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Alternative 5′-exons of the mouse cAMP-dependent protein kinase subunit RIα gene are conserved and expressed in both a ubiquitous and tissue-restricted fashion

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Abstract The activity of cAMP-dependent protein kinase is controlled by its regulatory subunits. Mouse RIα regulatory subunit expression is initiated from five different non-coding 5′-regions (exons 1a, 1b, 1c, 1d and 1e). This organization appears to be conserved among species. All mouse tissues accumulate exon 1a and 1b transcripts and most contain more 1b than 1a, except brain, heart and oesophagus. Exon 1d and 1e transcripts are found in several tissues, while exon 1c is testis-specific. All five transcripts are in RIα-rich tissues: gonads and adrenal glands.

Key words: cAMP-dependent protein kinase; 5′-Untranslated region; Alternative transcript

1. Introduction

The cAMP-dependent protein kinase (PKA, EC 2.7.1.37), the main mediator of cAMP signal transduction in eukaryotic cells, allows regulation of numerous cellular functions [1]. The inactive PKA is composed of a regulatory subunit dimer which tethers two catalytic subunits. Activation occurs when two molecules of cAMP bind to each regulatory (R) subunit releasing catalytic (C) proteins [2,3]. The monomeric C subunits can phosphorylate cytosolic targets or translocate to the nucleus, inducing gene expression mainly through the activation of cAMP response element binding proteins [4].

Two isoforms of the catalytic (Cα, Cβ) and four isoforms of the regulatory (RIα, RIβ, RIIα and RIIβ) subunits are highly conserved in mammals [5]. In man, the X-chromosome-encoded protein kinase was identified as a new catalytic subunit [6] in addition to the testis-specific Cγ isoform [3]. The catalytic α and β isoforms have similar properties. The two classes of R proteins, type I and type II, differ in molecular weight, binding affinity for cAMP derivatives and the presence, in type II molecules, of a consensus PKA substrate site [2]. The α isoforms are ubiquitously expressed whereas the β subunits are mainly in the nervous system [7].

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Abbreviations: PKA, cAMP-dependent protein kinase; 5′-RACE, 5′-rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UTR, untranslated region

The RIα protein is generally found in the cytosol, but it is localized at the plasma membrane of human erythrocytes [8] and in a multi-protein complex at the 'cap' site of activated T lymphocytes [9]. The RIα subunit, as well as its transcripts, is also accumulated at the neuromuscular junction of the skeletal muscle of adult mice [10]. For the human RIα gene, expression is initiated at different transcription start sites which lead to two distinct, alternatively spliced transcripts coding for identical proteins [11]. Here, we have isolated and characterized five non-coding 5′-regions (exons 1a, 1b, 1c, 1d and 1e) for the mouse RIα gene. The exon 1a- and 1b-containing transcripts are ubiquitously expressed whereas the exon 1c transcripts are predominantly accumulated in testis.

2. Materials and methods

2.1. 5′-Rapid amplification of cDNA ends (RACE)

Total RNA from mouse tissues was prepared by CsCl gradient ultracentrifugation of guanidine isothiocyanate-lysed tissues [12]. First strand cDNA was synthesized employing a reverse transcriptase kit (Gibco) and the exon 2 RIα-specific primer mRIα-e2.3 (Table 1). To amplify the 5′-ends of RIα cDNA, the polymerase chain reaction (PCR) anchor primer from the 5′/3′ RACE kit (Roche) was used in combination with two nested RIα-specific primers (mRIα-e2.5 and mRIα-e2.5 nested; Table 1). PCR products were subcloned into the vector pPCR-Script Amp SK(+) (Stratagene) and from 15 to 30 clones from each tissue were sequenced [13].

2.2. Isolation of the mouse RIα gene

Pools (500–1000 clones) from a P1 vector-based genomic library, derived from the mouse hepatoma cell line BWTG3 [14], were screened using pairs of RIα-specific primers which amplify either the 3′- (mRIα-3.5 and mRIα-3.3) or the 5′-region (mRIα-5.5 and mRIα-5.3) of the RIα gene (Table 1). DNA from the positive clones was characterized by restriction endonuclease mapping, field inversed gel electrophoresis and Southern blotting [13]. Fragments containing either 5′- or 3′-ends of the RIα gene were sequenced using ABI prism procedures (Perkin Elmer). Computer-assisted sequence analysis was performed using the facilities of the Pasteur Institute.

