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Dog CREM Transcription Factors: Cloning, Tissue Distribution, and Identification of New Isoforms

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CREM (cAMP Response Element Modulator) transcription factors are involved in the cAMP-dependent transcriptional regulation of CRE-containing genes. Multiple CREM transactivators and repressors are generated from a single gene by alternative splicings and use of an alternative intronic promoter. Here we report the cloning and sequencing of the full-length dog CREM cDNA, corresponding to the CREM$\tau\alpha$ splice variant. Amino acid sequence identity with mouse and human orthologs reached 94.5% and 91.0% respectively. Using the RNAse Protection Assay (RPA) method with three distinct probes, we analyzed the expression of the various CREM transcripts in several dog tissues. We showed that CREM transcription factors have a restricted tissue distribution and that the ratio between activators and repressors varies considerably from one tissue to another. Moreover, we amplified, by RT-PCR, a cDNA that corresponds to two new CREM isoforms and confirmed, by RPA experiments, the presence of these mRNAs in dog thyroid and in other tissues. These transcripts result from splicing of the γ domain and encode potential CREM transactivators (CREM$\tau\alpha\gamma$ and CREM$\tau 2\alpha\gamma$).

Regulation of gene expression, in response to activation of the cAMP pathway by an extracellular signal (hormone, neurotransmitter, . . .), is mostly mediated by transcription factors of the CREB/CREM/ATF family (1). These proteins, able to homo- or heterodimerize by formation of a leucine zipper structure, bind DNA on CRE (cAMP Response Element) sites. The CREM (CRE Modulator) gene shows a highly-structured exonic organization, each exon encoding an independent functional domain (2). Alternative splicing events of these exons control the synthesis of a variety of proteins from a classical promoter, P_1 (Fig.1a). Splicing out two exons encoding Gln-rich transactivation domains (Q1 and Q2) results in the expression of transcription factors that repress gene transcription, while proteins containing at least one of these regions are able to activate CRE-mediated gene expression (2). As the CREM gene encodes two mutually exclusive DNA-binding domains (DBD I and DBD II) with the same specificity of binding but slightly different affinities for CRE sites, each CREM isoform is thus also characterized by its DNA-binding domain (2,3). The transactivation potential of these various CREM transcription factors can be modulated through phosphorylation of a specific serine residue, located in the Pbox regulatory domain : several protein kinases, including the cAMP-dependent protein kinases (PKA), are involved in these regulations (4,5). On the other hand, an alternative intronic cAMP-inducible promoter (P_2) in the CREM gene, downstream from the transactivation regions, is used to produce powerful repressors of transcription, termed ICER for Inducible cAMP Early Repressor (Fig.1a) (6). CREM transcription factors have been implicated in various fundamental differentiation processes, like expression of several spermiogenesis-specific genes mediated by activator CREMτ (7,8) or rhythmic synthesis of melatonin driven by ICER repressors during circadian cycles (9,10).

In dog thyroid, activation of the cAMP pathway by thyrotropin leads to cellular proliferation and differentiation. In order to elucidate the eventual role of CREM transcription factors in these processes, we planned to conduct an exhaustive study on the expression of the CREM gene (manuscript in preparation). In this work, we cloned by RT-PCR and sequenced the cDNA corresponding to the dog CREM$\tau\alpha$ splice variant. We also investigated, by RNAse Protection Assay (RPA), the pattern of expression of the various CREM isoforms in dog thyroid as in several other dog tissues. Furthermore, during these experiments, we detected the presence of two new CREM transcripts in which the γ exon is excluded.

