Identification of PKA-responsive RNA Elements Controlling Alternative pre-mRNA Splicing

by

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A Thesis submitted to the Faculty of Graduate Studies of

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LIST OF ABBREVIATIONS

AR..... androgen receptor

ARRE.....transferrable PKA responsive RNA element

ARS.....activation-responsive sequence

CaMK.....calcium/calmodulin-dependent protein kinase

CaMK IV..... calcium/calmodulin-dependent protein kinase IV

CaMKK.....calcium/calmodulin-dependent protein kinase kinase

CaMKK β1.....calcium/calmodulin-dependent protein kinase kinase beta 1

cAMP..... 3', 5' cyclic adenosine monophosphate

CaRRE1.....CaMK IV responsive RNA element type 1

CBP..... CREB-binding protein

ChIP..... chromatin immunoprecipitation

CRE.....cAMP-response element

CREB.....cAMP responsive element binding protein

Crn..... crooked neck

DMD..... Duchenne muscular dystrophy

Dscam.....Down syndrome cell adhesion modecule

ERα..... estrogen receptor alpha

ERK.....extracellular signal-regulated kinase

ESE..... exonic splicing enhancer

ESS..... exonic splicing silencer

EST..... .....expressed sequence tag

GAD.....glutamic acid decarboxylase

hnRNP.....heterogeneous nuclear ribonucleoprotein

HOW..... held out wings

ISE..... intronic splicing enhancer

ISS..... intronic splicing silencer

KH..... hnRNP K homology

MAPK.....mitogen-activated protein kinase

MEME..... Mutiple Em Motif Elicitation

NMD.....nonsense-mediated decay

NMDAR1.....N-methyl-D-aspartic acid receptor 1

PKA.....cAMP dependent protein kinase A

PKCγ.....protein kinase C gamma

pol II..... RNA polymerase II

PR..... progesterone receptor

PSF.....PTB-associated splicing factor

PTB..... polypyrimidine tract binding protein

PTC.....premature translation-termination codon

PWS..... Prader-Willi syndrome

RRM..... RNA recognition motif

RUST..... regulated unproductive splicing and translation

Sam68..... Src associated in mitosis 68 Kd

SELEX.....systematic evolution of ligands by exponential enrichment

SFC..... splicing factor compartment

snoRNA..... small nucleolar RNA

SnRNP..... small nuclear ribonucleoprotein particle

SR proteins.....arginine/serine-rich proteins

STREX.....stress axis–regulated exons

SWI/SNF..... mating-type switch/sucrose non fermenting

Sxl..... Sex-lethal

Tra2..... Transformer 2

U2AF..... U2 auxiliary factor

UTR..... untranslated region

v5..... variant exon 5

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ABSTRACT

Alternative splicing is emerging as a central mode of genetic regulation, by which exons from a single pre-mRNA transcript are selectively combined to form distinct mRNAs. The splicing decision is differentially controlled in a tissue/cell type- or developmental stage-specific manner, and modulated by signal transduction pathways activated by extracellular stimuli. The signalling pathways regulate alternative splicing by targeting downstream cis-acting pre-mRNA sequences, but only a few of these sequences have so far been identified. In this study, the CaRRE1 (Calcium/Calmodulin-dependent protein kinase IV (CaMK IV) responsive RNA element type 1) was found also to be responsive to the cAMP-dependent protein kinase (PKA). Using a multi-cycle selection and amplification procedure based on cotransfection of a PKA-expressing construct and a library of minigene splicing reporters which contain random sequences in a test exon, I have identified transferrable PKA responsive RNA elements (ARRE) that confer splicing changes in heterologous minigenes in response to PKA. Shared by these ARRE sequences is a consensus heptamer RNA motif (the ARRE motif), CARAAHD (R=A/G, H=A/C/U and D=A/G/U). Database search found ARRE motif sequences in endogenous exons essential for PKA-regulated splicing. The ARRE also responds to CaMK IV but not to PKCγ. Moreover, the ARRE motif mediates PKA control of the alternative splicing of CaMKK β1 exon 16. Therefore, the ARRE also represents the first RNA element that mediates crosstalk between two signalling protein kinases.

I. INTRODUCTION

Various cell signalling pathways relay environmental cues to cellular targets for cells to respond accordingly. Interplay of the pathways occurs at multiple levels including transcription and posttranslational modification [1]. In comparison, the role of alternative pre-mRNA splicing in this interplay has not been studied.

3', 5' cyclic AMP (cAMP) is an ubiquitous second messenger activating the protein kinase A (PKA) pathway, which mediates the actions of many physiological stimuli such as hormones and neurotransmitters [2]. PKA exerts its effect through directly phosphorylating transcription factors [3], RNA binding proteins [4], ion channels [5] and other factors [6], as well as through crosstalking with other signaling pathways [7]. For example, PKA phosphorylates threonine 108 of calcium/calmodulin-dependent protein kinase kinase (CaMKK) to inhibit the latter's kinase activity [7]. PKA also converges with CaM kinases on the cAMP responsive element binding protein (CREB) to control gene transcription [3]. An antagonist of the dopamine D1 receptor inhibits dopamine-induced alternative splicing of the glutamic acid decarboxylase (GAD) gene, implying the control of alternative splicing by PKA since dopamine has been known to exert its effect by activating the PKA pathway [8]. PKA also colocalizes with SR protein splicing factors and regulates the splicing of a reporter minigene consisting of the adenoviral E1A gene [9]. However, the pre-mRNA elements in PKA-regulated alternative splicing of endogenous genes have not been identified.

CaMKKs play an important role in the CaMK pathway by directly phosphorylating the threonine 196 (Thr196) of CaMK IV [10]. The CaMKK β gene undergoes alternative splicing generating several variant proteins with distinct kinase activities. Particularly, inclusion of the alternative exon 16 (corresponding to 15 amino acids) of this gene confers increased CaMKK β activity [11]. It is not known whether the activity of CaMKK β can also be controlled by PKA through regulating the alternative splicing of the alternative exons of CaMKKβ.

Alternative splicing is a common way of gene regulation in metazoans. In mammalian genes, alternative splicing is often controlled by multiple intronic and exonic regulatory elements called intronic or exonic splicing enhancers or silencers, depending on their effect on splicing [12]. Well known splicing enhancer elements include the purine-rich elements [13-16]; splicing repressor elements include the pyrimidine-rich [17-20] and G-rich elements [21-26]. Recent evidence indicates that an element's effect on splicing can also be dependent on its location in the pre-mRNA, not only with varying strength but also to be either promoting or inhibiting, although the underlying molecular mechanisms are not fully understood [27,28].

Splicing regulatory RNA elements are mostly bound by trans-acting splicing factors to promote or inhibit the assembly of the spliceosome components onto the pre-mRNA [12]. For example, the purine-rich enhancer elements usually are bound by the arginine/serine-rich SR proteins [13-16], the pyrimidine-rich inhibitory elements by the polypyrimidine tract binding proteins (PTB) [17-20] and the G-rich elements by the

heterogeneous nuclear ribonucleoprotein (hnRNP) H [25,26] or A1 [21-24]. Trans-acting splicing factors, through their modifications by signaling pathways, have been found to provide the links between cellular signals and regulatory RNA elements known as cell signal-responsive RNA elements during signal-regulated splicing events [29].

Several cell signal-responsive RNA elements have been isolated through targeted mutagenesis of splicing reporter mini-genes. These include an A-rich element bound by Sam68 in the CD44 exon 5 [30], the CaMK IV-responsive elements (CaRRE) in the STREX exon of the Slo gene [31] and in exon 21 of N-methyl-D-aspartic acid receptor 1 (NMDAR1) [32], the hnRNP L and the PTB-associated splicing factor (PSF) bound activation-responsive sequence (ARS) [33-36] and the hnRNP A1 bound UAGG motif responsive to membrane depolarization [24]. Of these, Src associated in mitosis 68 Kd (Sam68) is directly phosphorylated by the extracellular signal-regulated kinase (ERK) of mitogen-activated protein kinase (MAPK) pathway [30]. At present, there are no PKA-responsive RNA elements identified in inducible alternative splicing.

Beyond the approach of targeted mutagenesis of individual splicing reporter genes for identifying splicing regulatory elements, SELEX (systematic evolution of ligands by exponential enrichment)-derived approaches have been used to globally screen splicing enhancers or silencers, allowing the identification of A/C-rich splicing enhancers bound by the YB-1 protein (Coulter et al., 1997). There has been no application of this strategy to the selection for signal-responsive RNA elements that regulate alternative splicing.

In this study, it was initially observed that the Calcium/Calmodulin-dependent protein kinase (CaMK) IV-responsive RNA element type 1 (CaRRE1) also mediates the regulation of the STREX exon by a second signal, the cAMP-dependent protein kinase (PKA) [2,37]. It extended the recent findings that PKA signaling is implicated in the control of alternative splicing [8,9,38,39], and prompted the attempt to obtain a global view of PKA-responsive splicing elements. Using a SELEX-based *in vivo* functional selection procedure [40,41], this work has systematically identified PKA-responsive RNA elements (ARRE) controlling alternative splicing, which share a consensus motif. Interestingly, like the CaRRE1, the ARRE responds to both PKA and CaMK IV, thus these elements can serve as the converging targets of the two different signal transduction pathways. Moreover, it was further found that an endogenous ARRE motif harbored in exon 16 of the CaMK kinase (CaMKK) β1 [11] mediates direct signal crosstalk between the PKA and CaMKK/CaMK pathways through PKA control of the alternative splicing of CaMKK β1, identifying the first RNA element involved in the direct interplay between two signal pathways at the level of splicing regulation.

II. LITERATURE REVIEW:

Regulation of Alternative pre-mRNA Splicing by Cell Signals

Introduction

Most eukaryotic mRNAs are transcribed as precursors containing intervening sequences (introns), which separate the exon sequences that will constitute the final mature mRNA products. Introns are removed in pre-mRNA splicing [42]. Alternative splicing, the process by which distinct combinations of exons are included in the mature mRNA, allowing a single gene to produce multiple protein isoforms [12], is emerging as a ubiquitous mechanism for modulating the capacity and function of the genome. It is considered one of the major ways proteomic complexity is encoded from a relatively small number of genes (http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml). In humans, about 40-60% of genes are alternatively spliced as estimated by analysis of expressed sequence tags (ESTs) [43]. The estimated number increased to at least 74% by a genomic survey with splice junction microarrays [44] and ~ 80% by using high-density oligonucleotide arrays on cytosolic poly(A)+ RNA from chromosomes 21 and 22 [45]. In addition to the prevalence of alternative splicing in the genome, the potential diversity from alternative splicing of a single gene is well exemplified by the Drosophila Down syndrome cell adhesion modecule (Dscam) gene, which can be potentially spliced into more than 38, 000 different mRNA isoforms [46].

Alternative splicing often affects the outcome of gene expression at both RNA and protein levels. Alternative splicing events taking place in translated regions of mRNAs may influence the coding sequence, and some of these events introduce premature translation-termination codons (PTCs) [47]. When alternative splicing in the coding regions does not produce a PTC, the changed primary sequence of the protein may affect protein structure, function and fate, including folding and conformation, post-translational modification, binding property, enzymatic activity, subcellular localization, stability and degradation [48,49]. The range of consequence can be from subtle modulation to complete loss or even antagonistic effect of protein function [48-51]. mRNA transcripts containing PTCs are usually degraded through the nonsense-mediated decay (NMD) pathway and are not productive of proteins [47,52,53]. In a proposed model: the regulated unproductive splicing and translation (RUST) [47], the generation of PTCs by alternative splicing serves as a mechanism of gene expression regulation, by which the level of a protein isoform is modulated through shifting the mRNA template towards degradation. When happening in 5' or 3' untranslated regions (UTRs), alternative splicing can introduce or eliminate RNA regulatory elements and thus influence the stability or translation rate of the transcript [54].

In spite of the abundance and significance of alternative splicing, we have just started to decipher the underlying factors and mechanisms. Recent studies in tissue/cell type-, sex- or developmental stage-specific alternative splicing have identified both cis- and trans-acting splicing regulatory components involved in this process [12]. Depending on their physical locations in the pre-mRNA transcript (in exons or introns) and effects on splicing (stimulating or inhibiting exon inclusion), cis-acting regulatory RNA elements

are called exonic or intronic splicing enhancers (ESE or ISE) or silencers (ESS or ISS). They usually function by recruiting trans-acting splicing factors (mostly proteins, or in some cases RNAs) that enhance (activators) or inhibit (repressors) the splicing machinery. Many splicing regulators are not exclusively enhancers/activators or silencers/repressors and can act either positively or negatively on splicing, depending on the position of action relative to an alternative exon, the surrounding pre-mRNA context and the cellular environment [27,28]. The mechanistic details that determine the positive or negative activity of the splicing regulators are not well understood. It has been realized that tissue/cell type-, sex- or developmental stage-specific alternative splicing events generally involve an elaborate combinatorial control through complex interplay between multiple cis-acting elements and trans-acting factors [12,52,55]. Beyond the current intensive effort to delineate how an individual regulator confers its promotion or inhibition effect on the splicing reaction, it is a more challenging future task to examine how the splicing machinery integrates the positive and negative inputs from the multiple regulators to make the splicing decision.

In addition to its control in a tissue/cell type-, sex- or developmental stage-specific fashion, alternative splicing is also inducible and regulated dynamically in response to extracellular stimuli and growth conditions [29,56]. Signal-responsive alteration of alternative splicing patterns seems to be a physiological adaptation process performed by the cell for fine tuning or adjusting its function according to environmental cues. Intensive studies have recently started towards deciphering this cellular response, but our knowledge is still rudimentary of the detailed mechanisms by which cell signaling

impinges on the splicing reaction. In a few cases, cis-acting components known as cell signal-responsive RNA elements have been identified that mediate inducible alternative splicing. One of these elements contributes to the inclusion of the v5 exon of CD44 in response to the Ras-Raf-MEK-ERK cascade, which phosphorylates its binding protein, Sam68 [30,57,58]. Upon T cell activation, an activation-responsive sequence (ARS) mediates the exclusion of CD45 exon 4 through recruiting hnRNP L and PSF to confer repressive effect on spliceosome assembly [33-36]. In response to membrane depolarization upon neuronal excitation, the UAGG-type silencing elements mediate the skipping of the CI cassette exon (or exon 21) of the NMDA receptor 1 (NMDA R1) [24]. Other neuronal depolarization-induced exon skipping events are well described by studies on the Ca^{2+}/calmodulin-dependent protein kinase (CaMK) IV-responsive RNA elements (CaRREs), whose trans-acting factors remain to be identified. In the Slo potassium gene a CaRRE type 1 (CaRRE1) element represses the STREX exon inclusion in response to CaMK IV [31], and in the NMDA R1 gene, a CaRRE type 2 (CaRRE2) element cooperates with a CaRRE1 to confer CaMK IV repression of exon 21 [32].

1. Mechanism of alternative pre-mRNA splicing in general

1.1. cis-acting RNA elements and the spliceosome dictate the basal splicing reaction

In a pre-mRNA splicing process, several most essential cis-acting elements (or constitutive elements) guide intron removal and exon ligation [12,42,59,60] (Fig. S1). At the 5' splice site, or the upstream exon-intron junction, a highly conserved GU dinucleotide is contained in a short consensus but degenerate sequence and demarcates the 5' end of the intron. In the intronic region at the 3' splice site, or the downstream intron-exon junction,

are short consensus but degenerate elements including, from 5' to 3', the branch point element containing a special conserved adenosine residue (A) and the polypyrimidine tract, followed by a highly conserved AG dinucleotide marking the 3' intron terminal.

Splicing of pre-mRNA takes place in a large RNA/protein macromolecular machinery called the spliceosome, which builds upon the above mentioned essential elements to catalyze intron excision and exon ligation through two consecutive transesterification reactions [12,42,59]. The assembly of the spliceosome involves sequential incorporation of five small nuclear ribonucleoprotein particles (SnRNPs) and many other proteins [12,42,59-62]. The spliceosome acts through a complex RNA-RNA, RNA-protein and protein-protein interactions to remove introns and join exons. But its active core is largely composed of RNA with ribozyme activity [63,64]. Along with the splicing pathway, several intermediate spliceosomal complexes are formed in a highly ordered way. This step-wise fashion of spliceosome formation provides multiple potential sites in the splicing process for regulation [12]. The splice site decision is mostly found to be regulated in the early stages of spliceosome assembly before the first transesterification step, but there is also evidence of regulation at a later stage. The Drosophila splicing repressor protein Sex-lethal (SXL) promotes the exclusion of exon 3 of its own gene. SXL blocks splicing at the second transesterification step through interacting with the spliceosome protein SPF45. [65].