2.3. Semi-quantitative reverse transcription (RT)-PCR

Oligonucleotides used in the RT-PCR reactions are given in Table 1. The first strand cDNA was synthesized at 55°C for 50 min using 5 μg of total RNA with Thermoscript (Gibco) enzyme (15 U) and 0.5 μg of oligonucleotide $dT_{(20)}$ in a total volume of 20 μl. For each set of samples, a reaction was prepared without reverse transcriptase as a control for reagent purity. Two μl of the cDNA preparations were used to independently co-amplify each alternative first exon with an RIα cDNA sequence common to all forms (exons 2 and 3) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [15] in the presence of 20 pmol of each primer, 0.2 mM deoxynucleotide triphosphates, 0.1 μl α[^{32}P]dCTP (3000 Ci/mmol) and 2.5 U Taq polymerase

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Table 1
Oligonucleotides used for 5′-RACE, library screening and semi-quantitative RT-PCR

Name	Sequences	Orientation	Location	Usage
mRIα-e2.3	CATACTGCCAGACGCCATGGTTC	−	+85/+106[a]	5′-RACE
mRIα-e2.5	GTTGCCATACTGCCAGACG	−	+5/+22[a]	5′-RACE
mRIα-e2.5 nested	CATACTGCCAGACGCCATGGTTC	−	−5/+17[a]	library screening
mRIα-5.5	CTCAGCTGTGGTAGATCCGCTA	+	−300/−279[a]	library screening
mRIα-5.3	CTCTGTCTTTTCTCCCTCCCTT	−	−131/−110[a]	library screening
mRIα-3.5	AATTGCCCTGCTGATGAATC	+	+977/+996[a]	library screening
mRIα-3.3	ATGTCCGCTTGAGGAATGTC	−	+1089/+1108[a]	library screening
RIα-e3	AGTCAGTACGGATGCCGGTTTTC	−	+206/+247[b]	RT-PCR
RIα-e2	TGTGCAGAAGCACAATATCCAGG	+	+62/+84[b]	RT-PCR
RIα-e1a	AGATCTCCGAACAGCTAAACAATCTAGAG	+	−3210/−3179[b]	RT-PCR
RIα-e1b	TATCGCAGAGTGGTAGTGAGGCTG	+	−2817/−2797[b]	RT-PCR
RIα-e1c	CATCTCTGCCCAGTCGTTTCTTC	+	−2267/−2245[b]	RT-PCR
RIα-e1d	TGAATTTAAGTAAGTCCCAGGCCAG	+	−84/−65[b]	RT-PCR
RIα-e1e	TGATGGCGACTCGGTCTTTAATC	+	−4327/−4305[b]	RT-PCR
GAPDH-up	ACCACAGTCCATGCCATCAC	+	+526/+545[c]	RT-PCR
GAPDH-down	TCCACCACCCTGTTGCTGTA	−	+958/+978[c]	RT-PCR

The sequences are given 5′ to 3′. Orientation '+' and '−' are forward and reverse. Position +1 corresponds to the location of the initiaton codon ATG of either the mouse RIα or the human GAPDH gene.
[a]Mouse RIα cDNA sequence (G.S. McKnight, personal communication).
[b]Mouse RIα 5′-sequence (EMBL accession number: AJ278427).
[c]Human GAPDH cDNA [15].

(EurobioTaq, Eurobio). Cycling conditions were 95°C for 30 s, 57°C for 30 s and 72°C for 30 s for 25 cycles except for the RIα-e1b primer (22 cycles) in order to remain in the exponential phase. PCR products were resolved in 6% non-denaturing polyacrylamide gels and quantified using a PhosphorImager (Molecular Dynamics). The amount of GAPDH was used to normalize the amount of the exon 2/3 RIα cDNA which was then set to 1 and the other products were corrected for their DNA size, GC content and cycle number (the correction factor for exon 1a is 0.859; exon 1b: 4.784; exon 1c: 0.833; exon 1d: 0.761 and exon 1e: 0.685).