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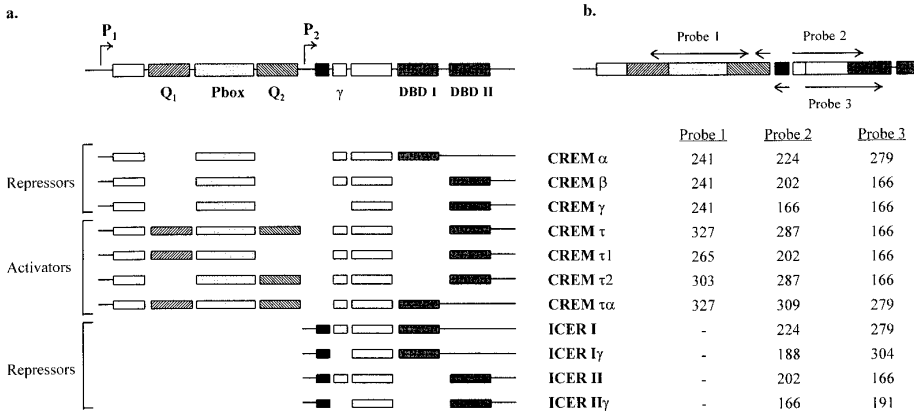

	Probe 1	Probe 2	Probe 3
CREM α	241	224	279
CREM β	241	202	166
CREM γ	241	166	166
CREM τ	327	287	166
CREM τ1	265	202	166
CREM τ2	303	287	166
CREM τα	327	309	279
ICER I	-	224	279
ICER Iγ	-	188	304
ICER II	-	202	166
ICER IIγ	-	166	191

FIG. 1. (a) Schematic representation of the CREM gene and its previously identified splice variants. Q_1 and Q_2 represent the Gln-rich regions, Pbox the regulatory domain, DBD I and DBD II the DNA-binding domains. P_1 and P_2 are the promoters of the CREM gene. Transactivation potential of each CREM isoform is indicated on the left. (b) Localization of the 3 RPA probes with respect to CREM exons and length of hybrids (in bp) formed between mRNAs and probes.

MATERIALS AND METHODS

1. Extraction of total RNA. RNAs were isolated from dog tissues, using the method described by Chomczynski and Sacchi (11). Concentration of RNA was estimated by spectrophotometry.

2. RT-PCR amplifications. Reverse transcriptions were carried out (at 37°C during 1 hour) on 1 μg total RNA (denatured 10 min at 65°C) in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.01 mg/ml acetylated BSA, 20 U RNAsin (Promega), 0.1 U hexamers, 1 mM dNTP and 200 U MMLV-RT (Gibco BRL). Before PCR amplifications, cDNAs were boiled at 95°C for 5 min. Reactions consisted of 1/10 of the reverse transcription mix with 0.5 μg of each primer, 200 μM dNTP and 2.5 U Pwo DNA polymerase (Boehringer Mannheim) in a buffer of 10 mM Tris-HCl (pH 8.85), 25 mM KCl, 5 mM ammonium sulfate and 2 mM $MgSO_4$. After a first denaturation step (2 min at 94°C), amplifications were performed during 40 cycles of 30 seconds at 94°C, 1 min at 55°C and 2 min at 72°C, followed by a final elongation of 10 min at 72°C. The amplified cDNAs were then sequenced using the Taq Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems). Primers used for PCR amplifications were chosen, based on the sequence of the mouse CREM gene (3,12):

B 5′-GGAATTCCATGCTGTAATCAGTTCATAG-3′ (1121,1101) (3)

D 5′-CGGGATCCGAGGACAAATGTAAGGCAA-3′ (-19,-1) (3)

F 5′-CGGGATCCGGTGCTACAATTGTACAGTA-3′ (604,623) (12)

G 5′-GGAATTCCTTCTTCCTGCGACACTCCCG-3′ (576,556) (3)

3. RNAse protection assay. RNA probes (Fig.1b) were synthesized by in vitro transcription (1 hour at 30°C) using 0.2 pmol template DNA (fragments of the dog CREM sequence) in a buffer containing 40 mM Tris-HCl (pH 8.0), 25 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 0.1 mg/ml BSA, 20 U RNAsin (Promega), 500 μM rNTP (A, G and C), 100 μCi UTP α ^{32}P (800 Ci/mmol, Amersham) and 25 U T_7 RNA polymerase (Gibco BRL). After a 10 U RNAse-free DNAse I treatment (15 min at 37°C, Boehringer Mannheim) and separation on a polyacrylamide gel, full-length probes were eluted in a 2M ammonium acetate/1% SDS solution (37°C, during 3 hours under vigorous agitation). After a denaturation step (10 min at 85°C), overnight hybridizations between total RNA (2 or 10 μg) and probes (2.5-5 10^5 cpm) were performed at 45°C in a buffer consisting of 80% formamide, 40 mM Pipes (pH 6.4), 1 mM EDTA (pH 7.5) and 400 mM NaCl. Hybrids were digested (1 hour at 30°C) by 50 μg/ml RNAse A (Sigma) and 4000 U RNAse T_1 (Gibco BRL) in 10 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 7.5) and 300 mM NaCl. Hybrids were then separated on a polyacrylamide gel (6% acrylamide/7M urea). Labelled-DNA size markers were run in parallel in order to identify the size of RNA hybrids (Fig.1b).

