In Vivo Selection of Kinase-responsive RNA Elements Controlling Alternative Splicing*

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Alternative pre-mRNA splicing is often controlled by cell signals, for example, those activating the cAMP-dependent protein kinase (PKA) or the Ca2+/calmodulin-dependent protein kinase IV (CaMKIV). We have shown that CaMKIV regulates alternative splicing through short CA repeats and hnRNP L. Here we use a splicing reporter that shows PKA/CaMKIV promotion of exon inclusion to select from exons containing random 13-nt sequences for RNA elements responsive to the kinases in cultured cells. This selection not only identified both PKA- and CaMKIV-responsive elements that are similar to the CaMKIV-responsive RNA element 1 (CaRRE1) or CA repeats, but also A-rich elements not previously known to respond to these kinases. Consistently, hnRNP L is identified as a factor binding the CA-rich elements. Analyses of the motifs in the highly responsive elements indicate that they are indeed critical for the kinase effect and are enriched in alternative exons. Interestingly, a CAAAAAAA motif is sufficient for the PKA/CaMKIV-regulated splicing of the exon 16 of the CaMK kinase β1 (CaMKK2) transcripts, implying a role of this motif in signaling cross-talk or feedback regulation between these kinases through alternative splicing. Therefore, these experiments identified a group of RNA elements responsive to PKA and CaMKIV from in vivo selection. This also provides an approach for selecting RNA elements similarly responsive to other cell signals controlling alternative splicing.

Alternative pre-mRNA splicing is a common means of gene expression regulation in metazoans (1, 2). The expression of splice variants is controlled not only in a tissue-, sex-, or developmental stage-specific way but also in response to stimulation of particular protein kinases by extracellular factors (3–5).

Many extracellular factors, including neurotransmitters and hormones, activate calcium and/or cAMP signals and downstream protein kinases. Besides membrane depolarization and the calcium/calmodulin-dependent protein kinase IV (CaMKIV)4 (5–11), the cAMP-dependent protein kinase A (PKA) has also been implicated in the regulation of alternative splicing. For example, dopamine induces alternative splicing in striatal neurons through the dopamine receptor that activates the PKA pathway (12–14). PKA phosphorylates and colocalizes with arginine-serine-rich (SR) splicing factors and regulates alternative splicing of an E1A minigene (15).

Alternative splicing in mammalian systems is controlled by multiple intronic and exonic regulatory elements called intrinsic or exonic splicing enhancers or silencers (1, 16, 17). The effect of a particular element can be either enhancer or silencer depending on its surrounding context in the pre-mRNA (5, 17–19). RNA elements that mediate cell signal-regulated splicing have also been isolated. These elements include the A-rich element in the CD44 exon 5, the CaMKIV-responsive RNA elements (CaRRE1 and CaRRE2) in the STREX (stress axis-regulated exon) of Slo and exons 5 and 21 of the NMDAR1 (N-methyl-D-aspartic acid receptor, type I) gene, the activation-responsive sequence in CD45, the G-tract in Bcl-x and the UAGG motif in NMDAR1 exon 21 (6–8).

Alternative splicing have also been defined through intensive mutagenesis analyses of individual splicing reporter minigenes.

SELEX (systematic evolution of ligands by exponential enrichment) has been used for A/C-rich splicing enhancers and G-rich silencers (23–25), but not for cell signal-responsive RNA elements. It would be particularly difficult to enrich kinase-responsive elements from spliced mRNA in cells with SELEX when a kinase, like CaMKIV, shows exon-exclusion activity through the CaRREs in the current splicing reporter systems (6–8).

In this report, we selected PKA- and CaMKIV-responsive RNA elements using SELEX based on the context-dependent promotion of exon inclusion by the kinases and characterized the selected elements.