1.2. Regulators of alternative splicing and mechanisms of their effects on splicing in general

The previously described constitutive splicing elements, with short and degenerate consensus sequences, generally contain essential but limited and insufficient information to determine if a spliceosome assembles on a site to carry out splicing. Many regulatory sequences are used by the cell to modulate spliceosome assembly, which are the ESEs, ISEs, ESSs and ISSs as mentioned above [12]. These regulatory cis-acting elements usually function through their trans-acting factors, mostly proteins or in some cases RNAs, to act on and stimulate or inhibit spliceosome assembly.

Major known RNA-binding proteins that regulate alternative splicing fall into two groups: SR proteins and hnRNPs, although there are also other types of splicing factors [12,29]. SR (serine-arginine rich) proteins are well known as splicing activators to enhance exon inclusion, often through ESEs, although they have also been found to inhibit exon inclusion as ISS-binding proteins [66]. SR proteins are characterized by their modular structural organization with one or two N-terminal RNA recognition motif (RRM)-type RNA binding domains and a C-terminal domain rich in arginine and serine dipeptides (RS domain). SR proteins have been suggested to operate and regulate splicing through both protein-protein and protein-RNA interactions [60,67-70]. SR proteins can interact with other RS domain-containing splicing factors such as U2AFs (U2 auxiliary factors), essential factors in the assembly of the spliceosome. One model of mechanism of splicing activation by ESE-bound SR proteins is that SR proteins recruit or facilitate, through protein-protein interactions, the binding of essential splicing factors such as U2AF to weak splice sites to promote or stabilize spliceosome assembly. A second proposed mechanism, based on the observation of RS domain-RNA interactions at the branch point

and the 5' splice site during spliceosome assembly, suggests that these interactions may contribute to the splicing activation by ESE-bound SR proteins.

Splicing repression often involves hnRNP proteins (heterogeneous nuclear ribonucleoproteins) containing RRM, RGG (arginine/glycine-rich) and KH (hnRNP K homology) domains with various functions [71]. Best known examples of hnRNPs acting as splicing repressors are hnRNP A1 [72] and hnRNP I (better known as polypyrimidine tract-binding protein, PTB) [19]. A variety of mechanisms are utilized in splicing repression [12]. Typical examples support that splicing repressors can counteract either the binding of essential splicing factors to the pre-mRNA [19] or the action of splicing enhancers on the basal components of the spliceosome [72].

Some RNA sequences can generate secondary structures that affect splicing. For example, some secondary RNA structures may sequester splicing elements or expose or bring them into close proximity for interactions with other splicing components and subsequently modulates the action of these elements on splice site selection [73].

1.3. Splicing regulators and human diseases

Mutations that eliminate or create cis-acting splicing elements or disrupt or alter the activity of trans-acting splicing factors are increasingly recognized as a cause of human diseases [74]. At least 15% of human disease mutations affect splicing [75]. Based on the correlation of disease-gene propensity with the length of the coding region and the

number of introns, approximately 60% of disease mutations in the human genome cause splicing defects [76].

Two examples illustrate how changes of splicing regulators play a role in human pathogenesis. A cis-acting element mutation in exon 31 of the dystrophin gene creates a specific binding site (UAGACA) for hnRNP A1. The ESS causes the skipping of exon 31, leading to disrupted dystrophin function and Duchenne muscular dystrophy (DMD) [77]. In another case, a small nucleolar RNA (snoRNA), HBII-52, binds to and masks a splicing silencer in the alternative exon Vb of the serotonin receptor 2C pre-mRNA to promote Vb inclusion. Prader-Willi syndrome (PWS) is a disease characterized by the loss of the chromosomal alleles of the SNVRF-SNRPN locus, which encodes for several snoRNAs including HBII-52. The lack of this splicing activator HBII-52 in PWS possibly causes of the loss of the high efficacy Vb-included serotonin receptor, which may account for the disease [78].

1.4. Combinatorial control of alternative splicing

Splicing regulatory elements are generally short and degenerate, and unlike the high-affinity sequene-specific DNA-binding transcription factors, splicing factors have lower binding affinities and sequence specificities [29]. Also SR and hnRNP proteins are mostly abundant in the cell [29]. Nevertheless, splicing occurs in specific transcripts with high fidelity and accuracy. Specific alternative splice site choices could be determined by combinatorial control through multiple relatively weak interactions between different cis-acting elements and trans-acting factors participating in the operation and regulation

of spliceosome assembly [12,55]. Tissue/cell type- and developmental stage-specific splicing decisions may be dictated by 'cellular codes' that are constituted by particular combinations of splicing factors specific to each cell type and development stage. In addition to the splicing factors expressed ubiquitously but with variable abundance among different cell types, tissue/cell type-specific splicing factors have been found to contribute to tissue/cell type-specific splicing patterns in less common cases, such as the Drosophila Sex-lethal (Sxl) [79] and Transformer 2 (Tra2) [80], the mammalian brain-specific splicing factor Nova-1 [81] and the neural-specific homolog of PTB (nPTB) [82].

2. Regulation of alternative splicing by signaling pathways transducing external stimuli

Extracellular signals modulate gene expression at different levels, including pre-mRNA splicing [83]. Evidence has accumulated since 1980s that alternative splicing can be regulated in response to various extracellular signals such as growth factors, cytokines, hormones, neurotransmitters, electrophysiological conditions and environmental nutrients or stresses [29,56]. Although in a number of cases, components involved in inducible alternative splicing events have been identified including signal cascades, signal-responsive cis-acting elements or trans-acting factors, mechanistic details in these systems are largely incomplete. The following section will describe one of the best characterized systems of signal-induced alternative splicing of specific transcripts, to illustrate how a signal transduction pathway is found to be coupled to the splicing of its target pre-mRNA.

2.1. Ras signaling-induced alternative splicing of CD44 pre-mRNA

CD44 is a cell membrane protein that can make multiple isoforms via alternative splicing. The CD44 gene contains ten clustered variable exons (v1 - v10), which are either retained or skipped during splicing [84,85]. The most frequently expressed CD44 mRNA isoform excludes the variable exons. The isoforms containing the variant exon 5 (v5) have been implicated in tumor pathogenesis and intensively explored. Ras signaling [86] has been found to control the inclusion of the v5 exon.

In an early study, a minigene splicing reporter model was established containing the mouse genomic sequence of CD44 v5 exon flanked by its intronic sequences. The minigene was transiently transfected into cells in the presence or absence of a stimulus (signaling pathway activator or cotransfected signaling protein-expressing construct), and the splicing patterns were analyzed by RT-PCR on extracted RNAs. It was found that active $p21^{ras}$ can induce the inclusion of the v5 exon. ESS and ESE RNA elements were identified in the v5 exon by deletion and replacement mutations in the minigene, which are 5' and 3' of the exon respectively, and are important for inducible exon inclusion [57]. In a following work, using a luciferase gene-based splicing reporter containing the v5 exon and pathway activators, inhibitors, constitutively active or dominant negative signaling protein expression constructs, the Ras-Raf-MEK-ERK signal cascade was identified as the pathway that controls v5 inclusion on T cell activation [58].

Other studies identified trans-acting splicing factors involved in Ras-responsive inclusion of the v5 exon. In vitro RNA-affinity precipitation and *in vivo* binding assay in

a yeast three-hybrid system showed that hnRNP A1 binds in vitro and *in vivo* to the v5 exon RNA sequence. Overexpression of hnRNP A1 by transfection of a hnRNP A1-expressing plasmid reduced v5 inclusion *in vivo* in resting-state cells. Replacement mutations of the ESS sequences in the v5 exon abolished the hnRNP A1 repressive effect, indicating the v5 ESS-dependence of the hnRNP A1 repressor effect. Upon Ras activation by contransfection of an active Ras-expressing construct with the hnRNP A1 construct, the hnRNP A1-dependent v5 repression was reversed. This reversion was not due to a downregulation of hnRNP A1 by Ras activation. Thus, the Ras effect was via its antagonistic action on the hnRNP A1 function rather than via an effect on hnRNP A1 expression [87].

In activated cells, the nuclear RNA binding protein SAM68 (Src associated in mitosis 68 Kd) [88] is implicated in the induction of v5 inclusion by the Ras-ERK pathway [30]. RNA-affinity precipitation and electrophoretic mobility shift assay (EMSA) showed that SAM68 preferentially binds to the 5' ESS element of the v5 exon and the optimal binding site was narrowed down by mutational analysis to 10 nucleotides containing the sequence AAAAUU, which is similar to a SAM68 binding sequence identified by SELEX. Cotransfection of SAM68 stimulated v5 exon inclusion in the minigenes previously used [57,58], in a signal-dependent manner, in response to phorbol ester, an activator of the Ras-Raf-MEK-ERK pathway. In this study, an inhibitor of MEK, the kinase of ERK, abolished the phorbol ester-dependent enhancement of v5 inclusion by SAM68. Importantly, SAM68 was found in this study to be phosphorylated by ERK. Mutation of the ERK phosphorylation site of SAM68 impaired its activity of Ras signal-dependent

enhancement of v5 inclusion, while phosphorylation of SAM68 by ERK promoted v5 inclusion in an in vitro splicing system. Finally, morpholino antisense knockdown of endogenous SAM68 abolished Ras-dependent endogenous v5 inclusion, confirming the essential role of SAM68 in this splicing regulation. Together, these data identified SAM68 as a first trans-acting factor that linked an extracellular stimulus to splicing pattern change with modulation of its activity by a signal transduction pathway [30].

The exact mechanism by which SAM68 contributes to alternative splicing of v5 exon has not been clearly established. Given that SAM68 binds to the 5' region of v5 exon close to the hnRNP A1-dependent region, one possibility is that the phosphorylated SAM68 bound to the v5 RNA element interferes with and relieves the repressive effect of hnRNP A1 on v5 inclusion, which might then facilitate splicing activators to act through the ESE [29].

In addition to SAM68, another splicing factor known as SRm160 was demonstrated in a more recent study to regulate Ras-dependent v5 inclusion [89]. Overexpressed SRm160 enhances v5 inclusion in the luciferase-based v5 minigene, with the effect dependent on Ras activation by phorbol ester or transfected Ras-expression construct. SRm160 was found to be a splicing activator through GAA-repeats [90]. Consistent with this, replacement mutation of a 10-nucleotide GAA-containing ESE sequence, ATGAAGAGGA, abolished the enhancing activity of SRm160 on v5 inclusion, suggesting that SRm160 may function through the GAA-containing sequence in the ESE region of the v5 exon. siRNA knockdown of SRm160 inhibited v5 inclusion in both the

v5 minigene and endogenous CD44 gene. Therefore, SRm160 is another essential splicing factor for Ras-ERK signaling-dependent v5 inclusion. Interestingly, SRm160 and Sam68 also coprecipitated in an immunoprecipitation assay. This coimmunoprecipitation was not affected by RNase treatment, suggesting that the association of these two proteins was not linked by RNA, and that the association may be through protein-protein interaction [89].

Taken together, the studies described above illustrate that the Ras signaling cascade appears to concurrently coordinate multiple splicing factors, positive or negative, that recognize several cis-acting RNA elements respectively to achieve a combinatorial regulatory effect on v5 inclusion. The mechanistic details for this are still unclear. However, beyond these findings that are within the confines of our views on how the components of the 'conventional' splicing machinery integrate to operate a splicing process, a recent observation on Ras regulation of the v5 exon has brought a more complex scenario, which involves the coupling of regulation of transcription to that of splicing [91].

2.2. Mechanisms of cell signal regulation of alternative splicing in general

2.2.1. Signal pathways elicit changes in splicing factors - a link between signaling and splicing

External stimuli-mediated splicing regulation is primarily operated through activation of intracellular signal transduction pathways that relay signal to the splicing machinery to modulate its assembly on the pre-mRNA. Signal-induced changes in splicing factors

appear to be the link between the signal and splicing machinery whose assembly also involves signal-responsive RNA elements that 'sense' the changes of their trans-acting partners and mediate the effects of the signal-modulated splicing factors on the formation of splicing complexes on the pre-mRNA.

Changes in splicing factors in response to a stimulus may include posttranslational modification, subcellular relocalization, and modulation of expression level (or stability) of splicing factors. Modification of splicing factors, especially change in phosphorylation status, has been most frequently found. Although relatively few examples have been reported for the other types of changes, more and more splicing regulation events involving these mechanisms may probably be observed. In addition, these different types of changes could concurrently occur on the same splicing factor in a signaling-splicing pathway. This is especially frequently seen in cases where subcellular relocalization of splicing factors is accompanied by changes in their phosphorylation status (see below for several examples). In some instances, it has been found that one change in the splicing factor may subsequently lead to another or one change may depend on another. For example, phosphorylation of a splicing factor can lead to its relocalization, which is indicated below in the case of PKA effect on the splicing repressor PTB. Changes in splicing factors have been extensively shown to alter their effects on the splicing machinery and thus affect the splicing decisions [29,56].

Cell signals most often alter splicing outcome by inducing changes in phosphorylation status of splicing factors [29,56]. SR proteins can be phosphorylated by

kinases such as Clk/Sty, SRPK, CDK11 and topoisomerase I [92-95]. Phosphorylation status changes of SR proteins were found in some studies to affect alternative splicing patterns [96-99]. However, it is not known how signal transduction pathways specifically differentiate their target SR proteins which have similar structures and seemingly redundant functions [68]. Splicing factors can also be modified by dephosphorylation. Still in the case of SR proteins, in response to Fas signaling, the activated protein phosphotase 1 (PP1) dephosphorylates SR proteins [100]. In another instance, when in M phase or upon heat shock of the cell, the splicing repressor SRp38 is dephosphorylated and activated with its splicing repression activity released from being sequestered in the phosphorylated form [101].

There are also other types of splicing factor modifications. Some hnRNP proteins can be methylated [102-104]. The arginine methyltransferase CARM1 is demonstrated by a recent study to be involved in methylation of several splicing factors including CA150, SAP49, SmB and U1C and can alter the inclusion of CD44 v5 exon in a minigene and in the endogenous CD44 gene [105]. Using a CD44 v5 exon-containing splicing reporter (other than those described above) [106], overexpressed CARM1 promoted v5 exclusion rather than its inclusion in CARM1-knockout mouse embryonic fibroblasts (MEFs) and in a Tet-inducible CARM1-expressing HEK293 cell line. Apparently, the CARM1 effect is opposite to that of Ras signaling. In addition, coexpressed CA150 synergized the CARM1 effect on promoting exon exclusion, whereas coexpressed CA150 mutant with a deleted CARM1 methylation site (the PGM motif) failed to show the synergistic activity. Therefore, CARM1 repression of the v5 exon seems to involve its methylation of CA150.

Furthermore, CARM1-knockout MEFs showed drastically reduced v5 exlusion relative to wild-type cells, and re-expressing CARM1 rescued the v5 exclusion characteristic of the existence of CARM1 activity. These data indicate that the methyltransferase CARM1 is essential for v5 exclusion in the endogenous CD44 gene in MEF cells. However, it would have been more interesting to test the roles CA150 or other splicing factors, which are the methylation targets of CARM1, in the CARM1 repression of endogenous v5 exon, in order to provide direct evidence whether methylation of splicing factors by CARM1 contributes to or is required for v5 exclusion in endogenous CD44 gene [105].

In addition to splicing factor modifications by phosphorylation and methylation, the essential spliceosomal splicing factor Prp8p has been shown to bind ubiquitin through a variant Jab1/MPN domain. Mutational analysis showed that several amino acid residues in the domain that were essential for splicing were also required for the binding of the domain with ubiquitin, suggesting a potential functional link between ubiquitination and splicing regulation [107]. Finally, sumoylation of hnRNP proteins was observed by a proteomic analysis but the functional effect of this modification is unknown [108]. Other types of modifications seen on transcription-related protein factors such as acetylation, nitration and carbonylation [109] have not been observed on splicing factors.