RESULTS AND DISCUSSION

1. Cloning and Sequencing of dog CREMτα Splice Variant

It is known from Southern blot experiments that the CREM gene exists in a variety of species including mouse, rat, hamster, human, pig, chicken and Xenopus (3,13); mouse and human sequences have been reported (3,12,13). In order to clone the full-length dog CREM cDNA, RT-PCR amplifications were carried out on dog thyroid total RNA with two sets of primers (D-G and F-B, see Materials and Methods), designed from sequences of the mouse CREM gene (3,12). The 1460 bp isolated cDNA, constructed from two overlapping PCR fragments, corresponds to the dog CREM$\tau\alpha$ splice variant and represents an open reading frame of 344 amino acids (Fig.2). Besides, by RT-PCR and RPA experiments (see below), we were able to demonstrate alternative splicing events characterizing the expression of the dog CREM gene and, by sequencing PCR products derived from various CREM mRNAs, we precisely localized its different exons (Fig.2). Comparison of the dog CREM sequence with mouse and human orthologs (3,12,13) revealed a very high degree of identity in amino acids : 91.0% between dog and human and 94.5% between dog and mouse (Fig.3). In fact, divergence of sequence is restricted to the amino-terminal part of the protein. The second Gln-rich region as well as the DNA-binding domains are totally con-

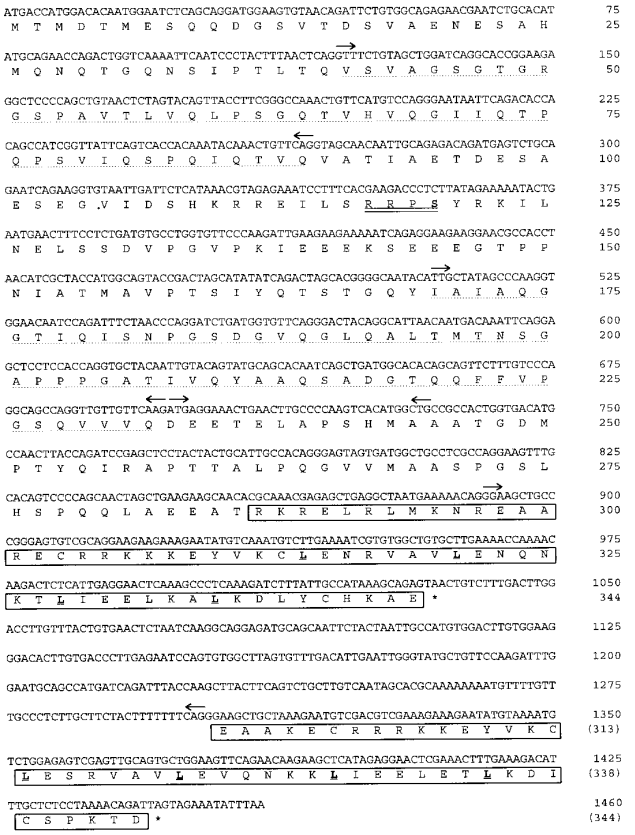

FIG. 2. Dog CREM$\tau\alpha$ cDNA and its predicted amino acid sequence. The nucleotide sequence is available in EMBL, GenBank and DDBJ databases under the accession number X99115. Gln-rich regions are dotted underlined and PKA phosphorylation site is double underlined. DNA-binding domains are boxed; within these domains, leucines involved in the leucine zipper structure are underlined. Sites of alternative splicing are noted by arrows. Numbers in parenthesis represent position of amino acids when the second DNA-binding domain is used.

served, further evidence of their fundamental role in the transactivation process (interactions with basal transcription machinery and recognition of specific DNA sequences, respectively) (2). Moreover the Pbox regulatory domain is also particularly well conserved in these three species, the major phosphorylation site (Ser 120), targeted by several protein kinases, being perfectly conserved in all of them. This suggests that phosphorylations regulating the activity of the dog CREM transcription factors should be virtually identical to those previously identified in mouse (4,5).

