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MOLECULAR CLONING AND CHARACTERIZATION OF THE PROMOTER REGION OF THE MOUSE REGULATORY SUBUNIT RIIβ OF TYPE II cAMP-DEPENDENT PROTEIN KINASE

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SUMMARY: The promoter and exon 1 of the regulatory subunit (RIIβ) of type II cAMP-dependent protein kinase were isolated from a mouse genomic library. The 5'-flanking DNA lacked TATA and CAAT sites but contained GC rich regions typically found in constitutively expressed house keeping genes. Fusion gene constructs, containing RIIβ 5'-flanking sequences and the bacterial CAT structural gene, were transfected into NB2a neuroblastoma cells and CHO cells. The NB2a cells expressed high levels of CAT activity. CHO cells expressed CAT activity at 5% of the level seen in the NB2a cells. Transfection of deletion constructs into both cell lines was used to define the core promoter and enhancer elements. The core promoter was situated between bp -291/-121. An enhancer element was located between bp -1426/-1018.

The cAMP-dependent protein kinases (EC 2.7.1.37) comprise a family of enzymes each member of which is composed of two regulatory (R) and two catalytic (C) subunits (1). Three isoforms of the C subunit (2-4) have been identified in mammalian tissue. Cα and Cβ are found in most cells. Cγ has been demonstrated only in testis (4). Four distinct isoforms of the R subunit have been cloned and the tissue distribution in mammalian cells has been determined (5-9). RIα, RIβ and RIIα are ubiquitously distributed (10-12) while RIIβ is expressed in specific cells and tissues. RIIβ is present in brain (10,13), neuroendocrine cells (14), ovarian granulosa cells (15) and Sertoli cells (16) and several cell lines (17-19). RIIβ expression is also induced by hormones and cAMP in a number of cells (20). The expression of a tissue specific isoform of R subunits suggests specialized cellular function for this R protein.

The abbreviations used are: cAMP, cyclic adenosine 3',5'-monophosphate; RI, regulatory subunit of type I cAMP-dependent protein kinase; RII, regulatory subunit of type II cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase; bp, base pairs; CAT, choramphenicol acetyltransferase; CHO, Chinese hamster ovary.

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To better understand the mechanisms responsible for the tissue specific expression of RIIβ and the regulation of RIIβ transcription, we cloned the RIIβ gene from a mouse genomic library. In this report we present the sequence of the of 5'-flanking DNA and the first exon of the mouse RIIβ gene, and the expression, in NB2a mouse neuroblastoma cells and CHO cells, of fusion constructs containing RIIβ DNA sequences and the CAT reporter gene.

MATERIALS AND METHODS

DNA SEQUENCE ANALYSIS: A mouse genomic library in λEMBL-3 (Clontech, Palo Alto, CA.) was screened according to Maniatis (21) using a bovine brain RIIβ cDNA probe (9). Positive clones were purified and restriction fragments were subjected to Southern blot analysis using cDNA probes corresponding to the 5'-end, middle and 3'-end of the RIIβ cDNA. A 3.1 kb fragment which hybridized with the 5'-cDNA probe (bp 1- 353) was digested with *Hind*III and subcloned into pGEM-3Z (Promega) and sequenced by the dideoxy chain termination method of Sanger et al. (22). The sequence was determined from both ends using the Sequenase 2.0 kit (USB Corp.).

CELL TRANSFECTIONS: Cells were grown in Dulbecco's modified Eagle's medium. NB2a (23) cultures were supplemented with 5% fetal calf serum and 5% horse serum. CHO cells were supplemented with 2.5% fetal calf serum, 10% horse serum and 1% non-essential amino acids. Cells were grown at 37°C in a humidified atmosphere containing 10% CO_2. Cells were transfected by the $CaPO_4$ precipitate method of Graham and van der Eb. (24). Transfections were performed a minimum of three times and the data are the means of duplicate determinations from three or more transfections.

ENZYME ASSAYS: Cells were lysed by a triple freeze-thaw cycle 60 hours after transfection. 15,000 x g supernatants were used for enzymatic assays. β-galactosidase activity was determined colorimetrically according to Herbomel et al. (25). For CAT activity a portion of the supernatant was heated to 65°C for 10 min and then CAT activity was determined by liquid scintillation spectrometry of acetylated products according to Seed and Sheen (26). Protein was determined by the method of Bradford (27).

