUC in GH3 cells (somatomammotroph phenotype) is higher than in AtT20 cells (corticotroph phenotype) thus suggesting that −641CART-LUC contains *cis*-elements that enhance expression in GH 3 cells.

The data obtained from these studies provide a basis for future studies on the mechanisms regulating the CART gene. The data presented (Figs 3 and 4) also confirm that the CART gene can be positively regulated via a cyclic AMP-dependent pathway. Further studies aimed at identifying which transcription factors and pathways are involved in regulating the CART gene may identify common signal transduction pathways that are shared by neuropeptide genes

such as proopiomelanocortin (POMC) and melanin-concentrating hormone (MCH). In the arcuate nucleus CART, POMC, and MCH are coexpressed, affect feeding (Elias *et al.* 1998; Broberger 1999; Vrang *et al.* 1999) and may also be transcriptionally regulated in a similar manner.

Acknowledgements

The authors acknowledge support of NIH grants MH58922, RR00165, DA00418, DA10732, DA15162 and helpful discussions with Drs T.J. Murphy and Larry Young.

These results are presented in an abstract submitted to the Society for Neuroscience 31st Annual Meeting (Abstract Number 246.12).

References

Adams L. D., Gong W., Vechia S. D., Hunter R. G. and Kuhar M. J. (1999) CART: from gene to function. *Brain Res.* **848**, 137–140.

Ahima R. S. and Hileman S. M. (2000) Postnatal regulation of hypothalamic neuropeptide expression by leptin: implications for energy balance and body weight regulation. *Regul. Pept.* **92**, 1–7.

Ahima R. S., Saper C. B., Flier J. S. and Elmquist J. K. (2000) Leptin regulation of neuroendocrine systems. *Front. Neuroendocrinol.* **21**, 263–307.

Andersen B., Weinberg W. C., Rennekampff O., McEvilly R. J., Bermingham J. R., Hooshmand F., Vasilyev V., Hansbrough J. F., Pittelkow M. R., Yuspa S. H. and Rosenfeld M. G. (1997) Functions of the POU domain genes Skn-1a/i and Tst-1/Oct – 6/SCIP in epidermal differentiation. *Genes Dev.* **11**, 1873–1884.

Barrett P., Morris M. A., Moar K. M., Mercer J. G., Davidson J. A., Findlay P. A., Adam C. L. and Morgan P. J. (2001) The differential regulation of CART gene expression in a pituitary cell line and primary cell cultures of ovine pars tuberalis cells. *J. Neuroendocrinol.* **13**, 347–352.

Brenz Verca M. S., Widmer D. A., Wagner G. C. and Dreyer J. (2001) Cocaine-induced expression of the tetraspanin CD81 and its relation to hypothalamic function. *Mol. Cell Neurosci.* **17**, 303–316.

Broberger C. (1999) Hypothalamic cocaine- and amphetamine-regulated transcript (CART) neurons: histochemical relationship to thyrotropin-releasing hormone, melanin-concentrating hormone, orexin/hypocretin and neuropeptide Y. *Brain Res.* **848**, 101–113.

Chang W., Zhou W., Theill L. E., Baxter J. D. and Schaufele F. (1996) An activation function in Pit-1 required selectively for synergistic transcription. *J. Biol. Chem.* **271**, 17733–17738.

Chijiwa T., Mishima A., Hagiwara M., Sano M., Hayashi K., Inoue T., Naito K., Toshioka T. and Hidaka H. (1990) Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP- dependent protein kinase, *N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *J. Biol. Chem.* **265**, 5267–5272.

Comb M., Birnberg N. C., Seasholtz A., Herbert E. and Goodman H. M. (1986) A cyclic AMP- and phorbol ester-inducible DNA Element. *Nature* **323**, 353–356.

Couceyro P. R., Koylu E. O. and Kuhar M. J. (1997) Further studies on the anatomical distribution of CART by in situ hybridization. *J. Chem. Neuroanat.* **12**, 229–241.