2. Tissue Distribution of CREM Isoforms

Recent studies on the CREM gene focused exclusively on the strong production of ICER transcription factors by neuroendocrine tissues after activation of the cAMP pathway, considering that other CREM isoforms, transcribed from the classical promoter, are expressed at a very low level (9,14). However, because they are already present in cells before any stimulation, these CREM transcription factors are responsible for the early regulation of gene expression occurring after cAMP accumulation and are thus of particular importance for this cascade. Previous work had shown that the expression of the CREM gene, from its classical promoter, is controlled by tissue-specific alternative splicing events (3,12). However, these investigations were carried out, by RT-PCR, on a few mice samples and considered only a restricted number of CREM isoforms. Therefore, we decided to study, in a more extensive way, the tissue distribution of these CREM splice variants. Using the RPA method with three distinct probes (Fig.1b), we thus looked for their expression in 13 dog tissues (Fig.4). In fact, the dog CREM gene is ubiquitously expressed but mRNA processing, and thus the nature of alternatively spliced CREM transcripts, greatly depends on the tissue examined. Indeed the relative intensity of RPA hybrids varied importantly among tissues. In adult testis, only activators are expressed, mostly CREMτ (Fig.4a and b), an observation extending to the dog data that have been documented in mouse for mature testis undergoing spermatogenesis (12). We show here that some tissues like thyroid, kidney, brain and cerebellum (and to a lesser extent heart, lymph node, liver and lung) present a wide distribution of CREM isoforms, consisting of activators as well as repressors (Fig.4a and b). Others preferentially express CREM repressors as observed for skeletal muscle, pancreas, stomach and spleen (Fig.4a and b). Using a third probe, we noted that splicing of the exon encod-

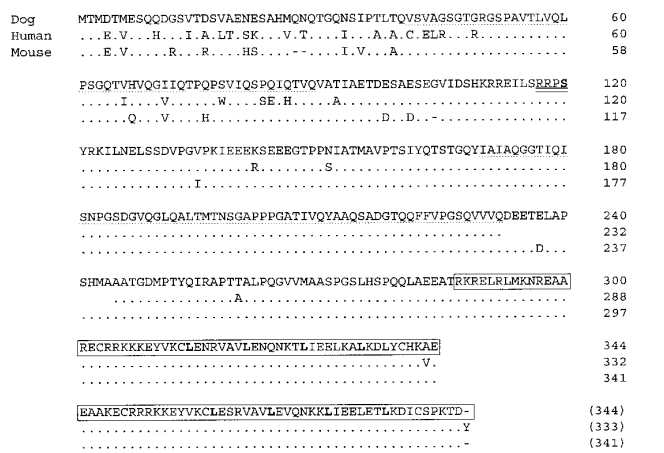

FIG. 3. Comparison of dog, human and mouse CREM sequences. Gln-rich regions are dotted underlined and PKA phosphorylation site is double underlined. The DNA-binding domains are boxed; leucines implicated in the leucine zipper structure are noted in bold type. Position of amino acids corresponding to the second DNA-binding domain is indicated in parenthesis. Points refer to identical amino acids with respect to the dog sequence and dashes represent amino acids absent in dog and mouse sequences.