RESULTS AND DISCUSSION

Cloning of the mouse RIIβ gene. We sequenced 1426 bp of the 5'-flanking DNA, 301 bp of the translated region in exon 1 and part of the first intron (Fig. 1). Exon 1 contains the nucleotides coding for amino acids 1-100 including the initiation Met. The 5'-flanking region lacks classical TATA and CAAT boxes but contains GC rich regions typically found in constitutively expressed house keeping genes (28). The region between nucleotides -1 to -400 upstream of the ATG initiation codon contains 5 putative SP1 (29) sites and an AP-2 (29) consensus sequence. 1180 base pairs upstream from the ATG there is an AP-1 (30) core consensus sequence. We do not yet know whether these putative transcription factor recognition sequences are functional *in vivo*. Tasken et al. (31) recently showed that regulation of RIIβ

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MOUSE -1426     AGCTTC AGTTTTGTAA GAAGAAAGGG TTTTGGAATT GGTTGTATAA CAATGTGAAT ATATTTAACT
      -1360 GCTGGATTGT ATTCTTAAAA TGGATAAAGC AGCAATTCTG TACTATGTTT ACTTTTCTAG TGAATTCCCT
      -1290 TATGAAAAAA AAAGATAAAT AAACATTGTA AACAAATGGG TAAGAAGCAA TTTTTAAAAA ATAAAACGAA
      -1220 ATGATATTTT TGATTTCTAG GTTTTGCGTT TCGTGAGTCA CATATTTTCC TTATGTGTGG GCAGAAAGGT
      -1150 AAGTGAGAAC AACTAAGGAA ATAAAATAAA ACTCAATAAA TAAATAAAAC TTAAGAATAA AATAAAACTC
      -1080 AAGTGAGTTT GCACTTGGAC TGCTTTACAA TGAAATGGGC ATCGTAGAAA ATAAAGTATG CATATTTATA
      -1010 CAGCGTTTAA TTTGGCAAAA GATAAATTTT TCTGTTTCAA AATTCTCAGA ATAGGCTCGT TTTAAAGACA
       -940 AGGGTTTTCT TTAAAAAGCA CAAATGTGTA GTTCTCTTTT CCTTTTTATT GCTATATTCA AATTTTGGAG
       -870 ATTTAATTGA AGTGACGCAA AGTGTCAGGA AAGGTTTTAA AAATAAGCAA CAAGAGGCTA ATCAGGTAAC
       -800 ACACCCTTAG TTGTCTGTCC AGCTGCCCTG ACTACCCTGT ATCCTGTCTT GAAGGATCAA CTAACTTTGT
       -730 TAAGTGCTAC ACAATAAATG CGGCCTTTGT CTCAGTAACC AGTAGGAAAT GTACTAAGCA GAGTGTGGCA
       -660 GAGCATCTGT AAGGCAAACC ATCACAGCCA CGAGGCTAAA AGGCGGAATT AAAGAAAATT TTGTGACAGA
       -590 CAAGTGGCAG CATCTCTTTT ATCCAGAAGA GAACCAGTAG GGCTGTGCGA CTAACAAAAG ACACCACGCA
       -520 CGTTTCTCTT GGGGTTGGAT TCTAGGAAAA GGAAAAGCCA CCAAGGTGGA GGCTGAAGTT TCAGCAGGAA
       -450 TTGCGCCGCA CCAACTCTGC GTGGGACAGG GAGAAAGGTG CGTGGGATTC GCTCAGGGAG CTCAGGTGGA
       -380 GCGCGCGGCC TCAGCCCCAC GCCCCAGGCT CAGGCTGCCT CCACCCTGGG GCCACTTGAG AGGCGGCAGT
       -310 GCTCCCGCCC GCCGGTTGCC ATGGTTTCCG GGGATCACGT GGGCGCGCGG GCGGAGGCGG GGCGCGCCGG
       -240 GCGGGGGGCG GGGCAGCGGC GGCGGGAGCA GGAGCGGGAG GAGCTGGAGA TGCTGCCAAC CCTCCCCGGG