Relocalization of splicing factors is another way to regulate splicing. Ischemia in the brain induces nucleus-to-cytoplasm translocation of the splicing factor tra2-beta1 in a hyperphosphorylated form [110], which is accompanied by the alternative splicing change of the ICH-1 gene. In addition, several tra2-beta1 binding proteins including SAM68

subsequently accumulate in the cytoplasm, which might also constitute part of the mechanism for regulating splicing by relocalization of splicing factors. The MKK(3/6)-p38-signalling cascade induced by osmotic or irradiation stress leads to concurrent decrease of hnRNP A1 in the nucleus, and its cytoplasmic accumulation increased with phosphorylation. These changes are accompanied by concomitant alternative splicing pattern changes in an adenovirus E1A minigene [111].

Signal induced nuclear import is also found to regulate splicing. The Ras-PI3K-AKT pathway activated by growth factors phosphorylates SR proteins 9G8 and SF2/ASF and stimulates their translocation from the cytoplasm to the nucleus, where they enhance the inclusion of the EDA exon of a minigene derived from the fibronectin gene [112]. The splicing factor crooked neck (Crn) associates with the RNA-binding protein held out wings (HOW), forming a complex that controls glial cell maturation in Drosophila [113]. In response to neuronal signal, the Crn/HOW complex translocates into the nucleus where it is involved in the alternative splicing of target gene transcripts for gliogenesis.

The mechanism underlying the coupling of posttranslational modification and subcellular relocalization of splicing factors in response to an extracellular stimulus is, in some cases, that the modification accounts for relocalization. This is typically examplified by PKA signaling-induced changes in the nucleo-cytoplasmic redistribution of PTB in PC12 cells. The nuclear export and accumulation of PTB in the cytoplasm as well as in growing neurites is dependent on direct PKA phosphorylation of PTB Ser-16 [4,114].

There are relatively few reports that showed signal pathways directly change the amount (expression) of splicing factors. One example is that in response to neuronal excitation by depolarization, increased hnRNP A1, which binds to the UAGG silencing motif, probably contributes to the exclusion of NMDA R1 exon 21 [24]. In another case, administration of progesterone in ovariectomized mice stimulated the expression level of the SR protein SC35 [115].

2.2.2. Signal regulation of alternative splicing by coupling transcription to splicing

It is an emerging concept that transcriptional regulators and coregulators, including steroid hormones and other types of regulators, also regulate alternative splicing, by coupling transcription to splicing. Steroid hormones such as estrogens, androgens and progestins bind to and activate their intracellular receptors that serve as transcription factors binding to the promoters of target genes where these transcription factors also act as "platforms" to recruit other proteins known as transcriptional coregulators. The resulting coregulator complexes affect various transcriptional steps [116]. Recent studies have indicated that transcriptional (co)regulators activated by steroid hormones also modulate splicing of target transcripts. Consistent with the earlier mentioned mechanism that extracellular signals regulate splicing by inducing changes in splicing factors, in some cases steroid hormones may possibly affect splicing in the same manner [115]. However, increasing evidence supports that steroid hormones are frequently implicated in modulating splicing through transcription-coupled mechanisms [106,117-119].

A major aspect of this coupling mechanism is thought to be based on the recruitment of splicing factors by the transcription complex in the vicinity of the nascent transcript where the recruited factors may act on the splicing machinery, and thereby influence splicing decisions. Estrogen receptor alpha (ERα), depending on its phosphorylation at Ser-118 by EGF-induced MAPK activation, binds directly with its transcription coactivator SF3a p120, which is also a spliceosomal splicing factor. Overexpressed SF3a p120 enhanced Estrogen-ERα induced exon skipping in a CD44-derived minigene (same as that used by Cheng et al., 2007) [106]. Consistent with the Ser-118 phosphorylation-dependent binding of ERα with SF3a p120, both expression of a dominant negative mutant of ERα with the substitution of Ser-118 by Ala (S118A) and siRNA knockdown of SF3a p120 abrogated the ERα effect on splicing, while MAPK activation enhances the ERα effect on splicing. These data suggest that association of Ser-118 phosphorylated ERα with SF3a p120 may be an essential mediator of the ERα regulation of splicing in the CD44 minigene. Furthermore, in endogenous oxytocin gene, estrogen-ERα signaling induced removal of intron 1 was enhanced by EGF-mediated MAPK activation. Both S118A ERα mutant and siRNA knockdown of SF3a p120 abrogated this enhancement effect, supporting that the recruitment of SF3a p120 by Ser-118 phosphorylated ERα can mediate endogenous splicing regulation by ERα [119]. Similarly, androgen receptor (AR) may potentially recruit U SnRNPs in the proximity of target transcripts through binding its coactivator ANT-1, a U5 SnRNP-associated protein [120].

In addition to binding essential spliceosomal splicing factors, transcription (co)regulators also interact with regulatory splicing factors. CAPER α and β are related to the $U2AF^{65}$ family splicing factors. They were found to bind ERα and progesterone receptor (PR) and serve as coactivators for transcriptional activation mediated by these hormone receptors. By testing the effects of overexpression of CAPER or their mutants or siRNA knockdown of these proteins, the CAPER proteins were shown to mediate PR regulation of the alternative splicing of a minigene derived from the human CT/CGRP gene as well as the endogenous vascular endothelial growth factor (VEGF) gene [118].

Transcriptional (co)regulators have also been shown to interact with protein kinases that potentially target the splicing machinery to modulate splicing. For example, the transcriptional coactivator BRG1 and corepressor N-CoR were found to coimmunoprecipitate with the mammalian PRP4 kinase (PRP4K). And PRP4K was also shown to be a U5 snRNP-associated kinase and interact with and phosphorylate PRP6, a spliceosomal protein important for spliceosomal maturation [117].

Transcription-coupled splicing can also be regulated by the kinetics of transcriptional elongation. Due to their relative weakness, use of splice sites of alternative exons might require longer time to interact with the spliceosome under a relatively low elongation rate of transcription [121-123]. This notion seems to be further confirmed by a recent study [91] that extends the previous knowledge (reviewed above) regarding how Ras signaling controls the inclusion of CD44 v5 exon.

The SWI/SNF (mating-type switch/sucrose non fermenting) complex, which was previously known for its function in chromatin remodeling, interaction with RNA polymerase II (pol II) and activation or repression of transcription [124], has been shown [91] to act as a regulator of alternative splicing. Overexpression of Brahma (Brm), the ATPase subunit of SWI/SNF, stimulates v5 inclusion and the effect is enhanced by Ras signaling. Brm complexes with spliceosomal components, the U1 and U5 snRNAs, and especially intriguingly, also with SAM68. Chromatin immunoprecipitation (ChIP) demonstrates that pol II accumulates on regions encoding the variable exons in the CD44 gene in a Brm-dependent manner. The accumulation is higher upon activation of the Ras pathway, which is consistent with the enhancement of Brm stimulation of v5 inclusion by Ras signaling. The study further showed that in the variable region Brm associates with pol II that is phosphorylated at its CTD Ser-5, a phosphorylation pattern known to decrease the velocity of pol II elongation. These findings further elaborate the picture of v5 stimulation by Ras signaling, implying one more potential control mechanism that activation of Sam68 through phosphorylation by ERK contributes to triggering the formation of a transcription-splicing complex containing Brm and pol II, where pol II elongation rate is decreased and the recruitment of the splicing machinery to v5 is facilitated. However, more work in the future will be needed for the eventual establishment of clear and complete mechanistic details implicated in such processes as Ras regulation of the v5 exon. The relationship of the emerging mode of coupling transcription to splicing regulation with those 'conventional' seems intriguing but elusive. Possibly even other mechanism(s)/factors of regulation may also be operating behind these existing observations, considering our realization that alternative splicing control

has been proving more complex than we have previously known based on some relatively simple traditional models of regulation.

3. Implication of PKA signaling in alternative splicing regulation

Many extracellular factors such as hormones and neurotransmitters transduce their signal by activating G-protein-coupled receptors that modulate adenylyl cyclase activity. The resultant changes in intracellular cAMP, in turn, affect the activity of cAMP-dependent protein kinase (protein kinase A, PKA) [2,37]. The PKA pathway ubiquitously regulates many cellular processes such as metabolism, cell growth and differentiation and synaptic neurotransmitter release. PKA is a holoenzyme composed of two catalytic (C) subunits bound by a regulatory (R) subunit dimer. cAMP binding to the R subunits dissociates the holoenzyme and the released C subunits phosphorylate a number of cytoplasmic substrates. A proportion of the C subunits also translocate into the nucleus, where the major known targets of nuclear C subunits are a group of cAMP-responsive nuclear factors called CREBs (cAMP-response element binding proteins), which bind and regulate the transcription of genes containing cAMP-response elements (CREs). However, little has been known about the possible function of PKA in splicing regulation until implicated by a few recent studies.

It was observed that dopamine treatment of striatal neurons, which induces cAMP/PKA signaling, regulates the alternative splicing of the glutamic acid decarboxylase (GAD) gene, and the signal effect depends on D1 receptor [8]. Subsequent studies in striatal neurons showed that both dopamine and forskolin (another PKA

inducer) regulate the splicing of ania6 pre-mRNA [38,39]. Furthermore, a most recent study found that the C subunits of PKA are localized to splicing factor compartments (SFCs) in the nucleus, regulate the alternative splicing of an adenoviral E1A minigene *in vivo* and phosphorylate several SR proteins in vitro [9].

III. STATEMENT OF HYPOTHESES

Previous studies have shown evidences that PKA signaling is implicated in the alternative splicing regulation of genes. However, little is known about the mechanism by which the PKA signal transduction pathway is coupled with splice site choice. In this study, it is proposed that there are PKA-responsive RNA elements controlling alternative splicing, which provide a link between the signal and the splicing reaction. Signal-responsive elements may be systematically identified using global approaches such as the SELEX (systematic evolution of ligands by exponential enrichment)-derived *in vivo* functional selection strategy. The PKA-responsive elements may share consensus RNA motif(s) as potential recognition targets by trans-acting splicing factors. The consensus motif may be found in endogenous genes by genomic search and mediate regulation of alternative splicing by the PKA pathway.

IV. MATERIALS AND METHODS

1. Plasmid construction and mutagenesis

All plasmids were constructed using standard cloning procedures [125] and mutations were made by overlapping extension PCR. The constructs and mutations were confirmed by DNA sequencing.

1.1. The 33SB minigene

The parental plasmids were pDUP33 [126] and the Exontrap vector pL53In [57], derived from human β globin and preproinsulin genes respectively. To facilitate cloning of test sequences, Sal I and BamH I restriction sites [41] were introduced into the 33-nucleotide (nt) middle exon of pDUP33 minigene (Fig. 1A). A DNA sequence from PCR amplification, actgactctctctgcctattggtctattttcccacccttagGTCGACGGTGGTGCCATGGCAGGCCGGATCCAGgttggtatcaaggttacaagacaggtttaaggagaccaat, comprising the modified DUP33 exon (in upper case, with Sal I and BamH I sites underlined) and its original 41-nt upstream and 40-nt downstream intron sequences, was tagged with Apa I and Bgl II restriction sequences, digested and ligated between the Apa I and BamH I sites of pL53In. In the resulting vector, 33SB, the previous pL53In intronic BamH I site is eliminated after the Bgl II and BamH I cohesive ends were ligated, leaving the BamH I sequence in the 33-nt exon as a unique site. To examine RNA sequences for splicing regulatory activities, 13-nt test sequences were cloned into the 33SB vector by replacing its sequence GTGCCATGGCAGG (Fig. 1A).

1.2. cTNT splicing reporter-derived constructs

The parental construct, the cTNT minigene (a kind gift from Dr. Tomas A. Cooper) [41,127], is derived from the cardiac troponin T gene, encompassing its genomic sequence from exon 1 to exon 6. The transcription of this minigene is driven by a Rous Sarcoma virus (RSV) promoter. Exon 5 is an alternative exon whereas the other exons are constitutively included during splicing. A natural splicing enhancer in exon 5 is replaced by a Sal I-BamH I cassette for cloning of test sequences. In this study, the Sal I-BamH I cassette was replaced with that from the 33SB vector or with those from the 33SB derived constructs containing cloned RNA elements. The inclusion patterns of the resulting exon 5 were analyzed by RT-PCR using primers cTNT1 and cTNT2 (see below for primer sequences), which target exons 4 and 6, respectively.

1.3 DUP175NK

The parental plasmid DUP175 was described previously [31]. Restriction sites were introduced into the middle exon of DUP175 for Nde I, 16 nt downstream from the 5' end of the exon, and for Kpn I, 40 nt downstream of the 5' end of the exon, replacing the original exon sequences of the same length with the restriction sites.

1.4. Minigene constructs of alternative exons containing the ARRE motif

Exons and partial flanking introns were amplified by PCR from human genomic DNA, tagged with Apa I and BamH I sites and cloned between the Apa I and Bgl II sites of DUP175 (part of the plasmid construction work was done by Guodong Liu), except

that for 175NK-CaMKK β1, the CaMKK β1 exon 16 with only its partial upstream intron was cloned between the Apa I and Kpn I sites of DUP175.

1.5. Flag-PKA ^{114} mutant expression construct (Flag-PKAm)

The kinase-dead Flag-PKA catalytic subunit was constructed by introducing 2 mutations, Lys73 to Glu73 and Lys169 to Glu169, residues critical for PKA kinase activity [37]. The DNA coding for the flag-PKA mutant was initially amplified as 3 fragments from the Flag-PKA construct using mutagenesis primers. Primers (mutated nucleotides in lower case) used for the flag-PKA mutant construct include FlagPKA1(5'-CGGGATCCGCCATGGACTACAAGGACGACGATGACAAAGCCGC CGCGGGCAACGCCGCCGCCGCCAAG-3'),
PKAK73Es(5'-GGGAACCACTACGCCATGgAGATCTTAGACAAGCAGAAG-3'),
PKAK73Ea(5'-CTcCATGGCGTAGTGGTTCCCACTC-3'),
PKAK169Es(5'-CATCTACCGGGACCTGgAGCCCGAGAATCTTCTCATC-3'),
PKAK169Ea(5'-CTcCAGGTCCCGGTAGATGAGGTC-3') and
PKA2 (5'-GCTCTAGACTAAAACTCAGTAAACTCCTTG-3').
Briefly, fragment A was amplified using primers FlagPKA1 and PKAK73Ea, which mutated Lys73 to Glu73. Fragment B was generated using primers PKAK73Es and PKAK169Ea to mutate Lys169 to Glu169. Fragment C was made using primers PKAK169Es and PKA2. The full-length flag-PKA mutant DNA was obtained by overlaping extension PCR, and then cloned between the BamHI and XbaI sites of

pcDNA3.1(+). The expression of the PKAm was confirmed by Western blot with an anti-Flag antibody. (cloning done by Guodong Liu and Western blot by Jiankun Yu.)

2. PC12 Cell Culture and treatment with PKA stimulators

PC12 is a rat tumor cell line derived from a pheochromocytoma of the adrenal medulla. It becomes terminally differentiated when treated with nerve growth factor and is a model system for neuronal differentiation. PC12 cells grown in DMEM supplemented with 10% horse serum and 5% fetal bovine serum (FBS) in six-well plates to 50-60% confluence were treated with ethanol (with concentration of 0.1% in media, v/v) as vehicle control, forskolin (10 μM) or cpt-cAMP (100 μM) for 7h before RNA extraction and RT-PCR analysis.

3. Culture of HEK293T cells, transfection with DNA constructs (except in the SELEX-derived selection procedure described below)

HEK293T is a human embryonic kidney cell line stably transfected with SV40 large T-antigen. It is a highly efficient cell line model for transient transfection experiments. HEK293T cells were cultured in DMEM with 10% NCS in 12-well plates to about 90% confluence. After media were changed into DMEM without serum transfection was done with lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. To test PKA or CaMK IV effect on splicing, 0.2 μg of a minigene splicing reporter construct was cotransfected with 0.6 μg of Flag-PKAm (Am), Flag-PKA (A), CaMK IV-dCTK75E (IVm) [31,128], or CaMK IV-dCT (IV) [31,128] plasmid for 24h. CaMK IV-dCT "contains a C-terminally truncated version of the mouse CaMKIV-encoding gene, truncated to

Leu-317, and an N-terminal FLAG epitope. It was cloned into the *Bgl* II site of the pSG5 simian virus 40 expression vector (Stratagene). The kinase dead mutant CaMK IV-dCTK75E was constructed by changing lysine 75 to glutamic acid in CaMK IV-dCT, which disrupts ATP binding at the catalytic site" [128]. To test PKCγ effect on splicing, 0.2 μg of a minigene splicing reporter construct was cotransfected with 0.6 μg of pPKCγ-EGFP vector (Clontech Labratories, Inc.) overnight. Cells were then washed, maintained in DMEM and treated with TPA (120 ng/ml) or DMSO (with concentration of 0.05% in media, v/v) as vehicle control for 24h. The translocation of PKCγ-EGFP from the cytoplasm to the plasma and nuclear membranes as a result of TPA activation of the PKC signal transduction pathway was visualized by monitoring EGFP fluorescence on cell membranes under a fluorescence microscope.