Dhillon H., Ge Y., Minter R. M., Prima V., Moldawer L. L., Muzyczka N., Zolotukhin S., Kalra P. S. and Kalra S. P. (2000) Long-term differential modulation of genes encoding orexigenic and anorexigenic peptides by leptin delivered by rAAV vector in ob/ob mice. Relationship with body weight change. *Regul Pept.* **92**, 97–105.

Douglass J. and Daoud S. (1996) Characterization of the human cDNA and genomic DNA encoding CART: a cocaine- and amphetamine-regulated transcript. *Gene* **169**, 241–245.

Douglass J., Mckinzie A. A. and Couceyro P. (1995) PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. *J. Neurosci.* **15**, 2471–2481.

Elias C. F., Lee C., Kelly J., Aschkenasi C., Ahima R. S., Couceyro P. R., Kuhar M. J., Saper C. B. and Elmquist J. K. (1998) Leptin activates hypothalamic CART neurons projecting to the spinal cord. *Neuron* **21**, 1375–1385.

Elias C. F., Lee C. E., Kelly J. F., Ahima R. S., Kuhar M., Saper C. B. and Elmquist J. K. (2001) Characterization of CART neurons in the rat and human hypothalamus. *J. Comp. Neurol.* **432**, 1–19.

Fagergren P. and Hurd Y. L. (1999) Mesolimbic gender differences in peptide CART mRNA expression: effects of cocaine. *Neuroreport* **10**, 3449–3452.

Farrow K. N., Manning N., Schaufele F. and Gutierrez-Hartmann A. (1996) The c-Jun delta-domain inhibits neuroendocrine promoter activity in a DNA sequence- and pituitary-specific manner. *J. Biol. Chem.* **271**, 17139–17146.

Girardin S. E., Benjannet S., Barale J. C., Chretien M. and Seidah N. G. (1998) The LIM homeobox protein mLIM3/Lhx3 induces expression of the prolactin gene by a Pit-1/GHF-1-independent pathway in. *Corticotroph Att20 Cells FEBS Lett.* **431**, 333–338.

Good D. J. (2000) How tight are your genes? Transcriptional and posttranscriptional regulation of the leptin receptor NPY, POMC. *Genes Horm. Behav.* **37**, 284–298.

Hansen T. V., Rehfeld J. F. and Nielsen F. C. (1999) Mitogen-activated protein kinase and protein kinase A signaling pathways stimulate cholecystokinin transcription via activation of cyclic adenosine 3′,5′-monophosphate response element-binding protein. *Mol. Endocrinol.* **13**, 466–475.

Harris M., Aschkenasi C., Elias C. F., Chandrankunnel A., Nillni E. A., Bjoorbaek C., Elmquist J. K., Flier J. S. and Hollenberg A. N. (2001) Transcriptional regulation of the thyrotropin-releasing hormone gene by leptin and melanocortin signaling. *J. Clin. Invest.* **107**, 111–120.

Haugen B. R., Mcdermott M. T., Gordon D. F., Rupp C. L., Wood W. M. and Ridgway E. C. (1996) Determinants of thyrotrope-specific thyrotropin beta promoter activation. Cooperation Pit-1 with another factor. *J. Biol. Chem.* **271**, 385–389.

Herdegen T. and Leah J. D. (1998) Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos Krox, CREB/ATF proteins. *Brain Res. Brain Res. Rev.* **28**, 370–490.

Hurd Y. L., Svensson P. and Ponten M. (1999) The role of dopamine, dynorphin, and CART systems in the ventral striatum and amygdala in cocaine abuse. *Ann. NY Acad. Sci.* **877**, 499–506.

Karin M., Castrillo J. L. and Theill L. E. (1990) Growth hormone gene regulation: a paradigm for cell-type-specific gene activation. *Trends Genet.* **6**, 92–96.