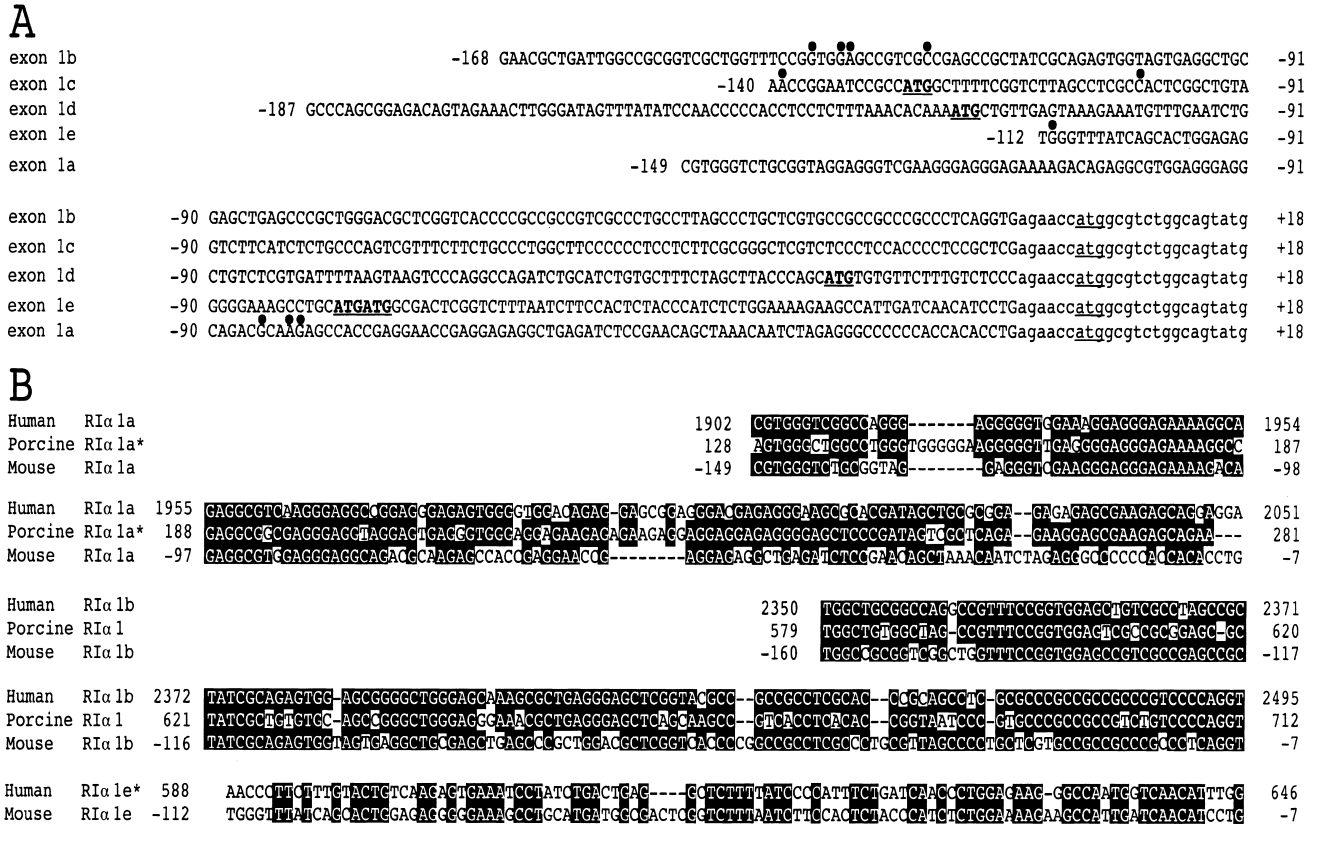


Fig. 1. Sequences of the mouse alternative RIα first exons and homology with human and porcine RIα sequences. A: Sequences of the four new 5′-UTRs of the mouse RIα gene are aligned with the known mouse 5′-UTR (exon 1a). Points indicate the putative major transcription start sites deduced from the number of 5′-RACE clones terminating at these nucleotides. Exon 2 is given in small letters, the translation initiation codon is defined as position +1. Potential upstream start codons are in bold and underlined. B: The 2600 bp human (GenBank accession number: Y07641) and 952 bp porcine (GenBank: X05943) 5′-regions are aligned with mouse exons 1a (−149 to −7), 1b (−160 to −7) and 1e (−112 to −7). The black boxes indicate identical nucleotides. The dotted lines correspond to gaps in the sequence alignment. Porcine exon 1a* and human exon 1e* are deduced from sequence analysis.