4. RNA preparation and RT-PCR analysis

Total RNA was extracted from cells following the transfection/treatment procedures with the GenElute Mammalian Total RNA miniprep Kit (Sigma-Aldrich, Inc.) according to the supplier's protocol. About 2.5 μg of RNA was used in a 10 μl reverse transcription (RT) reaction containing M-MLV reverse transcriptase (Invitrogen) and oligo-dT(18). Two μl of the RT product was used in a 25 μl PCR reaction for 30 cycles at an annealing temperature of 60℃. The RT-PCR products were resolved on 3.5% agarose gels containing ethidium bromide. Band intensities indicating the amounts of the splicing isoforms were quantified by Image J (developed at the National Institutes of Health). The RT-PCR primers used to characterize splicing patterns target the flanking exons, for endogenous STREX exon: QRA59, 5'-CACATTGGAGTCCATGTTGTC-3'; RBSlo1,

5'-AGTGCCTTCGTGGGTCTGTCC-3'; for 33SB derived splicing reporters: Insulin1, 5'-GATCCGCTTCCTGCCCCTG-3'; Insulin2a, 5'-ACCCAGCTCCAGTTGTGC-3', for cTNTβ derived splicing reporters: cTNT1, 5'-AGGAGTATGTGGAAGAAG-3'; cTNT2, 5'-GTCATCCTGAGTGTGGTTGGCAAAG-3'; for DUP175NK derived splicing reporters: DUP8a, 5'-CTCAAACAGACACCATGCATGG-3'; DUP10, 5'-CAAAGGACTCAAAGAACCTCTG-3', for endogenous CaMKK β1 exon 16: 93437S, 5'-GATCCTAGTGAAGACCATG-3'; 93437A, 5'-CCACCTTTCACAAGAGCAC-3'.

5. Selection procedure: A selection system to identify PKA-responsive RNA elements controlling alternative splicing

To construct a starting random library of splicing reporters (starting pool or round-0 pool), a 13-nt randomized cassette embedded in a primer, SXenh-N (GAAGATCTATTGGTCTCCTTAAACCTGTCTTGTAACCTTGATACCAACCTGGA TCCGGNNNNNNNNNNNNCACCGTCGACCTAAGGGTGGGAAAATAG) was designed to replace the 13-nt sequence of the 33SB vector. The PCR fragment amplified with SXenh-N and Insulin1 primers and 33SB as template was cut with Apa I and Bgl II and ligated with Apa I- and BamH I- digested pL53In. The resulting ligation product (457μl) contained ~ 6.86×10^7 reporter clones as calculated based on transformation of *E. coli* cells with a small aliquot of this starting pool (~ 7.5×10^4 colonies were obtained with 0.5 μl ligation product). The reporter pool was cotransfected using lipofectamine 2000 together with 10 μg of Flag-PKA (or CaMK IV-dCT) plasmid and 5 μg of an EGFP expressing construct into HEK293T cells at about 90% confluence in a 10 cm dish (7×10^6

cells). Twenty-four hours later the transfection efficiency was confirmed by examining EGFP expressing green cells to be >90% of cells under a fluorescence microscope. Total RNA was harvested using the GenElute Mammalian Total RNA miniprep Kit (Sigma-Aldrich, Inc.) followed by digestion with RQ1 DNase (Promega) to remove DNA, which is confirmed by the absence of amplified products in PCR using a RT sample without reverse transcriptase (Fig. 2B). RT-PCR was performed to selectively amplify middle exon-included mRNA using primers targeted to splice junctions (primer SXenh-J1, 5'-GGAGGACCCACAAGGTCG-3' and primer SXenh-J2, 5'-CCAGTTGTGCCACTGGAT-3'). The PCR products were cloned as Sal I-BamH I fragments back into the 33SB vector and the selection cycle went through multiple rounds, repeating the same procedure in the subsequent rounds as in the first one except that about 1×10^6 plasmid clones (confirmed by transformation test in *E. coli*) were cotransfected with 1.5 μg of Flag-PKA (or CaMK IV-dCT) plasmid and 1 μg of the EGFP expressing construct into 1×10^6 HEK293T cells.

6. Sequence analysis and database search

Sequences of PKA (or CaMK IV) responsive RNA element clones identified in the selection procedures were aligned using the on-line motif search tool Mutiple Em Motif Elicitation (MEME, http://meme.sdsc.edu/) to identify consensus motifs shared among multiple clones. The identified consensus motif sequences of PKA-responsive RNA elements were used to search the ASAPII database [129,130] to identify motif-containing exons (The ASAP search was done by Jiuyong Xie). The sequence of an exon of interest was searched using the UCSC BLAT

(http://www.genome.ucsc.edu/cgi-bin/hgBlat?command=start&org=human) and the genomic location of the exon was determined in the UCSC Genome Browser on Human Mar. 2006 Assembly. Based on the sequence information from the Browser, RT-PCR primers targeting exons flanking the exons of interest were designed for verification of the splicing patterns (The BLAT search and RT-PCR primer design were carried out by Guodong Liu). Using these primers, the splicing patterns of the exons of interest were analyzed by RT-PCR in PC12 cells (by Wenguang Cao).

V. RESULTS

1. The CaMK IV-responsive RNA element CaRRE1 also mediates splicing change in response to PKA.

As previously mentioned, the CaRRE1 element controls the inclusion pattern of the STREX exon during the splicing of the Slo gene in response to CaMK IV activation. It was interesting to examine whether the CaRRE1 also mediates PKA regulation of exon inclusion. Two 13nt sequences CaRRE1a and CaRRE1b containing the core CaRRE1 were transferred to an exon derived from the human beta globin gene and were tested of their ability to confer response to Flag-tagged PKA on this heterologous minigene (33SB, Fig. 1A & B). These elements confer strong response to PKA on the 33SB gene. The 33SB gene expresses 68% middle exon-included products when cotransfected with PKA mutant (Am, Fig. 1B, lane 1). The exon inclusion level is 81% when cotransfected with wild type PKA (A, lane 2). Replacement of the middle 13 nucleotides with either CaRRE1a or CaRRE1b reduced exon inclusion to 38% and 36% respectively in the presence of PKA mutant (lanes 3 and 7); however, in the presence of overexpressed wild type PKA (lanes 4 and 8), the exon inclusion level was strongly increased to 77% and 75%, respectively. Importantly, this increase was significantly reduced or abolished by mutations in the CaRRE1 sequences (lane 5, 6, 9 and 10), suggesting that the CaRRE1 is also a PKA-responsive RNA element controlling alternative splicing.

The similar enhancing effect on exon inclusion by CaRRE1a and 1b in response to

CaMK IV was also observed in 33SB (Fig. 1C). Interestingly, it has been known that the CaRRE1 represses exon inclusion in response to CaMK IV in the DUP175 splicing reporter and in the endogenous Slo gene as well [31]. Therefore, the CaRRE1 shows context-dependent effect on exon inclusion. This context-dependence nature of the CaRRE1 is similarly observed in other splicing regulatory elements as reported by a number of previous studies [27,28,32,131]. Importantly, the results suggest that the PKA and CaMK IV pathways can converge on a common target RNA element in regulating exon inclusion.

2. PKA-responsive RNA elements controlling alternative splicing enriched from a selection system.

The above CaRRE1 element-mediated enhancement of exon inclusion in 33SB, taken together with the context-dependent effect of constitutive regulatory elements, suggested that a RNA sequence that mediates the repressive effect of a protein kinase can also be tested as a kinase responsive enhancer in this vector. If so, a SELEX (systematic evolution of ligands by exponential enrichment)-derived approach can be used to enrich for elements that are responsive to protein kinases such as PKA and CaMK IV, with the goal of finding a consensus sequence. An *in vivo* functional screening strategy (Fig. 2A) was designed to enrich short RNA sequences that mediate kinase-regulated splicing. Since CaMK IV-responsive elements have been intensively examined, our focus was mainly on the PKA-responsive ones.

In this strategy, a pool of 13-nt random sequences were cloned into the vector 33SB to obtain a library of splicing reporters, which were cotransfected into HEK293T cells with the Flag-PKA plasmid. The spliced products were amplified by RT-PCR using primers selectively targeting exon junctions for exon-included products. The selectively amplified products were again cloned into the 33SB vector and cycled through multiple selection rounds. The resulting splicing reporter pool from each selection round was then assayed for PKA responsiveness in HEK293T cells (Fig. 2C). Compared to the starting, or round-0, pool, the subsequent five rounds of selected reporter pools showed enhanced PKA responses with a peak at round 3, suggesting the enrichment of reporter clones containing RNA elements that enhance exon inclusion in response to overexpressed PKA.

To verify the enrichment of PKA responsive clones, individual reporter clones from round-0, 2 and 3 pools were randomly picked and tested for PKA-responsiveness in HEK 293T cells. The percentages of clones with net changes by PKA of at least 10% higher than the vector background level are 38.9% (7 out of 18 tested clones), 44.4% (8 out of 18 tested clones) and 57.1% (16 out of 28 tested clones) in rounds 0, 2 and 3 respectively. Thus this selection indeed enriched PKA-responsive clones.

Three representative reporter clones 1, 14 or 15 with strong PKA-responsiveness from the round-3 pool are shown in Fig. 2E. These clones contain inserts that reduced exon inclusion compared to the vector 33SB (64%) when coexpressed with PKA mutant (Am, lanes 3, 7, 13). Interestingly, PKA overexpression significantly increased the exon inclusion with net changes ranging from 28% to 34% (lanes 3, 4; 7, 8; 13, 14). Moreover,

mutations in the inserts abolished or decreased the net changes by PKA (lanes 5, 6; 9, 10; 11, 12; 15, 16). This suggests that the selected insert sequences play essential roles in the PKA-dependent splicing changes.

3. Transferrable PKA-responsive RNA elements (ARRE) were identified and share a consensus motif

To test the PKA-responsive activities in a different pre-mRNA context, strongly PKA-responsive RNA elements as tested in the 33SB minigene were cloned into a second splicing reporter minigene, cTNTβ (Fig. 3). 14 out of 21 (67%) transferred elements mediated increased net change of exon inclusion by PKA compared to the PKA effect in the cTNTβ vector as control. The sequences and PKA response patterns in the cTNTβ context of the representative RNA elements from the 33SB clones 1, 14 and 15 are shown in Fig. 3A and 3B respectively. These data indicate that a major proportion of the strongly PKA-responsive RNA elements identified in the selection procedure still respond to PKA upon transfer to a different pre-mRNA context.

The transferable PKA-responsive RNA elements were then analyzed using the motif search tool Multiple Em Motif Elicitation (MEME, http://meme.sdsc.edu/) and a consensus heptamer motif, CARAAHD (R=A/G, H=A/C/U and D=A/G/U), was identified (Fig. 3C). Regarding the function of the consensus motif, as shown in Fig. 2D, mutations that disrupted the sequences corresponding to the consensus motif (underlined) in the three tested representative RNA elements abolished or decreased the PKA

responsiveness, indicating that the motif plays an essential role in PKA control of splicing. In summary, the signal-based functional selection procedure identified transferable PKA-responsive **R**NA **e**lements (ARRE) that share a consensus motif CARAAHD (the ARRE motif) essential for the activity of these elements.

4. The CaRRE1 contains an ARRE-like motif essential for PKA control of alternative splicing.

Next, the relevance of the ARRE to PKA control of splicing in endogenous genes was studied, first by exploring the possible link between the ARRE motif and the CaRRE1. Notably, CaRRE1a and CaRRE1b share a 9-nt common sequence (Fig. 1A), in the middle of which is a heptamer, cacaugg, which bears some similarity in sequence to some versions of the ARRE motif. To determine whether this heptamer candidate motif is critical for the CaRRE1 to act as a PKA-responsive element, mutations were introduced into it and it was found that the PKA responses were dramatically decreased or completely abolished (Fig. 1B, lanes 5, 6 and 9, 10). The evidence supported that the CaRRE1 heptamer sequence is an essential RNA motif for PKA dependent splicing changes, functionally similar to the ARRE motif. To further examine the functional correlation between the ARRE and CaRRE1 motifs 3-nt substitutions were introduced such that the resultant heptamer was CAAAAAG, an exact match with the ARRE motif (Fig. 3D, upper panel). The replacement of the CaRRE1 motif by the ARRE motif led to no detrimental effect on the PKA responsiveness, which was at levels similar to and higher than that of the wild-type CaRRE1 (Fig. 3D, lower panel). The increased effect

conferred by the substitutions in the CaRRE1 motif implies that the CaRRE1 motif may be a sequence variant version of the ARRE motif, which has a sub-optimal sequence and thus a sub-optimal activity compared to the exact sequence version of the ARRE consensus sequence.

5. Dual responsiveness to PKA and CaMK IV and signal specificity of the ARRE

The sequence similarity and functional correlation in mediating PKA response between the ARRE and CaRRE1 motifs, together with another finding of this study that the sequence of the ARRE motif resembles a consensus sequence of CaMK IV-responsive elements obtained from a similar signal (CaMK IV)-based selection procedure (Fig. 4A), strongly raised the possibility that the ARRE, like the CaRRE1, responds to dual signals of both PKA and CaMK IV. It was confirmed by the fact that as expected the representative PKA-responsive elements shown in Fig. 2E also respond to CaMK IV enhancing exon inclusion with CaMK IV compared to with CaMK IV mutant control (Fig. 4B). This is the second evidence to reveal that the PKA and CaMK IV pathways share common target RNA elements in controlling alternative splicing.

The responsiveness of the representative PKA-responsive RNA elements to both PKA and CaMK IV signals led to the question whether the responses are signal-specific for PKA and CaMK IV or these elements generally or ubiquitously respond to all kinds of cell signals, especially signaling kinases, in the same way as they respond to PKA and CaMK IV. To address this issue, the effect of another kinase, PKCγ, on the splicing

patterns of the representative PKA responsive clones (1, 14 and 15) was examined (Fig. 4C). Upon PKCγ activation the representative elements from clones 1, 14 and 15 failed to confer to the splicing pattern any significant change beyond the vector background level, showing that the behaviors of these elements with PKCγ were different from the way they respond to PKA and CaMK IV, therefore they are not ubiquitous signal responsive elements that function to mediate splicing regulation by all (kinase) signals in a common way.

6. The ARRE motif existing in some endogenous exons mediates PKA regulation of alternative splicing.

The ARRE was further characterized with regard to its function in endogenous genes. To find out whether ARRE sequences exist in endogenous genes where they regulate alternative splicing as well, the ARRE motif-containing exons were searched in Alternative Splicing Annotation Project II [129,130]. Seventeen such exons extracted from the search together with part of their flanking intron sequences were cloned between the two constitutive β-globin exons of the DUP175 vector [31], diagrams of representative constructs shown in Fig. 5A upper panel, and their splicing was examined in HEK293T cells in the presence of coexpressed PKA or mutant control. Representative splicing patterns are shown in Fig. 5A lower panel. Of these, 8 exons were constitutively skipped in the DUP construct in HEK293T cells (e.g. E12; Fig. 5A, lanes 1 and 2), and in this context the role of the predicted ARRE motif cannot be confirmed in these exons. Four exons showed aberrant splicing patterns (e.g. E14 and E16; Fig. 5A, lanes 5, 6 and 9, 10),

which were not suitable for clear characterization of the effect of the candidate ARRE motif sequences and were excluded from further analysis as well. Three exons displayed alternative splicing patterns, in which, however, no significant splicing change induced by PKA was observed (e.g. E13; Fig. 5A, lanes 3 and 4), suggesting that the predicted ARRE sequences did not exert a perceivable effect on the splicing in these pre-mRNA contexts in HEK293T cells. The remaining two exons, in E15 (Fig.5A, lanes 7 and 8) and E17 (Fig. 5A, lanes 11 and 12) constructs, exhibited PKA dependent splicing changes. To evaluate the function of the potential ARRE sequences in the PKA responses, the effects of mutations were tested. In E15, a peroxisome biogenesis factor 1 (PBF1) exon possesses two copies of putative ARRE motifs, close to each other (Fig. 5B). Mutation of either motif (E15m1 or E15m2, Fig. 5B) substantially attenuated the PKA effect (Fig. 5C, lanes 3, 4 and 5, 6), and disruption of both the motifs (E15m12, Fig. 5B) resulted in a sharper decrease in the PKA response (Fig. 5C, lanes 7 and 8). These experiments confirmed that the two copies of ARRE motif in this exon are both essential for PKA regulation and have synergistic effect on PKA induced splicing change. Thus the results here served as an example that the ARRE motif from the endogenous exon can have PKA responsive splicing regulatory activity. As for E17, mutation in the putative motif led to no change in the splicing pattern and PKA effect (data not shown), indicating that probably the motif in this pre-mRNA context does not contribute to the PKA regulated splicing and suggesting the possible existence of other type(s) of regulatory motif(s) that mediates PKA controlled splicing in addition to the ARRE motif identified by the selection procedure in the present work.