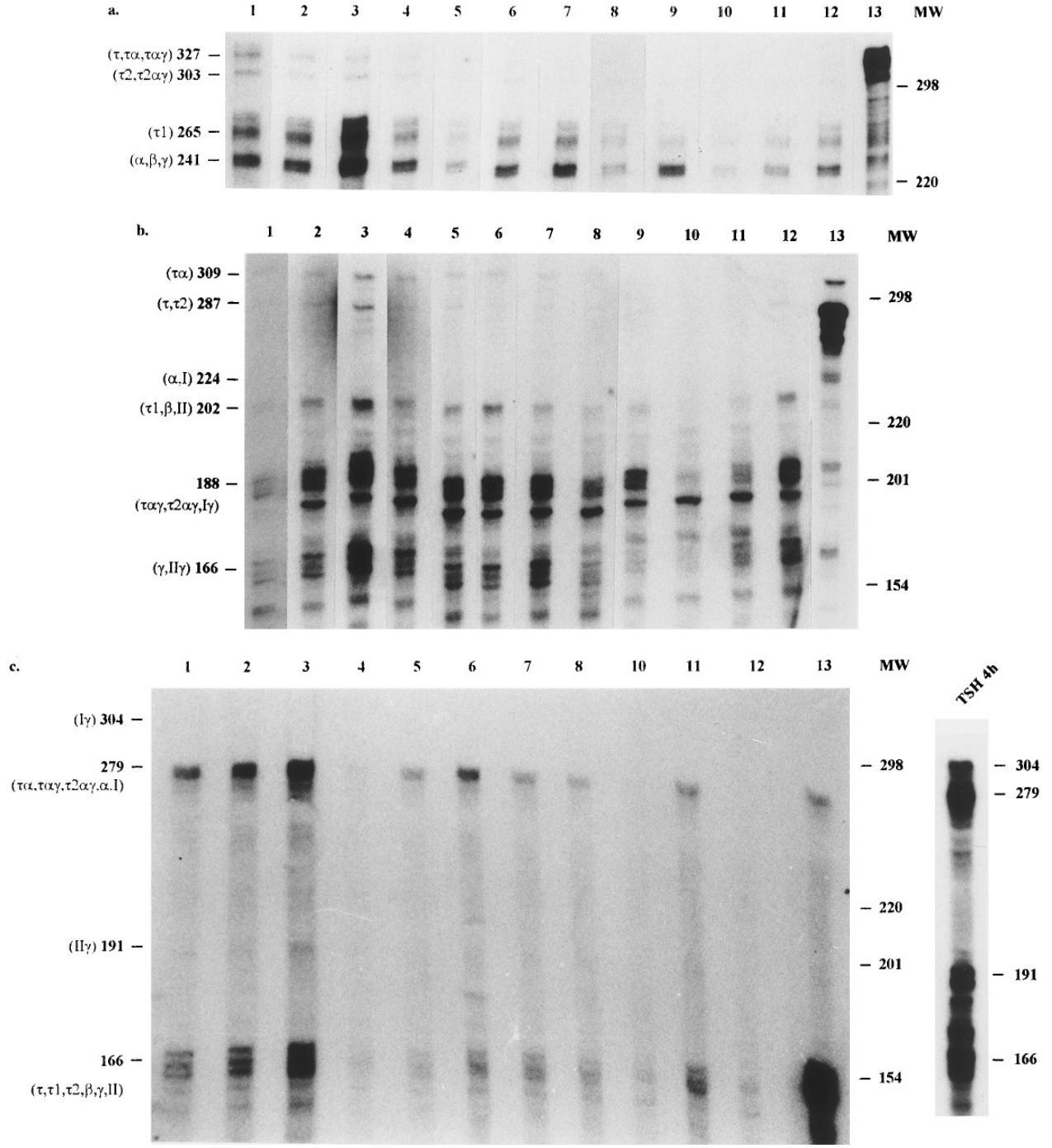

FIG. 4. Tissue distribution of CREM isoforms in dog, analyzed by RPA with probe 1 (a), probe 2 (b) and probe 3 (c). Investigation was focused on cerebellum (1), brain (2), kidney (3), thyroid (4), liver (5), lymph node (6), heart (7), lung (8), skeletal muscle (9), pancreas (10), stomach (11), spleen (12) and testis (13). TSH-stimulated dog thyrocytes are shown as positive control for RPA carried out with probe 3 (TSH 4h). For each condition of RPA experiments, 10 μg of total RNA were used except for testis (2 μg). The length of each specific hybrid and the corresponding CREM mRNAs (left) are deduced from DNA size markers (right). Other bands are to be considered as non-specific.