Koylu E. O., Couceyro P. R., Lambert P. D., Ling N. C., DeSouza E. B. and Kuhar M. J. (1997) Immunohistochemical localization of novel CART peptides in rat hypothalamus, pituitary and adrenal gland. *J. Neuroendocrinol.* **9**, 823–833.

Kristensen P., Judge M. E., Thim L., Ribel U., Christjansen K. N., Wulff B. S., Clausen J. T., Jensen P. B., Madsen O. D., Vrang N., Larsen P. J. and Hastrup S. (1998) Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature* **393**, 72–76.

Kuhar M. J. and Dall Vechia S. E. (1999) CART peptides: novel addiction- and feeding-related neuropeptides. *Trends Neurosci.* **22**, 316–320.

Kuhar M. J., Adams L. D., Hunter R. G., Vechia S. D. and Smith Y. (2000) CART peptides. *Regul. Pept.* **89**, 1–6.

Lin S. C., Li S., Drolet D. W. and Rosenfeld M. G. (1994) Pituitary ontogeny of the Snell dwarf mouse reveals Pit-1-independent and Pit-1-dependent origins of the thyrotrope. *Development* **120**, 515–522.

Look D. C., Pelletier M. R., Tidwell R. M., Roswit W. T. and Holtzman M. J. (1995) Stat1 depends on transcriptional synergy with Sp1. *J. Biol. Chem.* **270**, 30264–30267.

Mayr B. and Montminy M. (2001) Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat. Rev. Mol. Cell Biol.* **2**, 599–609.

Mcevilly R. J. and Rosenfeld M. G. (1999) The role of POU domain proteins in the regulation of mammalian pituitary and nervous system development. *Prog. Nucl. Acid Res. Mol. Biol.* **63**, 223–255.

Messersmith D. J., Kim D. J., Gu J., Dubner R. and Iadarola M. J. (1996) c-Jun activation of the DYNCRE3 site in the prodynorphin promoter. *Brain Res. Mol. Brain Res.* **40**, 15–21.

Messersmith D. J., Kim D. J. and Iadarola M. J. (1998) Transcription factor regulation of prodynorphin gene expression following rat hindpaw inflammation. *Brain Res. Mol. Brain Res.* **53**, 260–269.

Ohler U., Harbeck S., Niemann H., Noth E. and Reese M. G. (1999) Interpolated markov chains for eukaryotic promoter recognition. *Bioinformatics* **15**, 362–369.

Rhodes S. J., Krones A., Nelson C. and Rosenfeld M. G. (1996) Function of the conserved Pit-1 gene distal enhancer in progenitor and differentiated pituitary cells. *Mol. Cell Endocrinol.* **124**, 163–172.

Shaskus J., Greco D., Asnani L. P. and Lewis E. (1992) A bifunctional genetic regulatory element of the rat dopamine beta-hydroxylase gene influences cell type specificity and second messenger-mediated transcription. *J. Biol. Chem.* **267**, 18821–18830.

Shaywitz A. J. and Greenberg M. E. (1999) CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu. Rev. Biochem.* **68**, 821–861.

Thim L., Kristensen P., Nielsen P. F., Wulff B. S. and Clausen J. T. (1999) Tissue-specific processing of cocaine- and amphetamine-regulated transcript peptides in the rat. *Proc. Natl Acad. Sci. USA* **96**, 2722–2727.

Voss J. W. and Rosenfeld M. G. (1992) Anterior pituitary development: short tales from dwarf mice. *Cell.* **70**, 527–530.

Vrang N., Larsen P. J., Clausen J. T. and Kristensen P. (1999) Neurochemical characterization of hypothalamic cocaine- amphetamine-regulated transcript neurons. *J. Neurosci.* **19**, RC5.