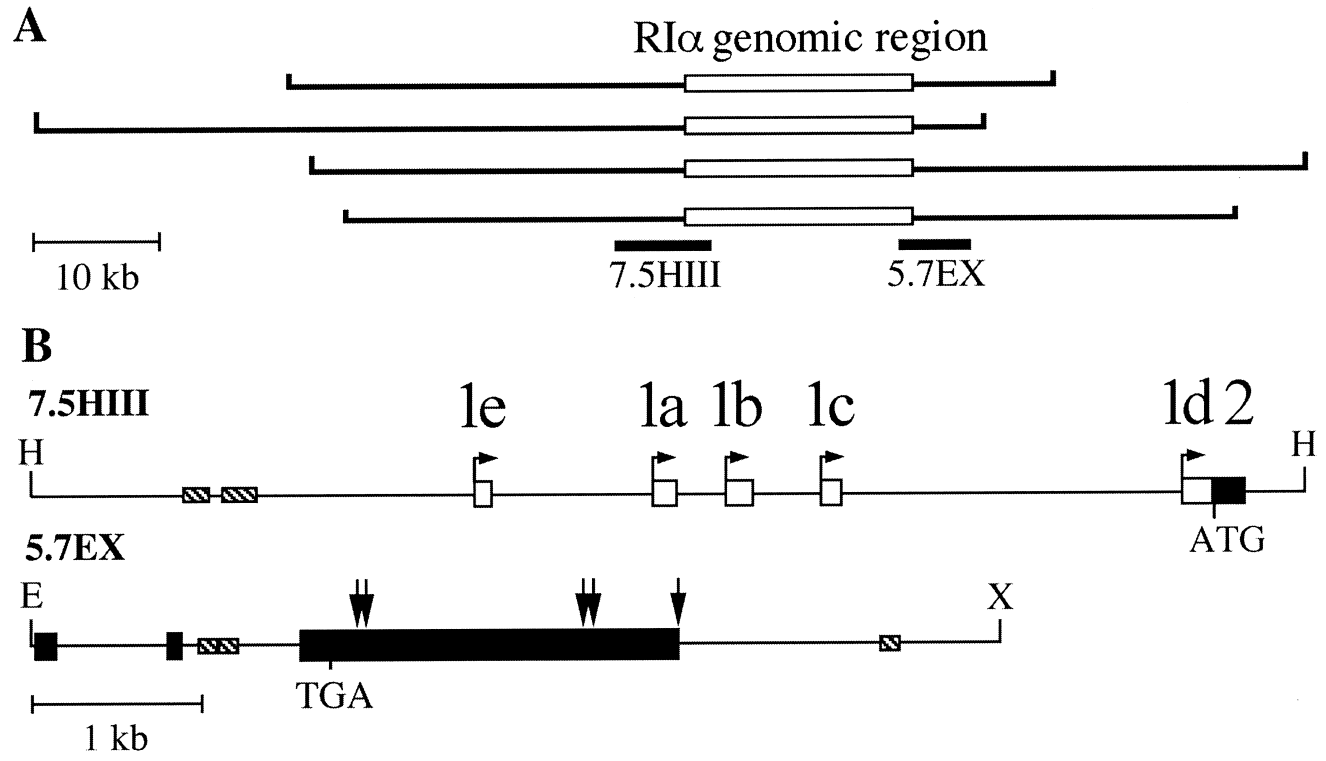


Fig. 2. Structure of the mouse RIα gene. A: The contig formed by the P1 clones, containing the mouse RIα gene. Sizes of inserts are 60 to 75 kb. Bars indicate the positions of the fragments shown in (B). B: Schematic representation of the 7.5HIII and the 5.7EX fragments, containing the 5′- and 3′-end of the RIα gene. 5′-UTRs are given as white boxes with the likely transcription start sites (see Fig. 1) represented by the bent arrows: exon 1a corresponds to nucleotides −3303/−3161 upstream of the codon ATG; exons 1b, 1c, 1d and 1e correspond to nucleotides −2870/−2710, −2322/−2189, −189/−1 and −4363/−4258, respectively. Black boxes represent coding sequences. Indicated restriction sites: E, *Eco*RI; H, *Hind*III; and X, *Xho*I. Arrows show the positions of polyadenylation signals, and hatched boxes indicate repeated sequences (B2/B4 and B1 short interspersed nuclear elements or MRE2 elements). The nucleotide sequence for the 5′- and 3′-regions of the mouse RIα gene have been deposited in the EMBL database under accession numbers AJ278427 and AJ278429, respectively.

3. Results and discussion

3.1. Novel 5′-untranslated regions (UTRs) for the mouse RIα transcripts

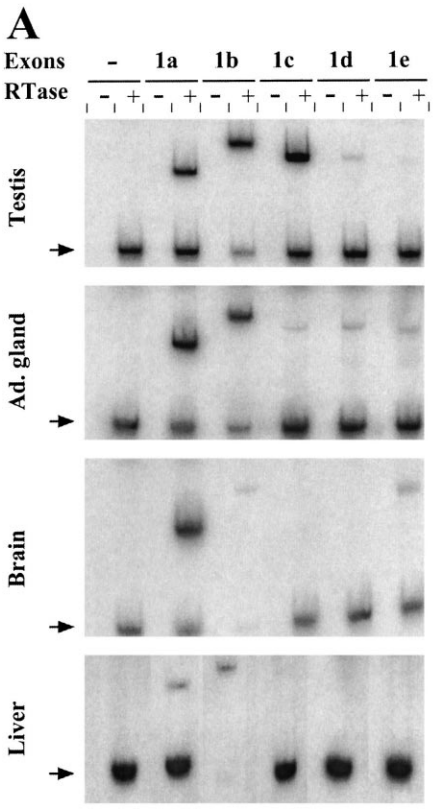
Using 5′-RACE, five distinct products, designated exons 1a, 1b, 1c, 1d and 1e, were obtained with RNA from mouse liver, muscle, brain, lung and testis (Fig. 1A). As expected, the RACE products were flanked by the reverse primer