For further assessment of the functional behaviors of the candidate ARRE motif sequences contained in the 17 exons that were tested in the DUP minigene constructs, PC12 cells were treated with PKA stimulators and RT-PCR was performed to investigate the endogenous splicing patterns of these exons with or without activated PKA signals (carried out by Wenguang Cao, a single experiment except for the following described Group 4). Based on their expression and splicing patterns observed in the experiment, the exons could be categorized into 4 groups (data not shown except group 4). Group 1 consists of 2 exons that were not detectable as expressed in PC12 cells. Group 2 consists of 11 exons involved in constitutive splicing patterns leading to only one splicing product where the exon was either completely skipped or included. The PBF1 exon belongs to this group. Group 3 consists of 2 exons that were alternatively spliced but the splicing patterns were unaffected by PKA stimulators. Finally, Group 4 consists of 1 exon, the CaMK kinase β1 (CaMKK β1) exon 16, that exhibited alternative splicing pattern changes upon treatment with PKA stimulators (Fig. 6A). Group 1 provided no information on the role of the potential ARRE motif sequences in PKA regulated alternative splicing in that the exons were not expressed or not alternatively spliced. Groups 2 and 3 suggested that the putative motif was not able to mediate a detectable PKA responsive splicing change in that context, and the group 4 exon raised the possibility that the candidate ARRE motif may be potentially involved in the endogenous splicing event regulated by PKA.

Interestingly, comparison of the results from the minigene experiments with those from the endogenous studies demonstrated some discrepancies between what these two

contexts revealed. For example, the ARRE motif sequences contained in a group 2 exon, the PBF1 exon, which was constitutively included into mRNA under endogenous conditions in PC12 cells, turned out to mediate PKA dependent changes of alternative splicing patterns in the DUP construct, E15, in HEK293T cells. In addition, the endogenous CaMKK β1 exon exhibits splicing changes upon treatment of PC 12 cells with PKA stimulators, but was constitutively skipped when cloned as a cassette exon in the DUP construct, E12. Overall, all the findings from the minigene-based and endogenous experiments suggest the complexity of the functional behaviors of the ARRE motif sequences that appear to depend on or are affected by the contexts where they exist.

In an attempt to recapitulate in a minigene the PKA control pattern of the endogenous CaMKK β1 exon 16 splicing, the possibility was considered that the 43-nt small size of this exon may have made it difficult to be included as a cassette exon during the splicing of the DUP-derived minigene E12. It is likely that transfer of a very short genomic sequence containing this exon into the DUP construct might have missed some endogenous enhancer sequence(s) outside of the transferred region that are needed to facilitate the inclusion of this small exon. Therefore, the CaMKK β1 exon 16 was fused to a DUP175 derived exon to obtain a larger middle exon in the DUP construct, which was expected to increase the inclusion level of the CaMKK β1 exon 16 sequence (Fig. 6B). It was confirmed that in the resultant splicing reporter 175NK-CaMKKβ1, the enlarged middle exon was mostly included, and a net PKA-induced change in exon inclusion (Fig. 6C, lanes 3 and 4) was obtained similar to the change for the endogenous gene (Fig. 6A). In the mutant construct 175NK-CaMKKβ1m, mutation that disrupted the

candidate ARRE motif CAAAAAA in the CaMKK β1 exon 16 sequence abolished the PKA response (Fig. 6C, lanes 5 and 6), concluding that the ARRE motif is indeed an essential RNA element that mediates PKA control of CaMKK β1 exon 16 inclusion. This finding represents direct evidence that the ARRE motif functions in an endogenous gene to mediate PKA regulation of alternative splicing.

Figure 1. The CaMK IV-responsive RNA element CaRRE1 also mediates splicing change in response to PKA.

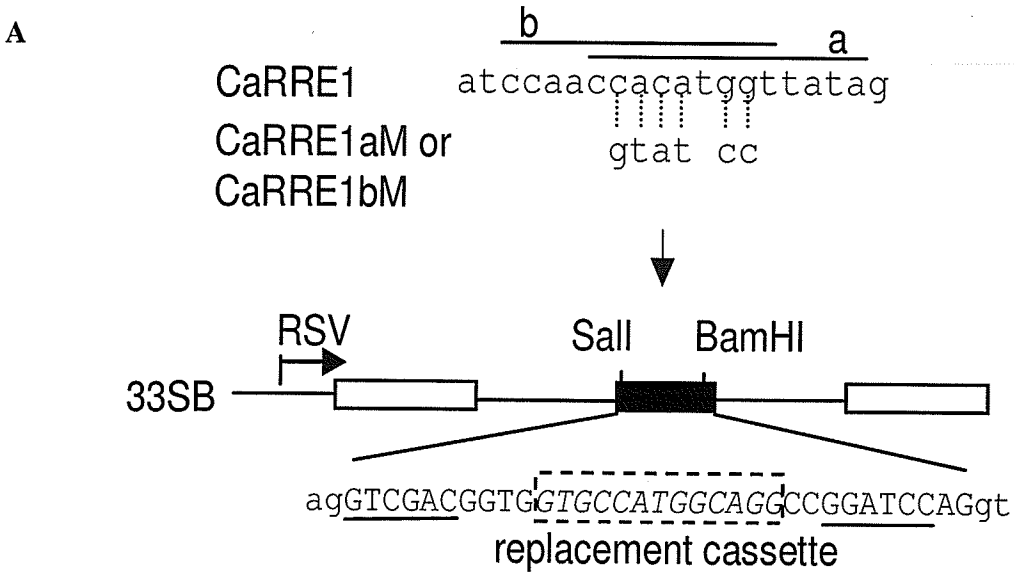
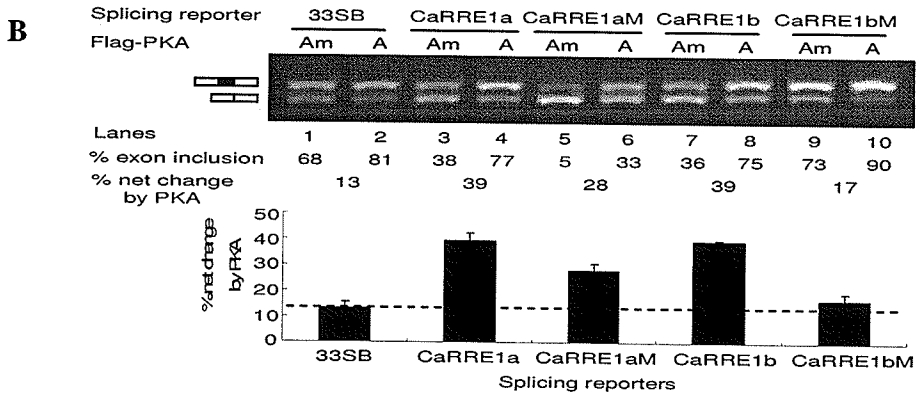


Fig. 1A. Cloning of 3' splice site (3' SS) sequences of the STREX exon into a minigene splicing reporter, 33SB. The CaRRE1 region shown is a 20-nucleotide (nt) sequence upstream of the STREX exon. From this region, two 13-nt test sequences, CaRRE1a (a) and CaRRE1b (b), were each transferred to 33SB. RSV: Rous Sarcoma Virus promoter, boxes: exons, lines: introns. Exon sequences are in upper case and intron in lower case. Restriction sites of Sal I and BamH I are underlined. Transferred sequences replace the 13-nt region replacement cassette (in dashed box). 13-nt mutant sequences, CaRRE1aM and CaRRE1bM are shown.

Figure 1. The CaMK IV-responsive RNA element CaRRE1 also mediates splicing change in response to PKA.



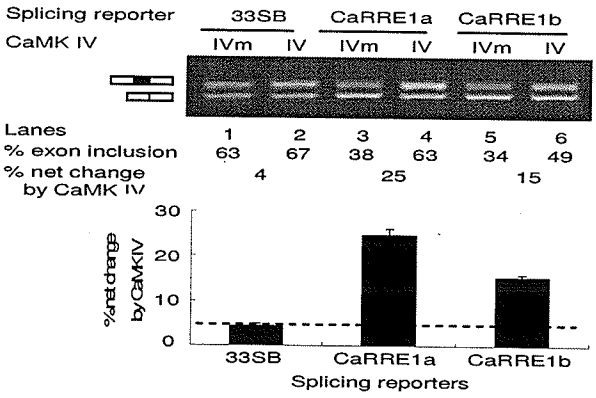


Fig. 1B and **1C**. Transferred CaRRE1 sequences mediate splicing changes in the minigene in response to PKA or CaMK IV. Upper panels: Splicing reporter constructs from the above were respectively cotransfected with Flag-PKA (A), Flag-PKAm (Am), CaMK IV-dCT (IV) or CaMK IV-dCTK715E (IVm) plasmid into HEK293T cells and mRNA from the transfected cells was analyzed by RT-PCR. RT-PCR products from the exon-included and -excluded mRNAs are diagramed as boxes with product lengths to the left of the gel. Percentage of the exon-included product in the total products is shown below each lane of the gel. Percentage of net change by PKA or CaMK IV (in exon inclusion) is shown for each splicing reporter. Lower panels: Percentage of net change by PKA or CaMK IV for each splicing reporter is bar-graphed (mean ± s.d., n=3). The dotted lines indicate the baseline of percentage values of the vector control. In all cases, significant difference of the CaRRE1a and CaRRE1b from the control is confirmed ($p < 0.01$).

Figure 2. A functional selection system identified PKA-responsive RNA elements controlling alternative splicing.

A

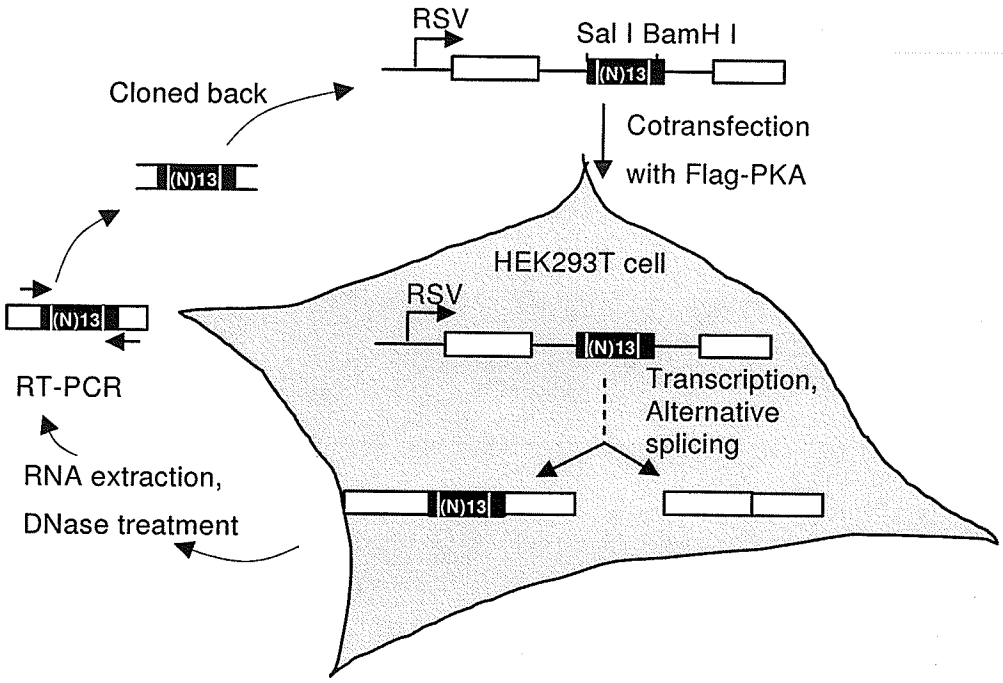


Fig. 2A. Diagram of strategy used to screen for the elements.

Figure 2. A functional selection system identified PKA-responsive RNA elements controlling alternative splicing.

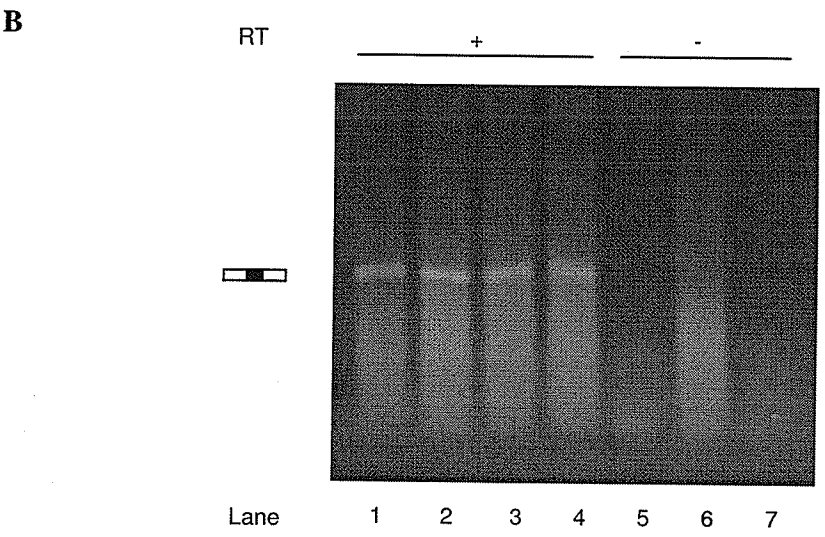


Fig. 2B. Elimination of DNA from RNA samples. Total RNA harvested from transfected cells was digested with RQ1 DNase (Promega) to remove DNA, followed by RT-PCR with (+) or without (-) reverse transcriptase (RT) to selectively amplify middle exon-included mRNA using primers targeted to splice junctions (SXenh-J1 and SXenh-J2).

Figure 2. A functional selection system identified PKA-responsive RNA elements controlling alternative splicing.