Wang Z. W., Zhou Y. T., Kakuma T., Lee Y., Higa M., Kalra S. P., Dube M. G., Kalra P. S. and Unger R. H. (1999) Comparing the hypothalamic and extrahypothalamic actions of endogenous hyperleptinemia. *Proc. Natl Acad. Sci. USA* **96**, 10373–10378.

Wingender E., Chen X., Hehl R., Karas H., Liebich I., Matys V., Meinhardt T., Pruss M., Reuter I. and Schacherer F. (2000) TRANSFAC: an integrated system for gene expression regulation. *Nucleic Acids Res.* **28**, 316–319.

Ying C. and Lin D. H. (2000) Estrogen-modulated estrogen receptor x Pit-1 protein complex formation and prolactin gene activation require novel protein synthesis. *J. Biol. Chem.* **275**, 15407–15412.

ELSEVIER

Molecular Brain Research 104 (2002) 81–85

MOLECULAR BRAIN RESEARCH

www.elsevier.com/locate/bres

Research report

CART promoter CRE site binds phosphorylated CREB

A. Lakatos[a,b], G. Dominguez[a,*], M.J. Kuhar[a,*]

[a]*Yerkes Regional Primate Research Center of Emory University, Division of Neuroscience, 954 Gatewood Rd. NE, Atlanta, GA 30329, USA*
[b]*Department of Biology, University Medical School of Pécs, Pécs, Hungary*

Accepted 7 May 2002

Abstract

It has been shown previously that: CART (cocaine- and amphetamine-regulated transcript) mRNA is tightly regulated in brain; protein kinase A (PKA) is involved in CART expression in GH3 cells; and a cyclic AMP-responsive element (CRE) site is present in the proximal promoter region of the CART gene. Thus, the goal of this study was to test if CRE binding protein (CREB) can bind to the consensus CRE site and if phosphorylation of CREB occurs in GH3 cells under conditions of enhanced CART gene expression. Electromobility shift assays showed that a 27-bp oligonucleotide containing the CART CRE site was indeed bound by nuclear factors. Western blotting showed that incubation of GH3 cells with forskolin, which enhances CART mRNA expression, caused an increase in phosphorylated CREB (P-CREB) levels. Supershift analyses indicated that the CART CRE oligo/protein complex interacted with a P-CREB antibody. Taken together, these data indicate that P-CREB is a likely regulator of CART expression in GH3 cells.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Signal transduction: gene expression

Keywords: CART; Promoter; CRE; CREB; EMSA; Cocaine

1. Introduction

CART peptide is a regulated peptide neurotransmitter involved in reward and reinforcement, feeding and other physiologic processes [15,17,18,23]. CART mRNA is reported to be regulated by psychostimulant drugs [4,8,9], leptin [1,6,16,25] and other factors. Accordingly, regulation of CART gene expression has been of interest. The promoter region of the gene has been sequenced, binding sites for transcription factors have been identified [7] and protein kinase A (PKA) has been shown to regulate CART mRNA expression in GH3 cells [3,7]. As a result of these prior findings, GH3 cells were chosen as our in vitro system to study CART transcriptional regulation. Because PKA is known to activate CREB, and because of the presence of a CRE site in the proximal promoter region [7], the goal of this study was to test if CREB binds to the CART promoter CRE site, and if phosphorylation of CREB occurs under the conditions of enhanced CART mRNA expression in GH3 cells.

2. Methods

2.1. Cell culture and treatment

GH3 cells, a rat pituitary adenoma cell line (ATCC, Manassas, VA) were maintained in F-12K Ham's (Kaighn's modification, ATCC) media supplemented with 12% horse and 2.5% fetal bovine sera (Gibco, Grand Island, NY). Approximately, 5×10^6 cells were seeded to a poly-Lysine coated 10-mm dish. After fasting the cells in F-12K Ham's media supplemented with 0.5% horse serum for 16–24 h,

*Corresponding authors. Tel.: +1-404-727-1737; fax: +1-404-727-3278.