ing the first DNA-binding domain is also highly tissue-specific (Fig.4c). Lymph node, brain, cerebellum and liver predominantly produce CREM transcription factors containing the first DNA-binding domain while testis, pancreas, stomach and spleen express more CREM isoforms with the second DNA-binding domain. On the other hand, expression of ICER transcription factors was barely detectable in all unstimulated tis-

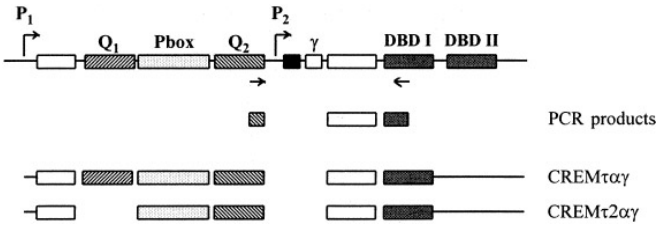

FIG. 5. New CREM isoforms identified by RT-PCR on dog thyroid total RNA. Position of PCR primers (F, forward and G, reverse) are indicated by arrows. From the exonic structure of the sequenced PCR cDNAs, at least two arrangements of the 5′ exons are possible, generating isoforms CREM$\tau\alpha\gamma$ and CREM$\tau 2\alpha\gamma$.

sues tested (no hybrid at 304 bp and 191 bp obtained with probe 3, Fig.4c). As positive control, we added a RNA sample prepared from cultured dog thyrocytes stimulated by TSH for 4 hours (Fig.4c); in this condition, we demonstrated an important induction of ICER expression (manuscript in preparation). Thus, the alternative intronic promoter of the dog CREM gene has a lower basal activity than the P_1 promoter and becomes active only after specific cell stimulations.

3. Identification of New CREM Isoforms

The detection, in dog tissues, of a 188 bp hybrid with probe 2 (Fig.4b) was quite intriguing because the expression of the corresponding mRNA (ICER Iγ) was not confirmed by RPA experiments carried out with probe 3 (absence of a 304 bp hybrid, Fig.4c). The presence of the 188 bp hybrid suggests the expression of an as yet unidentified CREM variant, in which the γ exon would not be incorporated in the transcript. Indeed, parallel RT-PCR experiments performed on dog thyroid total RNA with set of primers F-G (see Materials & Methods), followed by sequencing of the cDNAs, revealed the existence of at least one new CREM isoform (Fig.5). These CREM transcripts are characterized by the absence of the 36 bp γ exon, that is spliced out during mRNA processing. Only 3 of the previously described isoforms present this feature (CREMγ, ICER Iγ and ICER IIγ) and all are repressors of transcription. These new CREM isoforms most likely correspond to the CREM$\tau\alpha$ and CREM$\tau 2\alpha$ variants lacking the γ domain (CREM$\tau\alpha\gamma$ and CREM$\tau 2\alpha\gamma$). Even if the exact arrangement of the 5′ exons was not established, due to the position of the forward PCR primer, these new CREM isoforms are supposed to act as activators of transcription because of the presence of Q_2 transactivation domain. Moreover, their expression would easily explain the presence of the 188 bp hybrid in different dog tissues.

In conclusion, we have shown that the dog CREM gene is very close to the mouse and human orthologs. Its expression generates a great number of transcripts by tissue-dependent alternative splicings of exons encoding Gln-rich transactivation regions and the first DNA-binding domain. The physiological significance of the existence of multiple CREM splice variants as well as of their coexpression in cells still remains unclear. In any case, cAMP-mediated regulation of gene expression will result from the balance between CREM activators and repressors. Moreover, the affinity of CREM transcription factors for specific CRE sites (depending on the sequence of their DNA-binding domain) will determine a restricted pattern of cAMP-regulated genes in each tissue. Thus, the cAMP-dependent transcriptional regulation of a CRE-containing gene can differ from one tissue to another, depending on the particular distribution of the various CREM transcription factors in the tissues considered. On the other hand, the identification of the first potential CREM transactivators lacking the γ domain gives the opportunity to further study the unknown function of this region.

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