MOUSE  -170 CTGTGCTCGC TCTGCTCTGC CGCCGCACGG AGCAGCCTCG CCGGGGGCCC AGTGCGCCGC GCTCGCAGCC
HUMAN            GACGCG CGCCGGGAGC CGGCGGCCGG GCCAGCCGGC GCCGGGGCCC AGTGCGCCGC GCTCGCAGCC

MOUSE  -100 GGTAGCGCCC GGGGCGTCGC TCGGGAGCCG CGCAGCCCGA GACCGGACCG CGGATAGGGG GCGAGGGCGG
HUMAN       GGTAGCGCGC CAGCCGTAGG CGTCGCTCGG CAGCCGCGGG GCCCTAGGCG TGCCGGGGAG GGGGCGAGGG

                                                +1
MOUSE   -30 CGTCCAGGCG CCTCGGCGTG CACGGGCAGG ATG AGC ATC GAG ATC CCC GCG GGG CTC ACG GAG
HUMAN       CGGCCAGGCG CCTGCCGCCC CGGAGGCAGG ATG AGC ATC GAG ATC CCG GCG GGA CTG ACG GAG

MOUSE   +34 CTG CTG CAG GGC TTC ACG GTG GAG GTG CTG AGG CAC CAG CCC GCC GAC CTG CTG GAG
HUMAN       CTG CTG CAG GGC TTC ACG GTG GAG GTG CTG AGG CAC CAG CCC GCG GAC CTG CTG GAG

                                                                                  Ala
MOUSE   +91 TTC GCG CTG CAG CAC TTC ACG CGG CTG CAG CAG GAG AAC GAG CGC AAG GGC GCC GCG
HUMAN       TTC GCG CTG CAG CAC TTC ACC CGC CTG CAG CAG GAG AAC GAG CGC AAA GGC ACC GCG
                                                                                  Thr

                                                        Ala
MOUSE  +148 CGC TTC GGC CAT GAG GGC AGG ACC TGG GGG GAC GCG GGC GCA GCC GCG GGG GGC GGC
HUMAN       CGC TTC GGC CAT GAG GGC AGG ACC TGG GGG GAC CTG GGC GCC GCT GCC GGG GGC GGC
                                                        Leu

            Ile                                                     Arg                 Asn
MOUSE  +205 ATC CCC AGT AAG GGT GTC AAC TTC GCC GAG GAG CCC ATG CGC TCC GAC TCC GAG AAC
HUMAN       ACC CCC AGC AAG GGG GTC AAC TTC GCC GAG GAG CCC ATG CAG TCC GAC TCC GAG GAC
            Thr                                                     Gln                 Asp

                                        Ala     Gln
MOUSE  +262 GGC GAG GAG GAG GAG *** *** GCC GCG GAA GCA GGG GCG TTC AAC G
HUMAN       GGG GAG GAG GAG GAG GCG GCG CCC GCG GAC GCA GGG GCG TTC AAT GCT
                            Ala Ala Pro     Asp

MOUSE  +302 gtgaggaccg gccccctctc gccctgagcc ccggccgccg cgtccgcctc actctcgaaccc    +363
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Figure 1. Nucleotide sequence of the promoter and first exon of the mouse RIIβ gene. Both strands of the DNA were sequenced by the chain termination method. Nucleotides are numbered relative to the initiation ATG which is +1. The asterisks designate 6 nucleotides which are present in the human cDNA but are absent from the mouse genomic DNA. The putative core sequences representing the binding sites for transcription factors are underlined. The AP-1 site is TGAGTCA. The AP-2 site is CCCCAGGC. SP1 sites are GGGCGG and CCCGCC. The cDNA sequence for the human RIIβ is from Levy et al. (8).

expression by FSH and cAMP in Sertoli cells requires protein synthesis and that RIIβ mRNA is induced at slower time course compared to c-fos mRNA. We did not detect a CRE in the 5'-flanking sequence of the mouse RIIβ gene. The 5'-flanking sequence

does, however, contain AP-1 and AP-2 consensus sequences which could mediate the hormone responsive induction by cAMP. Enhanced transcription of RIIβ mRNA could be regulated by c-fos binding to the AP-1 site in the RIIβ gene. This would be consistent with the slower time course of RIIβ mRNA synthesis. We cannot, however, rule out the presence of a CRE at some other location in RIIβ gene.