E-mail addresses: gdoming@rmy.emory.edu (G. Dominguez), mkuhar@rmy.emory.edu (M.J. Kuhar).

0169-328X/02/$ – see front matter
PII: S0169-328X(02)00321-2

ls were treated with 20 μM forskolin (Sigma, St MO) for 0, 0.5, 1, 3, and 6 h.

Vestern blot analysis

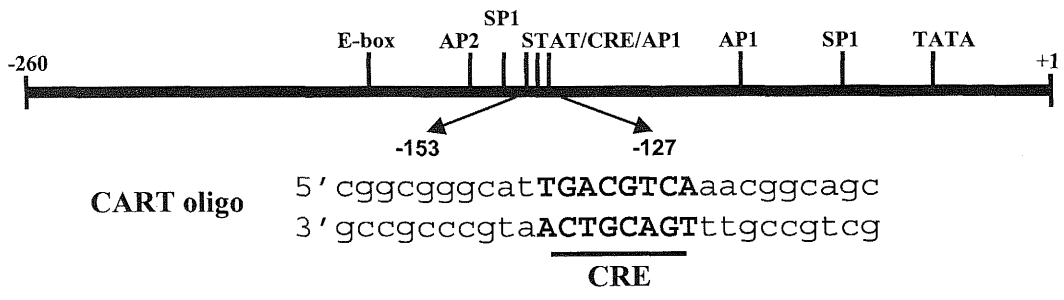
owing completion of the forskolin treatment, total was extracted in 100 μl of lysis buffer containing iton X-100, 50 mM Tris–HCl (pH 7.5), 300 mM 5 mM EDTA, 0.02% sodium azide, 2 nM methlysulfonyl fluoride (PMSF), 0.2 mM sodium anadate, and 5 nM okadaic acid. Equal amounts of mple buffer (12.5 mM Tris–HCl (pH 7.2), 5% ol, 0.4% SDS, 0.2 M 2-mercaptoethanol, 0.5 mg phenol blue) were added to 25 μg of protein lysate, was then boiled for 5 min and loaded onto a 10% pre-cast SDS–Tris–Glycine gel (Invitrogen, ad, CA). The gel was run at 120 V for 2 h and then cally transferred overnight (50 V at 4 °C) onto ilon–P membrane (Millipore, Bedford, MA) using a r buffer consisting of 1% SDS–1× Tris–Glycine. ot was incubated in blocking buffer (5% milk, 0.1% Tween-20 in PBS, PBS-T) for 1 h. After the initial ng step, the blot was incubated in blocking buffer ontained anti-CREB antibody at a 1:500 dilution Cruz Biotechnology, Santa Cruz, CA) for 1 h at temperature. After incubating with the primary dy the blot was washed at room temperature three (5 min per wash) with PBS-T (pH 7.6) and then ted with a horseradish peroxidase (HRP)-conjugated lary antibody, anti-rabbit IgG, at a 1:2000 dilution Cruz Biotechnology) in blocking buffer.

EB binding was detected using a chemiluminescent on kit (ECL+Plus, Amersham Pharmacia Biotech, nghamshire, UK) as recommended by the manufac- The same blot was washed with 0.2 M NaOH to e previous protein complexes and re-incubated over- at 4 °C with anti-phosphorylated-CREB antibody EB) (Cell Signaling, Beverly, MA) in blocking . The blot was washed as described above and ted with an HRP-conjugated secondary antibody, nti-mouse IgG, at a 1:2000 dilution for 1 h at room rature and developed using a chemiluminescent ion kit as described above.