mRIα-e2.5 nested at the 3′-end and the tail of poly(dATP) at the 5′-end. The 3′-termini of all PCR products correspond to the first 24 bp of exon 2 from mouse RIα cDNA (G.S. McKnight, personal communication). Exon 1a corresponds to the previously described 5′-UTR. The other 5′-regions diverge in the sequence upstream of the known splice junction between exons 1 and 2, indicating that they represent alternative first exons of mouse RIα mRNA. The differences in the 5′-extremities of the RACE products indicate that multiple transcription initiation sites are used (Fig. 1A), as described for the human and porcine RIα genes [11,16].

Alignment of the mouse first exons with the two 5′-UTRs of human RIα mRNA [11] revealed 67 and 69% identity, respectively, with exons 1a and 1b, implying that they are homologous to human 1a and 1b 5′-UTRs (Fig. 1B). In addition, exon 1b is homologous to the first exon of porcine cDNA. However, in the sequence upstream of the porcine first exon [16], a GA-rich sequence shows similarities to human and mouse 1a exons. Similarly, analysis of the 2.6 kb of the 5′-region of the human gene revealed the presence of a sequence that shares 72% identity with mouse exon 1e (Fig. 1B). Taken together, these results indicate that alternative first exons of the RIα gene are a conserved feature among mammals. While exons 1a, 1b and 1e exist in the human and porcine genomes, sequences homologous to exons 1c and 1d remain to be identified for species other than the mouse.