Expression of fusion constructs containing the RIIβ promoter and the CAT structural gene in NB2a cells and CHO cells. We constructed fusion genes containing RIIβ 5'-flanking sequences and the bacterial CAT structural gene and transfected the constructs into NB2a cells and CHO cells. Both the NB2a cells and the CHO cells were able to transcribe CAT from the vector. Basal activity of the promoterless vector was low in both cell lines, but there was about a 4-fold higher level of expression in NB2a cells (Table I). When the 1377 bp of the RIIβ gene were fused to the CAT plasmid, expression of CAT increased 10-fold in the CHO cells and nearly 40-fold in the NB2a cells (Table I). A series of deletion constructs were prepared and transfected into both cell lines. Deletion of 408 bp from the 5'-end resulted in a 40% to 50% reduction in CAT activity suggesting the presence of an enhancer element between bp -1426 and -1018 (Table II). Further deletion of DNA to -475 restored CAT activity in the neuroblastoma cells and the CHO to levels comprable to those using the full length plasmid. Deletion of the DNA to -291 resulted in a 2-fold increase in CAT expression in the CHO cells relative to the full length DNA, while CAT activity in NB2a cells remained comparable to the plasmid containing the full length DNA (Table II). These data suggest that there is a negative regulatory element located between bp -1018 and -475.

TABLE I

EXPRESSION OF pRIICAT IN CHO and NB2a CELLS

Cell Type	Construct	Units CAT/Unit β-galactosidase
CHO	pCAT plasmid	0.0122 ± .002
	pRIICAT	0.1148 ± .011
NB2a	pCAT plasmid	0.0514 ± .009
	pRIICAT	1.9163 ± .084

The *Hin*dIII/*Sac*II (-1426/-49) fragment was ligated into a promoterless pCAT vector and transfected into CHO and NB2a cells. A β-galactosidase vector containing the SV40 promoter was cotransfected with the CAT vector. CAT activity is expressed as units CAT/units β-galactosidase activity ± SEM (n=4). One unit of CAT is defined as the amount of enzyme that transfers 1 nmol of acetate to chloramphenicol in 1 min. One unit of β-galactosidase is the amount of enzyme that hydrolyzes 0-nitrophenyl-β-D-galactoside to produce an absorbance of 1 at 420 nm in 1 hr.

TABLE II

EFFECT OF NUCLEOTIDE DELETIONS OF pRIICAT ON CAT ACTIVITY

		Activity (% of pCAT plasmid)	
		NB2a Cells	CHO Cells
Promoterless Plasmid	...CAT	100	100
-1426	-49...CAT	3400	1000
-1018	-49...CAT	2100	500
-475	-49...CAT	2800	950
-291	-49...CAT	3200	1900
-121	-49...CAT	350	300
-1426	-291...CAT	100	100
-1426	-121...CAT	3200	1100

The 5'-flanking DNA was cut with the indicated restriction endonucleases. The fragments were inserted into a pCAT promoterless vector and transfected into NB2a and CHO cells. A β-galactosidase vector containing the SV40 promoter was cotransfected with the CAT vector. -1426/-49...CAT, *Hin*dIII/*Sac*II; -1018/-49...CAT, *Nsi*I/*Sac*II; -475/-49...CAT, *Bst*XI/*Sac*II; -291/-49...CAT, *Nco*I/*Sac*II; -121/-49...CAT, *Apa*I/*Sac*II; -1426/-291...CAT, *Hin*dIII/*Nco*I; -1426/-121...CAT, *Hin*dIII/*Apa*I. Results are expressed as CAT activity relative to the promoterless pCAT plasmid which is 100%.

Constructs containing bp -121/-49 showed a low level of CAT activity in both cell lines. Transfection of plasmids containing bp -1426/-121 displayed CAT activity comparable to the full length DNA, while plasmids containing bp -1426/-291 were not able to support CAT expression. These results indicate that the core promoter for the mouse RIIβ gene resides between bp -291 and -121.

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