2.3. Electrophoretic mobility shift assay (EMSA)

DNA–protein interactions were studied by EMSA. Nuclear protein extracts were prepared from forskolin-treated GH3 cells as described by Szeberenyi [22] with the following modifications. These included the addition of phosphatase inhibitors okadaic acid (5 nM) and sodium orthovanadate (0.2 mM), as well as protease inhibitors PMSF (2 mM), leupeptin (2 μg/ml), pepstatin A (1 μg/ml), and aprotinin (0.1 μg/ml) (Sigma, St Louis, MO). Total nuclear protein (15 μg) was incubated with 2 ng of ^{32}P-5′ end-labeled oligonucleotide, containing the CART CRE site (Fig. 1), in binding buffer composed of 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 5% glycerol, and 0.05 μg/μl Poly[d(I-C)] (Roche, Indianapolis, IN). Total nuclear protein extract and labeled oligonucleotide (approximately 1×10^5 cpm) were incubated for 10 min at room temperature. In some cases, a 20- or 100-fold molar excess of a specific competitor (non-labeled CART oligonucleotide), or 100-fold molar excess of a non-specific competitor OCT1, 5′TGTTGAGGGGACTTTCCCAGGC (Promega, Madison, WI) was added to the mixture, to assess the specificity of the DNA–protein binding complex, prior to the addition of the labeled oligonucleotide. After a 10-min incubation with unlabeled oligonucleotide, the ^{32}P-labeled CART oligonucleotide was added and incubation was continued for 20 min.

For the supershift assay, 2 μg of CREB antibody (Santa Cruz Biotechnology) was incubated with the nuclear extract and the ^{32}P-labeled CART oligonucleotide for 45 min omitting the previously described 10-min incubation. Supershift analysis was also done using 4 μg of P-CREB antibody (Santa Cruz Biotechnology). P-CREB antibody was incubated with the nuclear protein extract in binding buffer for 30 min at room temperature, followed by addition of the ^{32}P-labeled CART oligonucleotide and incubated for another 45 min. The DNA–protein complexes were separated by electrophoresis on a 6% non-denaturing (80:1) polyacrylamide gel (1× TBE, 2.5% glycerin). Gels were run (120 V) in the presence of 0.5× TBE buffer for 1.5 h at 4 °C. Dried gels were exposed to Kodak BioMax MR film (Eastman Kodak Company, Rochester,



Proximal CART promoter region showing transcription factor binding sites, and sequence of the CART CRE oligonucleotide used in EMSA and ift assays. The CRE oligonucleotide is −153 to −127 according to Dominguez et al. [7].

NY) or exposed for 24 h and analyzed using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

3. Results

A 27-bp double-stranded oligonucleotide containing the CRE site, corresponding to nucleotides −153 to −127 of the CART promoter [7], was synthesized and the 5′ end labeled with ^{32}P (Fig. 1). In order to test if this CRE site bound protein factors from GH3 cells, nuclear extracts from cells treated with 20 μM forskolin for varying amounts of time were tested in electromobility shift assays. Treatment with forskolin under these conditions has been shown to enhance CART gene expression [7]. The ^{32}P-labeled CART promoter oligonucleotide clearly bound to nuclear factors in the gels, resulting in the detection of a shifted DNA/protein complex (Fig. 2). The binding increased with forskolin treatment time, and addition of excess unlabeled CART oligonucleotide resulted in a reduction of the binding of the radiolabeled CART oligonucleotide. These experiments indicate that the GH3 cells, in response to forskolin treatment, produced increased binding to the CART promoter oligonucleotide.

Western blotting studies were carried out to test if treatment with forskolin under the same conditions produced changes in CREB and/or P-CREB levels. A prominent 43-kDa band was detected using the CREB and P-CREB antibodies. Even though the antibodies can detect CREB, CREM, and ATF-1, the molecular weight of the major species detected corresponds to that of CREB and not to CREM or ATF-1. It was found that only P-CREB