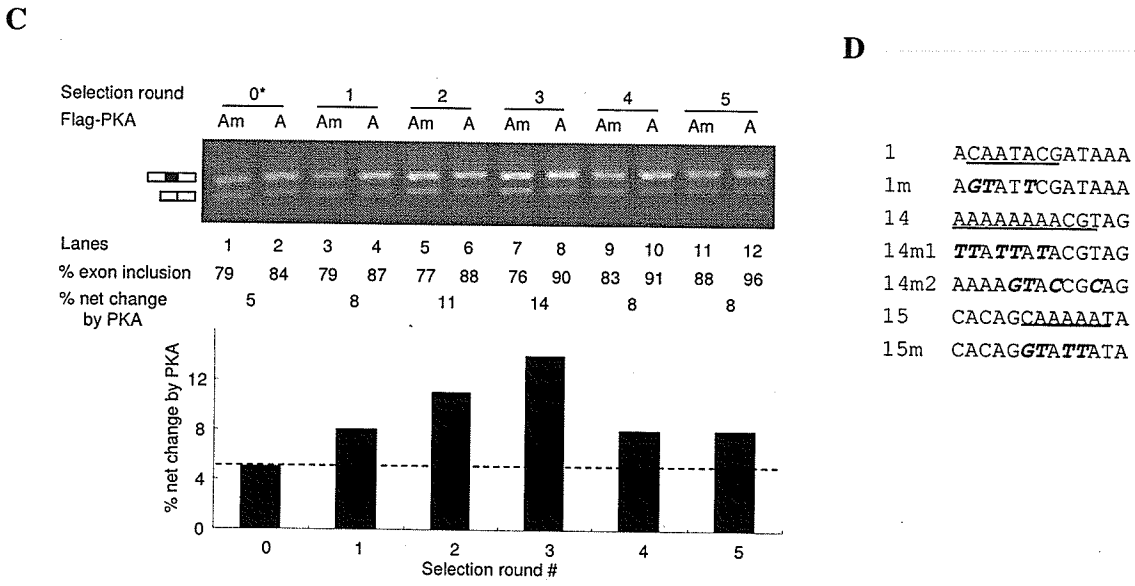


Fig. 2C. PKA-responsiveness of splicing reporter pools. Upper panel: RT-PCR assay of mRNA from HEK293T cells cotransfected with each of the splicing reporter pools from the selection rounds in A and either Flag-PKA (A) or Flag-PKAm (Am) plasmid. Asterisk: round-0 pool = the random library starting pool. Lower panel: Graph of percentages of net change by PKA for pools. The dotted line indicates the baseline percentage value of pool without selection. **Fig. 2D**. Sequences of RNA elements contained in representative strongly PKA-responsive splicing reporter clones #1, 14 and 15 from round-3 pool with mutant sequences shown below each element sequence. Underlined are sequences of a consensus motif to be described in Fig. 3C. Nucleotides of mutation are in bold.

Figure 2. A functional selection system identified PKA-responsive RNA elements controlling alternative splicing.

E

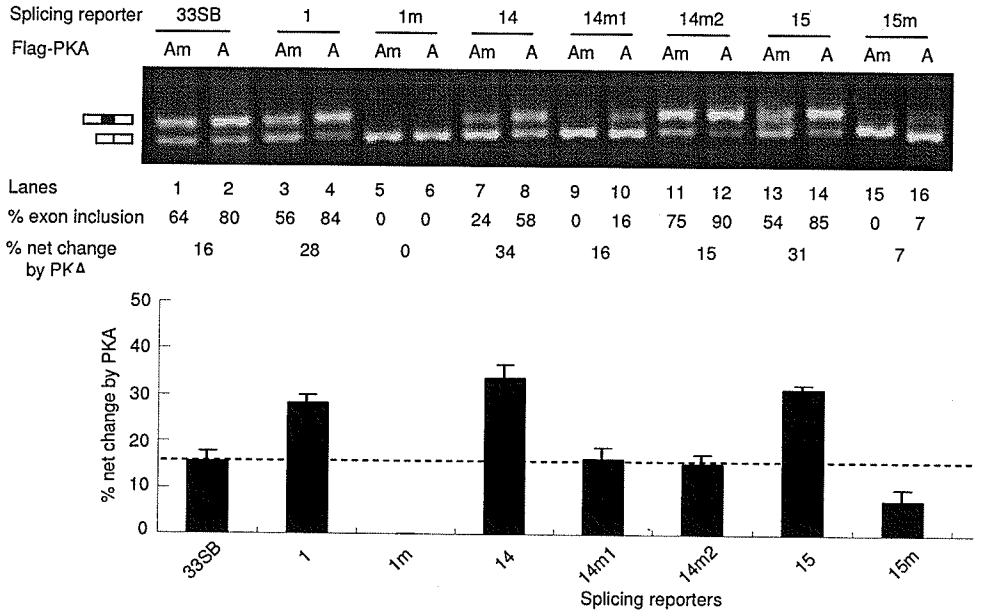


Fig. 2E. PKA-responsiveness of reporter clones and their mutants. Upper panel: reporter clones and mutants were cotransfected with either Flag-PKA (A) or Flag-PKAm (Am) plasmid into HEK293T cells and mRNA from the transfected cells was analyzed by RT-PCR. Lower panel: Graph of percentages of net change by PKA for reporter clones or mutants. The dotted line indicates the baseline percentage value of the vector (n=3).

Figure 3. Identification of transferrable PKA-responsive RNA elements controlling alternative splicing and sequence analysis of the elements to search for consensus.

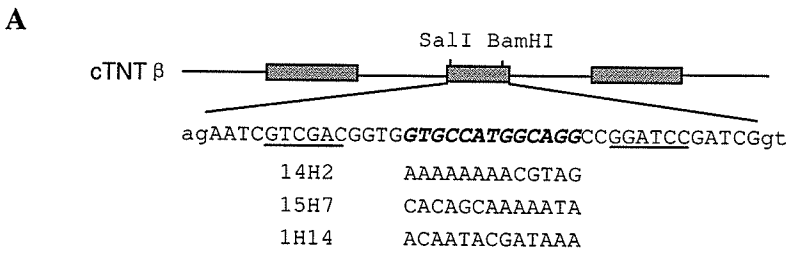


Fig. 3A. Transfer of strongly PKA-responsive RNA elements identified by the selection system to a heterologous splicing reporter gene, cTNTβ. Boxes: exons, lines: introns. Exon sequences are in upper case and intron in lower case. Restriction sites of Sal I and BamH I are underlined; in bold case and italic is the sequence same as the replacement cassette of the 33SB vector, which is replaced respectively by strongly PKA-responsive RNA elements identified in Fig. 2. Names of representatives of the resulting cTNTβ-derived splicing reporter constructs and the corresponding sequences of transferred RNA elements (from the 33SB-derived reporter clones # 1, 14 and 15 as shown in Fig. 2E) are listed.

Figure 3. Identification of transferrable PKA-responsive RNA elements controlling alternative splicing and sequence analysis of the elements to search for consensus.

B

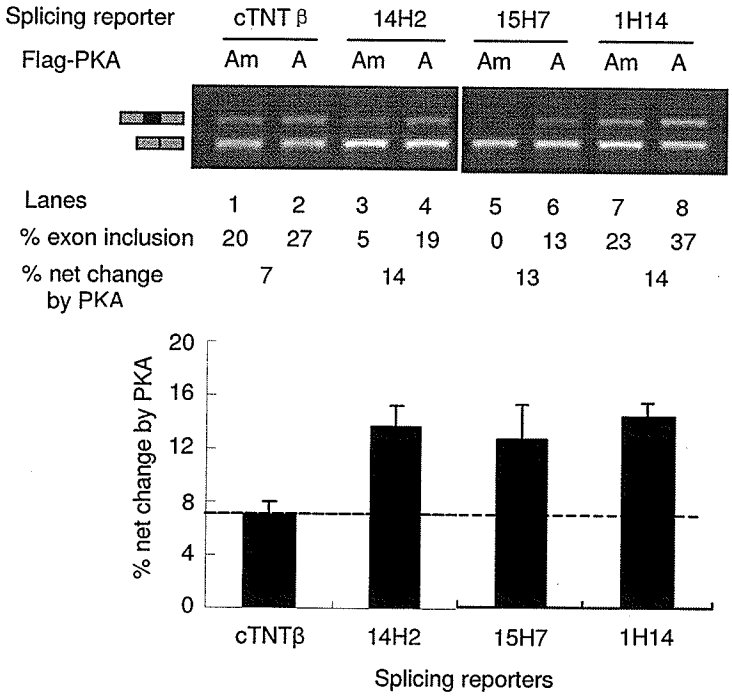


Fig. 3B. Upper panel: Agarose gel of the splicing reporter assay for PKA responsiveness as in Fig. 1B. Lower panel: Graph of percentages of net change by PKA for splicing reporters ($p < 0.05$ for 15H7, and $p < 0.01$ for 1H14 and 14H2.).

Figure 3. Identification of transferrable PKA-responsive RNA elements controlling alternative splicing and sequence analysis of the elements to search for consensus.

C

Clone		Sequence								% net change by PKA	
										a	b
3-1H1	CACCG	C	A	A	A	A	A	T	A	29	17
14H2	AAA	A	A	A	A	A	C	G	TAG	34	14
43H4	A	C	A	G	G	A	A	A	AAAAA	36	12
44H5	A	C	A	A	A	A	A	A	AAAAT	41	15
15H7	CACAG	C	A	A	A	A	A	T	A	31	13
2-16H8	AAA	C	A	G	A	C	T	T	AGA	51	18
3-8H9		C	A	C	A	A	T	G	ATATGA	51	19
2-6H10		A	A	A	A	A	T	A	CCAAAA	28	19
6H11	CAGC	C	A	A	A	A	C	A	G	44	16
16H13	AA	C	A	G	G	A	A	A	GATA	32	16
1H14	A	C	A	A	T	A	C	G	ATAAA	28	14
4T4	AACAAA	C	A	C	A	A	T	T		35	19
S9T5		C	A	A	A	A	C	G	GTGCAA	46	15
3-3T6	AAT	C	A	G	A	T	C	A	GAG	27	14

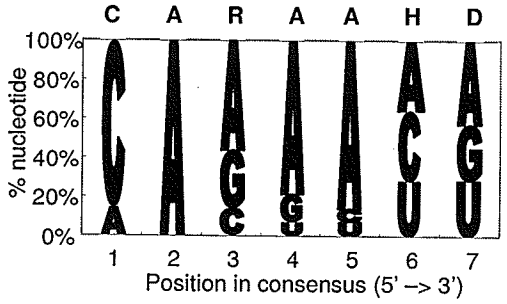


Fig. 3C. A consensus RNA motif shared by transferable PKA-responsive RNA elements. Upper: Sequences of fourteen cTNTβ-derived splicing reporters tested in B that confer a higher PKA response over the vector cTNTβ, with the nucleotides for the consensus motif aligned in the middle, clone names to the left and net changes of exon inclusion by PKA in 33SB (a) or cTNTβ minigene (b) to the right. Lower: Representation of the frequency of each nucleotide at each position of the heptamer consensus motif. The height of each letter is proportional to the nucleotide frequency, and the nucleotides are displayed from top to bottom in decreasing frequency. The consensus RNA motif sequence is summarized and shown above the graph (R=A/G, H=A/C/U, and D=A/G/U).

Figure 3. Identification of transferrable PKA-responsive RNA elements controlling alternative splicing and sequence analysis of the elements to search for consensus.

D

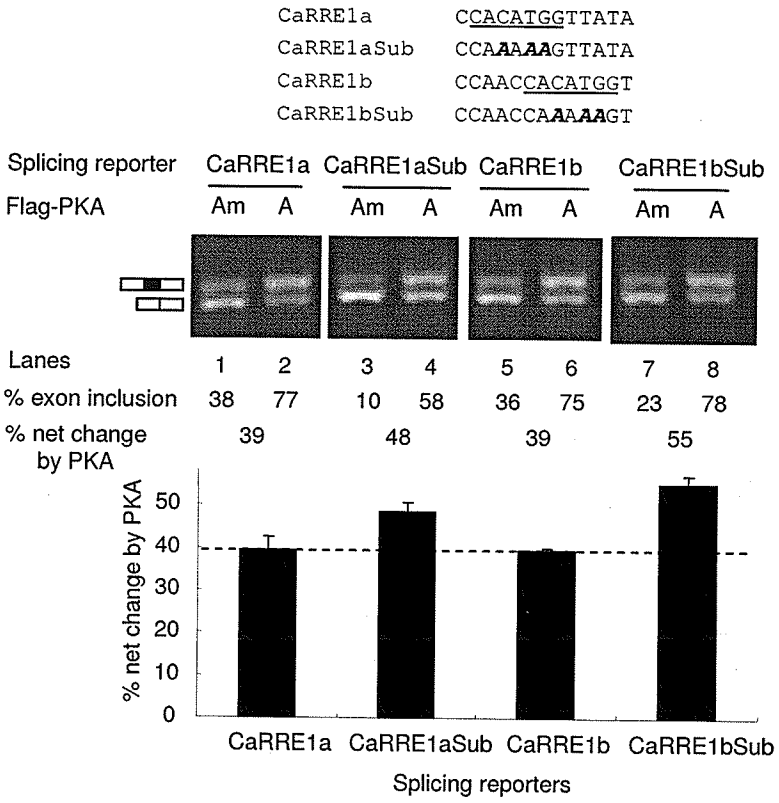


Fig. 3D. Effect of the consensus motif on PKA responsiveness when replacing a similar heptamer motif in the CaRRE1 element. Top: The underlined heptamer CACATGG in the 33SB-CaRRE1a and -CaRRE1b constructs are replaced with CAAAAAG (an exact match with the consensus motif) in the 33SB-CaRRE1aSub and -CaRRE1bSub constructs. Nucleotides of substitutions are in bold. Middle: Agarose gels of the splicing reporter assay for PKA responsiveness as in Fig. 1B. Bottom: Graph of percentages of net change by PKA for splicing reporters (n=3).

Figure 4. Similarity to CaMK IV-responsive RNA elements and kinase specificity of the PKA-responsive RNA elements.

A

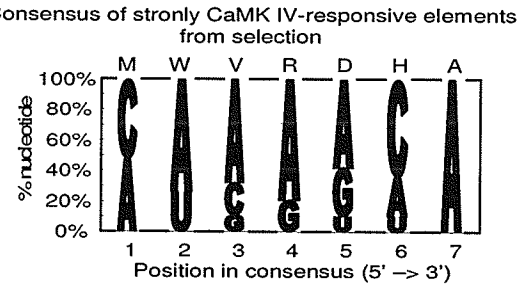


Fig. 4A. A consensus RNA motif of strongly CaMK IV-responsive elements identified from 21 clones by the same selection procedure as that performed to identify PKA-responsive elements except that coexpressed CaMK IV was applied as signal in place of coexpressed PKA.

Figure 4. Similarity to CaMK IV-responsive RNA elements and kinase specificity of the PKA-responsive RNA elements.

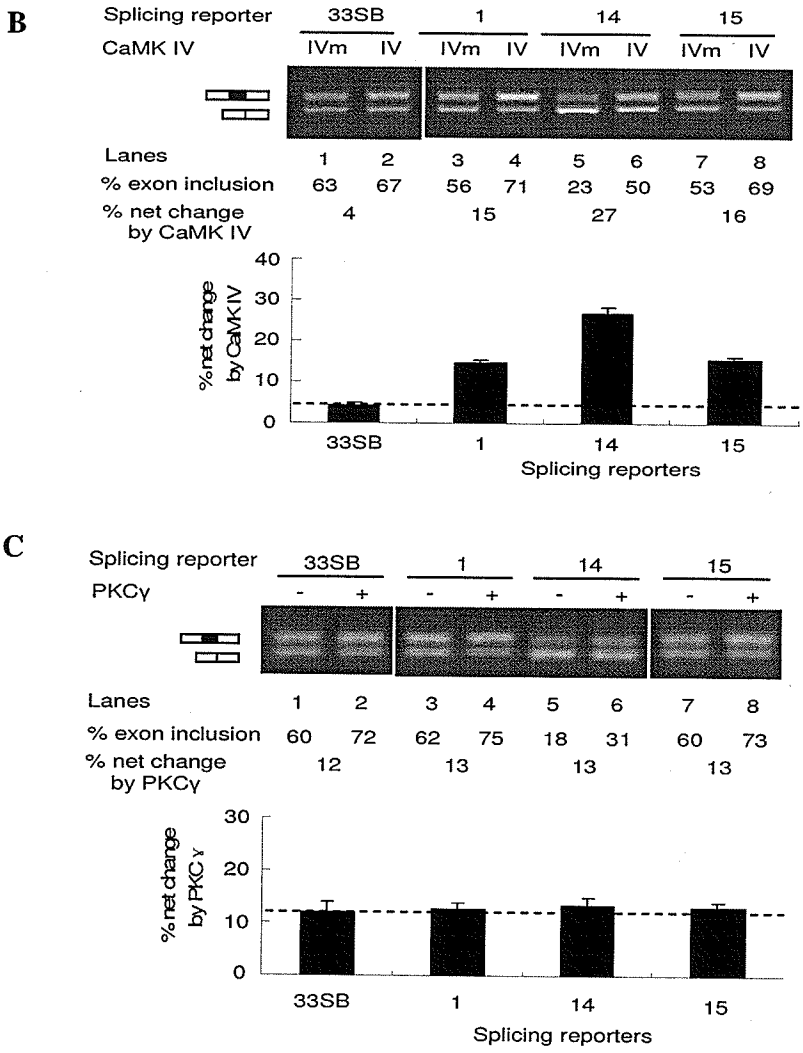


Fig. 4B and **4C**. Tests of CaMK IV and PKCγ responses of representative PKA-responsive reporter clones. Upper: splicing reporters as in Fig. 2E were contransfected respectively into HEK293T cells with CaMK IV-dCT (IV) or CaMK IV-dCTK715E (IVm) plasmid (in A) or PKCγ-EGFP vector (in B) followed by activation of PKCγ with TPA (+) or with DMSO (vehicle) as PKCγ signal control (-). RNA from the cells were analyzed by RT-PCR. Percentage of net change by CaMK IV or PKCγ is shown for each splicing reporter. Lower: Percentage of net change by CaMK IV or PKCγ for each splicing reporter is bar-graphed (n=3).