Additional ATG codons exist within exons 1c, 1d and 1e upstream of the known translation initiation codon in exon 2 (Fig. 1A). However, these ATG codons are not in-frame with the major open reading frame and translation from them would lead to early termination. Kozak [17] has shown that the presence of such upstream ATG codons is non-random in the 5′-UTR of vertebrate genes and could serve to inhibit cap-dependent translation [18]. Furthermore, the 5′-UTR of a transcript may have consequences for translation due to the formation of stem–loop structures that inhibit translation initiation as described for ornithine decarboxylase RNA [19]. Potential stem–loop structures, conserved among the mouse, porcine and human RIα genes (not shown), can be predicted for exon 1b, suggesting that these structures are important. However, their impact on translational efficiency is unknown.

3.2. The 5′-region of the mouse RIα gene

The screening of a mouse genomic library identified several clones forming a contig which covers 100 kb and contains the entire RIα gene dispersed over 25 kb (Fig. 2A). Sequencing revealed all 5′-UTRs within a 7.5 kb *Hind*III fragment (Fig. 2B). Transcription from the most distal first exon, 1e, is ini-



B

	Total	1a	1b	1c	1d	1e	1b/1a
Testis	222.6	30.6 ± 8.2	108.3 ± 35.0	81.3 ± 9.7	1.8 ± 0.8	0.6 ± 0.2	3.5
Ovary	202.7	57.2 ± 10.0	138.5 ± 8.4	0.7 ± 0.1	0.5 ± 0.1	5.8 ± 1.0	2.4
Ad. gland	201.3	78.2 ± 6.4	118.5 ± 3.9	1.0 ± 0.1	1.3 ± 0.2	2.3 ±0.5	1.5
Uterus	107.4	13.6 ± 3.7	93.8 ± 15.1				6.9
Brain	89.2	41.6 ± 2.2	45.5 ± 6.6			2.1 ± 0.5	1.1
Heart	86.8	47.9 ± 7.4	38 ± 0.5		0.3 ± 0.1	0.6 ± 0.1	0.8
Kidney	86.4	16.6 ± 1.4	69.1 ± 10.4	0.7 ± 0.2			4.2
Lung	84.4	19.2 ± 1.0	61.7 ± 4.9			3.5 ± 0.3	3.2
Parathyroid	67.6	8.2 ± 2.0	59.4 ± 5.9				7.2
Oesophagus	66.7	44.1 ± 1.0	20.8 ± 2.7		0.4 ± 0.1	1.4 ± 0.2	0.5
Cerebellum	62.3	24.8 ± 5.8	35.9 ± 3.7			1.6 ± 0.5	1.4
Lg. intestine	51.3	7.4 ± 2.0	43.6 ± 5.0			0.3 ± 0.1	5.9
Sm.intestine	46.9	2.9 ± 0.3	44 ± 2.6				15.2
Spleen	41.8	7.1 ±1.7	34.2 ± 3.0			0.5 ± 0.1	4.8
Sk. muscle	19.8	7.8 ± 1.1	12.0 ± 2.0				1.5
Stomach	11.7	4.2 ± 1.7	7.5 ± 4.0				1.8
Max.gland	7.0	0.9 ± 0.2	6.1 ± 1.4				6.8
Liver	1.4	0.1 ± 0.0	1.3 ± 0.2				10.8

Fig. 3. Amounts of the different RIα transcripts in mouse tissues. A: Examples of autoradiograms showing results of co-amplifications of exons 2/3 of RIα (167 bp), indicated by arrows, of different first exons (1a, 282 bp; 1b, 345 bp; 1c: 312 bp; 1d, 315 bp; and 1e, 315 bp) and of GAPDH (420 bp; used for normalization and not shown). Each autoradiogram is representative of four independent reactions. Autoradiograms were exposed for 16 h, except liver (1 week). B: Amounts of exon 1a, 1b, 1c, 1d and 1e RIα transcripts. For each sample, three to five independent reactions were carried out and data are expressed as mean values ± S.D. Abbreviations: RTase: reverse transcriptase; Ad.: adrenal; Lg.: large, Sm.: small; Sk.: skeletal and Max.: maxillary.