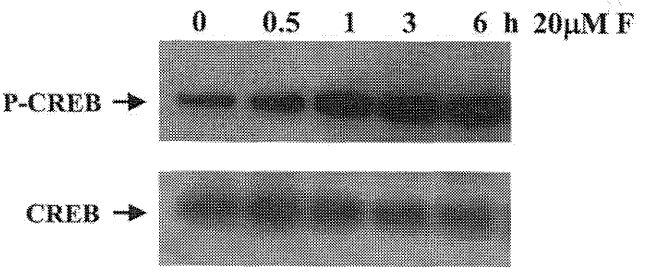


Fig. 3. Western blot analysis of CREB and phosphorylated CREB (P-CREB) in GH3 cells at various times after treatment with forskolin (F). The prominent 43-kDa CREB band is shown. See text for details.

levels increased significantly with duration of treatment with forskolin, whereas CREB levels remained unchanged (Fig. 3). P-CREB levels were increasing at all times, including up to 6 h, similar to the binding factor observed in Fig. 2. These results suggested that the nuclear protein binding to the CART promoter oligonucleotide could be P-CREB.

Accordingly, using supershift assays, we tested if the protein binding to the CART promoter CRE site would interact with CREB and P-CREB antibodies. Indeed, it was found that CREB and P-CREB antibodies supershifted the CART oligo/protein complex (Fig. 4). In addition, molar excess of unlabeled cold CART oligonucleotide competed for binding with the radiolabeled CART oligonucleotide, whereas an unrelated oligonucleotide (OCT1) did not, indicating specificity in the CART oligo/protein complex. The CART oligo/CREB/antibody complex was found in both untreated cells and in cells treated for 3 h with 20 μM

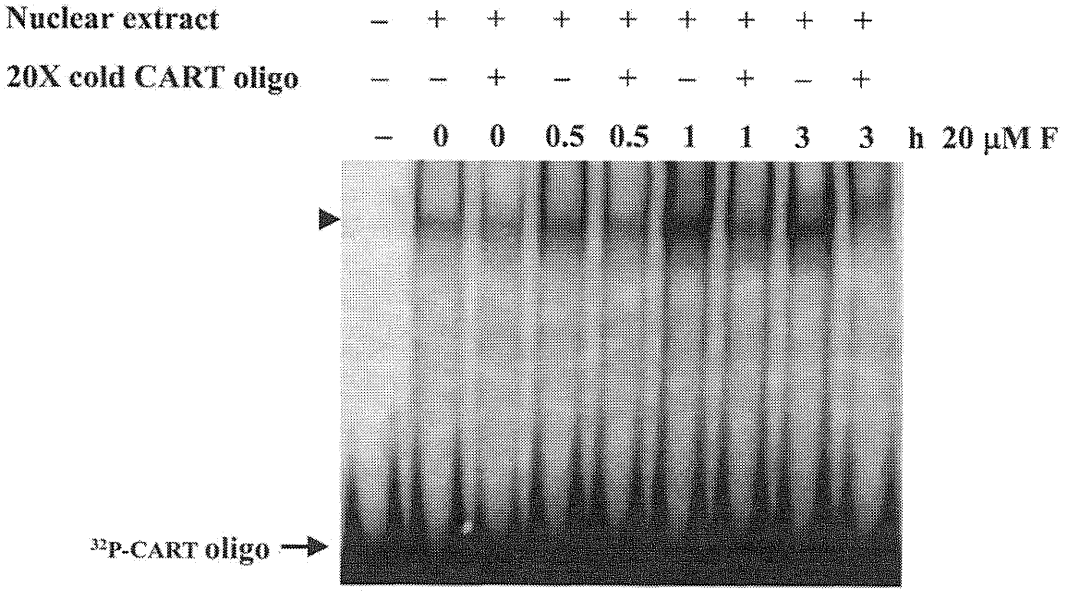


Fig. 2. Binding activity in nuclear extracts of GH3 cells of a CART CRE oligonucleotide by EMSA. Autoradiogram shows the effect of incubation with forskolin (F) for various times on CART oligonucleotide binding to nuclear extracts. Composition of each reaction is given at the top. An arrowhead indicates CART oligo/protein complex. See text for detail.