Figure 5. Minigene splicing assays of the activities of the consensus motif found in endogenous exons.

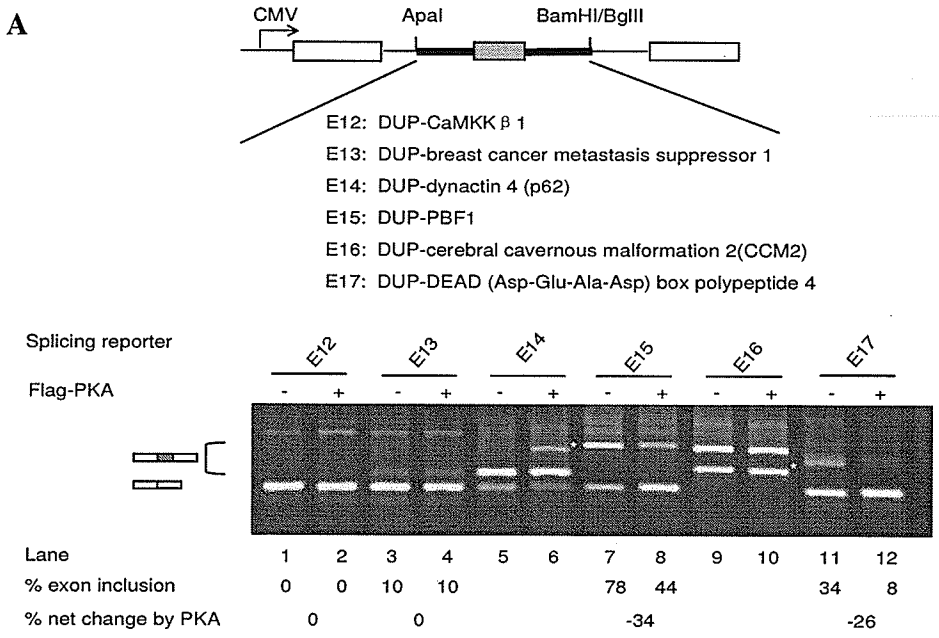


Fig. 5A. Construction of motif-containing exons extracted from database search into minigene splicing reporters and test of PKA effect on their splicing. Upper panel: Endogenous exons containing the consensus motif found by searching the ASAPII database were cloned into DUP175 minigene. Resulting constructs are diagramed. CMV, promoter; Boxes, exons (empty boxes, DUP175 exons 1 and 3; filled box, cloned endogenous exon); lines between exons, introns (light lines, part of DUP175 introns; heavy lines, part of endogenous introns flanking the cloned exon). Listed are names of representative constructs with names of endogenous genes in which the endogenous exons of interest are found. Lower panel: The above constructs were transfected into HEK293T cells with (+) or without (-) cotransfected Flag-PKA plasmid. Extracted RNA was assayed by RT-PCR. Splicing patterns of representative constructs are shown. Asterisks: Products of unknown identity. Percentages of exon inclusion and net change by PKA are shown forE12, E13, E15 and E17 with normal splicing products.

Figure 5. Minigene splicing assays of the activities of the consensus motif found in endogenous exons.

B

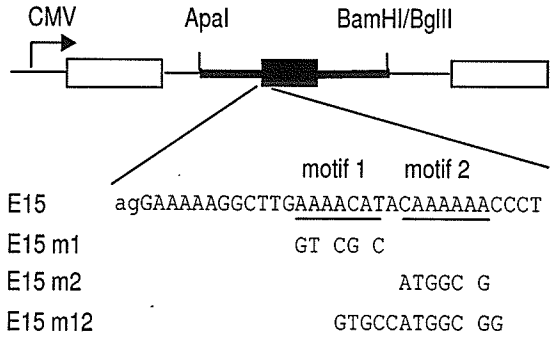


Fig. 5B. Diagram of E15 and its mutant constructs with their names and partial sequences shown below, intron sequence in lower case and exon sequence in upper case. Sequences of two candidate consensus motifs, motifs 1 and 2, are underlined. For sequences of mutant constructs only nts of mutation are shown, and aligned with their wild-type counterparts in E15 sequence.

Figure 5. Minigene splicing assays of the activities of the consensus motif found in endogenous exons.

C

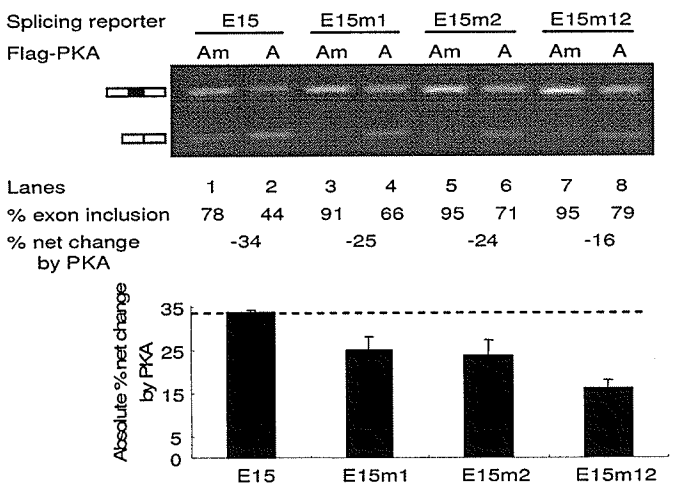


Fig. 5C. Effects of motif mutations on the PKA response of E15. Upper panel: Agarose gel of the splicing reporter assay for PKA responsiveness as in Fig. 1B. Lower panel: Graph of absolute percentages of net change by PKA for splicing reporters.

Figure 6. The activity of the consensus motif on mediating PKA control of alternative splicing of CaMKK β1 exon 16 in PC12 cells.

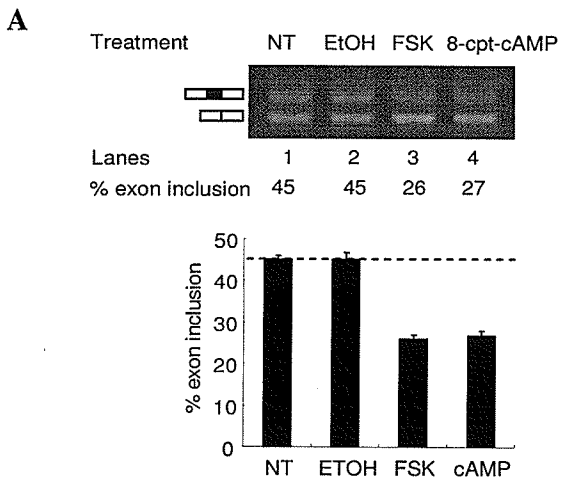


Fig. 6A. Changes in endogenous splicing patterns of CaMKKβ1 exon 16 upon treatments with PKA stimulators in PC12 cells. NT, non-treated; EtOH, ethanol (vehicle of PKA stimulators); FSK, forskolin, an adenylyl cyclase activator; 8-cpt-cAMP, a membrane-permeable cAMP analogue. RT-PCR products from the CaMKKβ1 exon 16-included or -excluded mRNAs are diagramed as boxes with product lengths to the left of the gel. Percentage of mRNA containing the CaMKKβ1 exon 16 in the total products is shown for each lane of the gel. Lower panel: Bar graph indicates mean ± s.d. of exon 16 inclusion level, n=3. The dotted line indicates the % exon inclusion level in the control samples. (Observation initially made by Wenguang Cao).

Figure 6. The activity of the consensus motif on mediating PKA control of alternative splicing of CaMKK β1 exon 16 in PC12 cells.

B

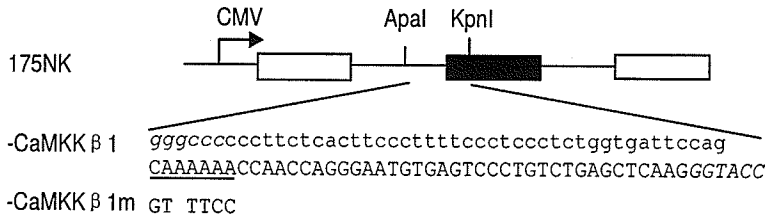


Fig. 6B. Minigene constructs to assess the effect of the KARRE in CaMKKβ1 exon 16 on PKA response. CaMKKβ1 exon 16 with part of its upstream intron was cloned into DUP175NK between the Apa I and Kpn I sites (175NK- CaMKKβ1). CMV, promoter; boxes, exons; lines between exons, introns. The cloned sequence between the restriction sites is shown, intron sequence in lower case and exon in upper case. The motif sequence, CAAAAAA, is underlined. In the mutant construct, 175NK-CaMKKβ1m, nts of mutation are displayed, and aligned with their wild-type counterparts.

Figure 6. The activity of the consensus motif on mediating PKA control of alternative splicing of CaMKK β1 exon 16 in PC12 cells.

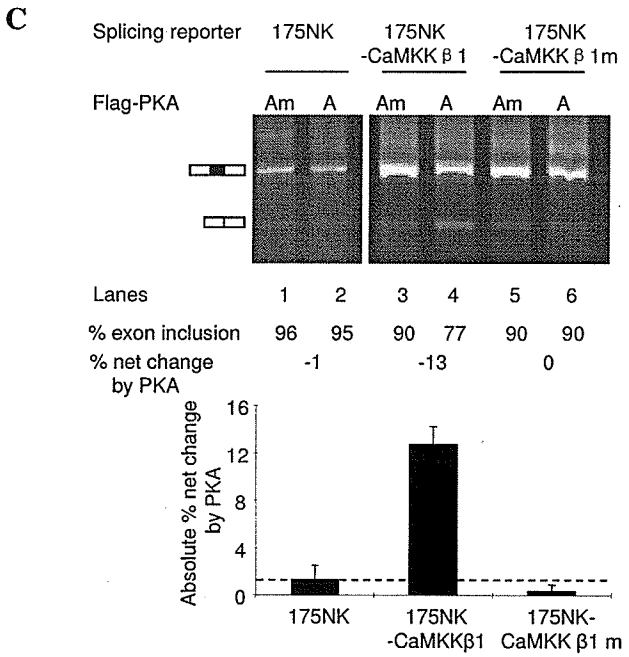


Fig. 6C. Splicing patterns of the splicing reporters with or without PKA. Upper panel: Agarose gel of the splicing reporter assay for PKA responsiveness as in Fig. 1B. Lower panel: Graph of absolute percentages of net change by PKA for splicing reporters (n=3).

Figure 7. Convergence and cross-talk of the PKA and CaMK signal transduction pathways through alternative splicing control.

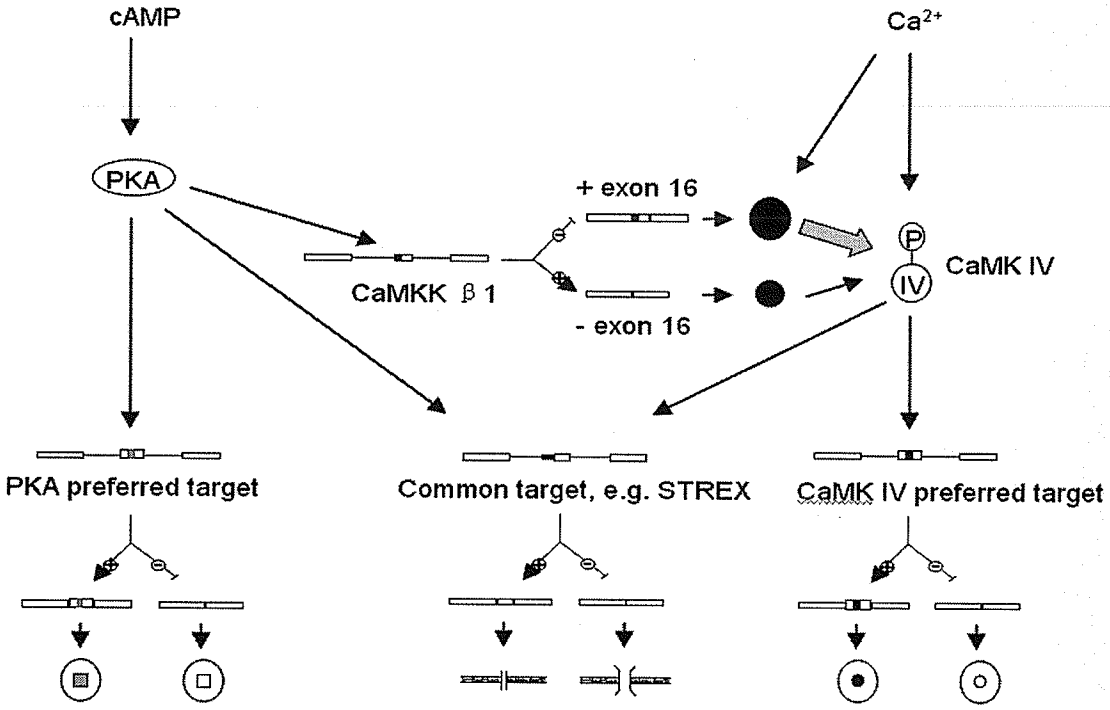


Fig. 7. Boxes, exons; lines between exons, introns; highlighted dark/grey regions in exons or heavy region in intron, signal responsive RNA elements; ellipses, proteins; P, phosphate group. Symbols at bottom: ellipses with filled or empty squares or circles inside, protein isoforms encoded by the exon-included or -excluded mRNAs; in the case the STREX exon, the mRNAs encode Slo BK potassium channels of distinct gating properties in the cell membrane. +/-, enhancing/silencing effect. PKA activated by cAMP or CaMK IV activated by Ca^{2+} control their downstream cellular events including the splicing regulation of target pre-mRNAs through signal-responsive RNA elements. PKA and CaMK IV signals converge upon a common target, the STREX exon, via the CaRRE1 element, while they may also respectively control their own specific target splicing events using RNA elements that exclusively respond to either of the signal. CaMKKβ1, as an upstream kinase in the CaMKK/CaMK cascade, phosphorylates and contributes to full activation of CaMK IV. PKA controls the differentiated expression of CaMKKβ1 protein isoforms through the regulation of CaMKKβ1 exon 16 inclusion mediated by a PKA responsive RNA element.

Figure S1. RNA elements involved in the basal splicing reaction.

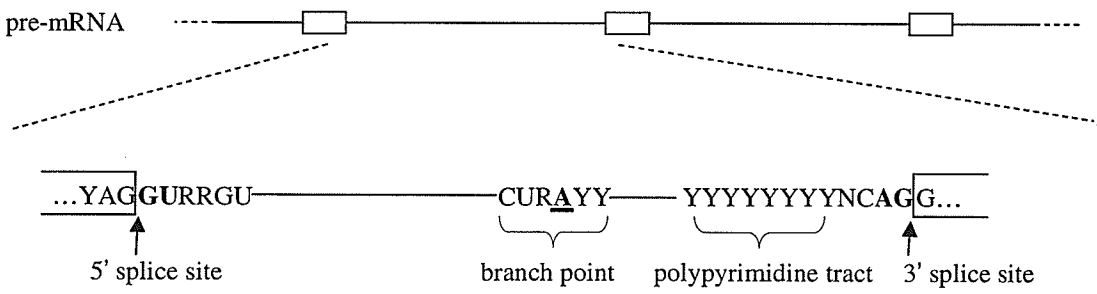


Fig. S1. Boxes, exons; lines between exons, introns. The conserved sequences are shown including the elements from the 5'splice site, branch point, polypyrimidine tract and 3' splice site. Degenerate nucleotide sequences: Y, C/U; R, A/G; N, A/C/G/U. The highly conserved dinucleotides, GU at 5' splice site and AG at 3' splice site, are shown in bold. The special branch point nucleotide A is underlined. Diagram is not drawn in real proportion.