tiated at −4.3 kb from exon 2, whereas exons 1a, 1b, 1c and 1d are, respectively, initiated at −3.3, −2.9, −2.3 and −0.2 kb. Since the alternative first exons are not contiguous, their expression must be controlled by different promoters located directly upstream of their respective transcription start sites. Unexpectedly, exon 1d is not separated from exon 2 by intronic sequences and this is true for all mouse laboratory strains tested (not shown). This implies a splicing event within the contiguous exon 1d–exon 2 sequence when transcription starts upstream at exons 1a, 1b, 1c and 1e. A precedent for such a mechanism has been described for the rat γ-glutamyl transpeptidase gene [20]. The four other exons, at their 3′-ends, are homologous with the splice donor site consensus sequence AG/GTRAGT, and a splice acceptor site CAGA is located at the 5′-end of exon 2 [21].

Sequencing of around 6 kb of the 3′-region (5.7EX) revealed that the last 556 bp of the coding region are divided into three exons of 121, 81 and 2215 bp in length, separated by introns of 657 and 694 bp (Fig. 2B). Moreover, three regions contain polyadenylation signals whose usage would give rise to the three transcripts of 1.5, 2.7 and 3.2 kb observed on Northern blots [22].

3.3. *Differential expression of alternative RIα transcripts in mouse tissues*

Fig. 3 shows examples of autoradiograms and calculations of the amount of RIα transcripts detected in mouse tissues using semi-quantitative RT-PCR. Note that the lanes containing exon 1b product show weaker signals than the other lanes, since only 22 instead of 25 amplification cycles were performed in order to remain in the exponential phase of the PCR reaction. The amounts of RIα transcripts observed among the tissues differed over a 100-fold range. In Fig. 3B, results are grouped according to the total amounts of transcripts. These data are consistent with those obtained by Northern blots (not shown) indicating that RT-PCR provides a faithful measure of RIα transcripts in tissues. In addition, 5′-exon utilization in the different tissues is compatible with the distribution of 5′-RACE products (not shown).

RIα is expressed at moderate to high levels in all tissues tested except liver, maxillary glands, stomach and skeletal muscle. Endocrine tissues (testis, ovaries and adrenal glands) express the highest levels and represent the only tissues where all transcripts are detected. Exon 1a and 1b transcripts are present in all tissues. With the exception of heart and oesophagus, 1b transcripts are most abundant.

Transcripts containing exon 1c were found at high levels only in testis (35% of the total). This provides a first example where alternative first exon usage could serve to increase expression of the ubiquitous RIα subunit in a tissue-specific manner.

Exon 1e transcripts are present in the central nervous system (brain and cerebellum), endocrine glands (adrenal glands, ovaries, testis) and lung, corresponding to 2–4% of the total

RIα. Since all these tissues are composed of numerous cell types, it remains possible that exon 1e transcripts are the major form in a minority cell type. In addition, exon 1e transcripts are readily detected in RNA from myoblast and hepatoma cells (not shown). It is noteworthy that exon 1d transcripts were only detected in tissues which express RIα at high levels (ovaries, testis, adrenal glands) or which contain more exon 1a than 1b transcripts (oesophagus and heart). Since exon 1d transcripts never exceed 2% of the total and their sequence is contiguous with exon 2, it cannot be excluded that they correspond to incompletely spliced intermediates. It should be noted that the sizes of the RT-PCR products correspond exactly to the lengths anticipated, excluding the presence of exon 1d sequences in the other transcripts.

No alternative transcripts have been reported for the other PKA regulatory subunits. RIα is the only regulatory subunit which is crucial for the progression of embryogenesis in the mouse [23]. The initiation of transcription at several sites, generating transcripts differing in their 5′-UTR could render the expression of this important gene less vulnerable to mutations which could modify its expression.

Future work will be devoted to the investigation of the possible biological significance of the five different 5′-UTRs for RIα transcripts. The sequences (or their cognate promoters) could provide information for transcript localization, for tissue-specific expression and/or for translation efficiency and RNA stability.

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