VI. DISCUSSION

The PKA signal transduction pathway regulates a variety of cellular processes and plays an important role in the regulation of gene expression. The majority of human genes undergo alternative splicing, however, only a few have implicated PKA in the control of alternative splicing [8,9,38,39]. Our present work extends the recent studies by adding to the spectrum of genes whose splicing is regulated by PKA as well as identifying the molecular components of the PKA regulated alternative splicing.

Context dependence of the activities of the splicing regulatory RNA elements, the CaRRE1 and ARRE

The CaRRE1, previously known to confer CaMK IV regulation of the STREX exon, is shown in this study to mediate PKA control of splicing as well. (Fig. 1). Interestingly, however, in the transiently transfected 33SB minigene, in response to PKA or CaMK IV signal, the CaRRE1 enhances exon inclusion, a signal response pattern distinct from that seen in the endogenous gene, where CaMK IV represses exon inclusion (Fig. 1 and reference [31]). It suggests that transiently transfected minigenes do not always faithfully reproduce the responses of the endogenous genes. In addition, comparison of the results of the ARRE in different minigenes shows different activities/effects of the ARRE on exon inclusion (Figs. 2, 5 and 6). These observations are consistent with the recent findings that splicing regulatory RNA elements can have variable or even opposite effects depending on their locations, pre-mRNA and cellular contexts (Hui et al., 2005; Xie et al.,

2005; Goren et al.,2006; Lee et al., 2007). However, the detailed mechanism(s) underlying these phenomena remain to be understood.

Screening of PKA-responsive RNA elements controlling alternative splicing with an *in vivo* functional selection system.

Although the CaRRE1 is established in this work as a PKA-responsive RNA element controlling alternative splicing (Fig.1), further information is needed on the essential nucleotide sequence most important to the PKA response. Most splicing regulatory RNA elements exhibit degeneracy in their sequences that are recognized by trans-acting splicing factors. It is likely that degenerate sequence variants similar to the CaRRE1 sequence share a consensus functional RNA motif essential for PKA response. Moreover, other types of PKA responsive elements could exist that are distinct from the CaRRE1. To address these issues, a global view of potential PKA-responsive elements will be helpful. A systematic identification of the PKA-responsive RNA elements was necessary.

For global identification of splicing regulatory RNA elements, several *in vivo* functional selection approaches have been previously developed. Cooper and coworkers used a randomized splicing reporter library to transiently tranfect cultured cells and cloned exonic splicing enhancers by recovering exon-included products from selective RT-PCR amplification [41]. However, systematic identification of splicing silencers is technically much more challenging in that silencer-containing sequences are excluded from the mature mRNA and thus are hard to recover from the transient transfection-based strategy. A further elaborated approach in which stably transfected cell lines were used to

extract genomic sequences that contain splicing silencers [132]. However, this approach is time consuming as it involves a large number of stable cell lines. The present study applied a modified version of the Cooper approach [41] by taking advantage of the context dependence of the CaRRE1 in the 33SB minegene, where exon inclusion can be enhanced by a signal responsive element (Fig. 2A). Namely, exon-included products were selectively amplified by RT-PCR using primers that specifically target exon junctions and were cloned from cells cotrasfected with a 33SB-based random library of splicing reporters and a PKA expressing construct with the expectation that elements promoting exon inclusion in response to the coexpressed PKA could be enriched. The enrichment of splicing enhancer-containing products was designed to be strengthened by multiple cycles of selection.

Both of the two previous selection approaches [41,132] performed splicing in "quiescent" cells that were not stimulated by specific signals of interest. In the present system outlined in this thesis, the major modification made to the Cooper approach is the overexpression of active PKA, which is expected to facilitate the enrichment of PKA-responsive elements. However, the constitutive enhancer elements, which promote exon inclusion without the need for PKA signal, were not excluded. Consequently, the enrichment of PKA-responsive elements versus constitutive ones was expected to depend on the competition between these two types of enhancer elements for relative abundance in the whole selected reporter population. As shown in Fig. 2C, the selection rounds 1 – 3 led to continuously increasing PKA responses of selected reporter pools with a peak at round 3. However, the PKA responses then dropped in the following rounds 4 and 5. The

response patterns appear to reflect the relative abundance of responsive versus constitutive enhancers in the whole reporter populations, which is suggested by the analysis of the percentages of responsive clones as described in the text of the Results section. It is likely that the accumulation kinetics of responsive (faster?) and constitutive (slower) enhancer elements differed during the selection rounds: in rounds 1 – 3 the accumulation rate of selected PKA-responsive elements was higher than constitutive ones, whereas it is the opposite case in the following rounds. Thus, in rounds 4 and 5 when the accumulation of responsive elements reached close to saturation the relatively stronger accumulation of constitutive ones now probably accounted for the decreased ratio of the abundance of responsive elements to constitutive ones and subsequently decreased reporter pool responses to PKA. The mechanism(s) is not very clear that underlies the differential accumulation rates of responsive versus constitutive elements during the selection rounds. The overexpressed PKA can affect not only the splicing of the transcripts containing different RNA elements but potentially also the transcription and stability of those transcripts.

Nevertheless, for the technical purpose, the overall enrichment of PKA-responsive elements in the selected element population versus constitutive elements was well supported by the increased PKA response of the reporter pool from each selection round (Fig. 2C) and PKA-responsive elements were finally validated by assaying individual reporter clones from selection rounds with strongest PKA responses (Fig. 2E). The present selection system thus provides a starting example of a system by which

signal-responsive elements can be globally identified, although the more in-depth knowledge about how the system works remains to be elucidated.

The selection system has identified PKA-responsive RNA elements (the ARRE) sharing one common motif, CARAAHD. The heptamer motif in the CaRRE1, essential for the PKA response, shows sequence similarity (Fig. 1A and B). Possibly the CaRRE1 represents a sequence variant of the ARRE type element as implied by both the sequence similarity and two additional supporting evidences: (1) replacing the CaRRE1 motif with the ARRE motif did not abolish the PKA response (Fig. 3D); (2) like the CaRRE1, all the tested representative ARRE clones showed CaMK IV responsiveness (Fig. 1C and 4B). However, these data are still not sufficient to conclude that the CaRRE1 and ARRE belong to the same type of PKA-responsive elements. Additional information such as the identity of the trans-acting splicing factors recruited by CaRRE1 and ARRE would help clarify their similarities and differences. In addition, the existence of more than one type of CaRRE elements that respond to the same signal, CaMK IV [31,32], indicates that there can be various types of elements controlling splicing in response to a single signal.

As mentioned above, the activities of splicing regulatory RNA elements are affected by where they reside. Various previous screening procedures have identified splicing regulatory elements using different minigene splicing reporters in various cell cultures or cell extracts they used [12], where the elements function well and thus are probably favored. Although some identified elements represent overlap from different procedures, it is more often the case that distinct elements have been preferentially selected out in using

different reporter constructs [12]. One possibility is that this is at least in part due to the potential bias of a pre-mRNA in a specific minigene or the bias of a specific cell type that may favor some type(s) of elements but weigh against others. Therefore, the selection system in this study may not have covered all possible types of PKA-responsive RNA elements. Additional similar screening tests using different minigene splicing reporters in different types of cells may lead to an expanded view of the classes of these elements.

Functional ARREs from endogenous exons

To confirm that the ARRE motif sequences found in endogenous genes regulate alternative splicing as well, the splicing patterns of motif-containing exons in response to PKA were characterized by splicing assays using minigenes. The functional roles of the ARRE motif sequences were assessed by mutational analysis (Figs. 5 and 6). In 2 of the 17 tested exons, the ARRE motif was confirmed to play an essential role in mediating PKA-dependent splicing pattern changes (Figs. 5B, 5C, 6B and 6C). In a recent report [32], the CaRRE 1 type elements (including a CaRRE1-like element) were verified to mediate CaMK IV regulation in 3 out of 11 assessed exons, and the validation of the function of the CaRRE 2 in endogenous exons was not available for any of these exons.

One reason for the seemingly low predictiveness of a PKA response by the ARRE motif sequence may be that alternative splicing in response to PKA (or other cellular signals such as CaMK IV) is possibly dictated by multiple splicing regulators through combinatorial control. The alternative splicing pattern has been shown to be determined by complex interplays between multiple cis-acting RNA elements and trans-acting

splicing factors in a tissue/cell or development stage-specific manner depending on the composition of the cellular splicing regulators. The ability of the ARRE described here and the CaRREs in other studies [31,32] to transfer a signal response in certain heterologous minigenes indicates that the transferred element encompasses at least the minimal sequence for an intact functional unit that serves as the downstream target of the signal pathway on the pre-mRNA transcript. Therefore, the transferrable element possesses an intrinsic activity of signal response on its own. However, it is not necessarily the case that this activity is always expressed as a detectable "phenotype", a splicing change, in any pre-mRNA. As illustrated by experimental observations from the CaMK IV responses of the CaRREs, the overall outcome of signal modulation of splicing depends on the contributions of multiple splicing regulators, and to fulfill its activity in the form of a visible splicing change the signal-responsive element may require other elements to cooperate with it [31,32]. The CaRRE1 effect on the STREX exon in another minigene is affected by other elements in the STREX exon, and particularly, requires an auxiliary exonic silencer (pyrimidine-rich). Moreover, although either of the CaRRE1 or CaRRE2 element from the exon 21 (E21) of the NMDAR1 gene is sufficient for CaMK IV repression in the DUP175 exon, both are required for efficient exon repression by CaMK IV in a E21-derived minigene. In addition to the cooperation between the CaRREs, other exonic silencer elements (UAGG) were also found to facilitate the repression of E21 by CaMK IV. In opposition to the cooperation, antagonistic effect from other elements can also exist to counteract the activity of the signal-responsive element, such as the purine-rich enhancer D34 in the STREX exon [31]. In the scenario of natural genes with multiple exons and extremely long introns, much more complex interactions between

diverse elements than in the simplified minigenes can occur and the activity of a signal-responsive element may be even more liable to be overwhelmed or blocked by other elements depending on the net effect of these interactions. As a result, the activity intrinsically possessed by the element can be prevented from being detectable.

Notably, among the 17 tested exons, there are 13 very short exons. The original purpose was to minimize the amount of potential splicing regulatory elements other than the candidate ARRE to obtain simplified minigene systems for clearer characterization of the ARRE. However, the potential disadvantage of this design is that such small exon sizes are a strong negative signal for the splicing machinery to skip the exon unless enough enhancer elements are present in the pre-mRNA transcript to facilitate the exon inclusion [126]. Unfortunately, it did turn out that most of these small exons were constitutively skipped. Since the ARRE identified in this study mostly function as silencers in the contexts of both endogenous genes and the DUP minigenes, the complete skipping of these test exons failed to allow detection of the potential PKA effect the ARRE might have conferred. This is well illustrated in the case of the ARRE from the CaMKKβ1 exon 16, where the ARRE activity became expressed as a detectable splicing change when put in an enlarged exon in the DUP minigene (Fig. 6B and C compared with Fig. 5A). Therefore, taken together, the combinatorial control mechanism proposed here might explain the low prediction of PKA response by the ARRE sequences.

Another possibility that may account for the observation that the presence of an ARRE motif is not highly predictive of a subsequent PKA regulated splicing event is that

the ARRE was initially identified as 13-nt elements that share a consensus heptamer motif, which is essential for the activity of the elements. To facilitate a practical search of the genome for identification of PKA target exons, only the heptamer consensus motif was used instead of the whole 13-nt sequences. In the 13-nt elements, the sequences that flank the heptamer motif look diverse and lack consensus, indicating that the flanking sequences are more degenerate or flexible in maintaining the activity of these elements. However, it is likely that for some ARRE elements, certain combinations of the sequences of the heptamer motif and the flanking fragments may be needed for the elements to function efficiently. In this regard, in some of the ARRE-containing exons I tested, the ARRE motif is possibly not active enough to mediate a PKA response due to the lack of a combination with suitable flanking sequences that is important for those sequence versions of elements. Since the heptamer motif is the consensus of multiple ARRE sequences essential for this type of elements to function, it is nevertheless a representative target of PKA signaling and a key component for dissecting the molecular mechanism of PKA regulated splicing.

The mechanism of the ARRE function

Little is known about the mechanism by which PKA regulates alternative splicing. The ARRE provides a starting point to dissect the molecular components in PKA regulation of splicing. An immediate next step will be to identify the trans-acting factors that link the ARRE with the PKA signal transduction pathway. As mentioned previously, PKA phosphorylates SR proteins. SR proteins are known to bind purine-rich elements that do not resemble the ARRE in sequence, so it does not seem that PKA acts on the

ARRE through SR proteins. The high A content and substantial level of C-residues in the ARRE appear similar to the known A/C-rich splicing enhancers [41,133] and CA-repeat element [27], which modulate splicing by recruiting splicing factors YB-1 and hnRNP L, respectively. In a preliminary test, overexpressed YB-1 or hnRNP L did not significantly change splicing in the representative PKA-responsive reporter clones (data not shown), which, taken together with the extent of the sequence similarity of the ARRE to the A/C-rich and CA-repeat elements, suggests that YB-1 and hnRNP L may not be the trans-acting factors involved in the function of the ARRE. Experiments where overexpression or depletion of these splicing factors are carried out under the condition of PKA activation would provide stronger evidence. Probably the ARRE is a class of novel RNA elements distinct from these known elements, and functions by recruiting unknown splicing fator(s) induced or altered by PKA.

Convergence and crosstalk between PKA and CaMK pathways through kinase-responsive RNA elements controlling alternative splicing

It is particularly interesting that the ARRE couples the PKA and CaMK signal transduction pathways. The two pathways converge on the ARRE (also the CaRRE1) to regulate the splicing of common target transcripts (Fig. 2E and 4B). The correlation of the PKA and CaMK IV in converging on a splicing target extends the previous findings that they also target a common transcription regulatory element, the cAMP(/Calcium)-responsive DNA element (CRE). The CRE functions through the phosphorylation of the CRE binding protein (CREB) at its Ser133 by PKA or CaM

kinases [3,134]. Phosphorylated CREB recruits the CREB-binding protein (CBP) to the promoter and regulates the transcription activity of target genes [135].

In addition to serving as the converging target of the PKA and CaMK IV pathways in their regulation of alternative splicing, the ARRE also represents the first RNA element identified to mediate direct cross-talk between different signal pathways through splicing control. CaMKK β1, upstream of CaMKs in the CaMKK/CaMK cascades, phosphorylates and contributes to full activation of CaMK IV (and also CaMK I) [11]. Alternative inclusion of exon 16 generates two protein isoforms of CaMKK β1, but it is not known how the shift of the expression patterns of the CaMKK β1 isoforms is regulated. The present study demonstrates that the ARRE motif mediates the cross-talk between the PKA and CaMKK/CaMK pathways through PKA control of the alternative splicing of CaMKK β1 exon 16 to modify the signaling protein components of CaMKK/CaMK pathway (Fig. 7). Furthermore, the ARRE directly involves both splicing regulation and signal network in that one signal pathway (PKA) regulates a splicing event, which, in turn, modulates another signal pathway (CaMKK/CaMK). The latter in turn regulates other downstream events including alternative splicing, forming an interactive regulatory network consisting of regulated alternative splicing events and modified signal pathways that are induced by each other.

VII. CONCLUSIONS

1. The PKA signal transduction pathway regulates the alternative splicing of the CaMKK β1 gene, in addition to the GAD and ania6 genes reported by previous studies.

2. There exist PKA-responsive RNA elements that mediate PKA control of alternative splicing.

3. A signal-based *in vivo* functional selection procedure is successful in systematic identification of signal-responsive splicing elements.

4. PKA-responsive RNA elements controlling alternative splicing are identified, including the CaRRE1 in the Slo gene and the ARRE that originates from the selection procedure.

5. The ARRE motif found in endogenous pre-mRNA sequence mediates PKA regulation of alternative splicing.

6. The CaRRE1 and ARRE contain similar RNA motifs essential for PKA response, and can both serve as the common targets of the PKA and CaMK IV pathways in splicing regulation.

7. An endogenous ARRE motif found in exon 16 of the CaMKK β1 gene mediates direct signal crosstalk between the PKA and CaMKK/CaMK pathways through PKA control of the alternative splicing of CaMKK β1.

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