**EXPERIMENTAL PROCEDURES**

Plasmid Construction and Mutagenesis—The Flag-PKA (A), Flag-CaMKIV-dCT (CaMKIV or IV) or -dCTK75E (CaMKIVm or IVm, kinase-dead) and PKCα are as described (6, 26, 27). Particularly for the CaMKIV and PKA plasmids, their activity or inactivity was confirmed by monitoring the phosphorylation levels of their target proteins CREB Ser133 and PTB Ser16, respectively (6, 27). In the Flag-PKA mutant (Am), the Lys73 and Lys169, phosphate-binding residues (28), were mutated to

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*This work was supported by the Canadian Institutes of Health Research (CIHR, MOP 68919) (to J. X.).
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4 The abbreviations used are: CaMKIV, calcium/calmodulin-dependent protein kinase IV; PKA, cAMP-dependent protein kinase; nt, nucleotide; DMEM, Dulbecco's modified Eagle's medium; RT, reverse transcription; TPA, 12-O-tetradecanoylphorbol-13-acetate; STREX, stress axis-regulated exon; SELEX, systematic evolution of ligands by exponential enrichment; PKC, protein kinase C; PTB, polypyrimidine tract-binding protein; CaRRE1, CaMKIV-responsive RNA element 1; CaMKK2, CaMK kinase β1.
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Glu\textsuperscript{73} and Glu\textsuperscript{109}, respectively (numbered according to NCBI protein accession no. AAA39936, corresponding to Lys\textsuperscript{72} and Lys\textsuperscript{168} in the previous report [28]), using primers Flag-PKA1 (5'-CGGGATCCATGCACTGAAAGCAGATGAC-AAGGCCGCCGCCGAC 3'), Flag-PKA (A), or pPKC (5'-CATCTACGGGACCCCAGGAATCCTTCTCATTACGTC-3'), PAKK16Es (5'-CATCTACGGGACCCCAGGAATCCTTCTCATTACGTC-3'), and PAKK2 (5'-GCTCTAGACTAA AACTCAGTAAACTCCTTG-3')

Splicer protein minigene 33SB is a chimera of pDUP4-1 and pl53In (29, 30). The Apal/BglII fragment of pDUP4-1 was cloned into the Apal/BamHI sites of pl53In (a gift from Dr. Harold König), and Sall and BamHI sites restriction sites were introduced into the 33-nucleotide (nt) middle exon (23), resulting in an insert fragment: actgactctttcgtctttgatttttcccatcgtgtggtttcccaactc-3', and the splicing reporters, the Sall-BamHI fragment of the splicing minigene (a kind gift of Dr. Thomas A. Cooper) (23), was substituted with that of 33SB-derived constructs containing cloned elements.

Cell Culture and Treatment—HEK293T cells were cultured in DMEM with 10% newborn calf serum in 12-well plates. Transfection was done with Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. To test PKA or PKC\textsubscript{Cy} effect on splicing, 0.2 \mu g of a minigene splicing reporter construct was cotransfected with 0.6 \mu g of Flag-PKAm (Am), Flag-PKA (A), or pPKC\textsubscript{Cy}-EGFP (Clontech Laboratories, Inc.) plasmid for 16–24 h. For PKC\textsubscript{Cy} effect, cells were then washed, maintained in DMEM, and treated with TPA (120 ng/ml) or DMSO (TPA vehicle control) for 24 h. The translocation of PKC\textsubscript{Cy}-EGFP from cytoplasm to the plasma membrane as a result of PKC activation of the PKC signal was monitored under a fluorescence microscope (27). PC12 cells were grown in DMEM supplemented with 10% horse serum and 5% fetal bovine serum. GH\textsubscript{3} cells were cultured as described previously (9).

RNA Preparation and RT-PCR Analysis—Total RNA was extracted from cells following transfection/treatments with the GenElute Mammalian Total RNA Miniprep Kit (Sigma- Aldrich, Inc.) according to the supplier’s protocol. About 2.5 \mu g of RNA was used in a 10-\mu l reverse transcription (RT) reaction containing M-MLV reverse transcriptase (Invitrogen) and oligo-dT\textsubscript{18}. 2 \mu l of the RT product was used in a 25-\mu l PCR reaction for 26 cycles at an annealing temperature of 60 °C. The RT-PCR products were resolved in 3.5% agarose gels containing ethidium bromide. For examination of endogenous exons, about 200 ng of RNA was used in a 10-\mu l RT reaction. 1 \mu l of the RT product was used in a 13-\mu l PCR reaction for 35 cycles at an annealing temperature of 55 °C, and the RT-PCR products were resolved in 3% agarose gels. Band intensities of the splice variants were quantified with the NIH Image J software 1.37v (developed at the United States National Institutes of Health). The percentages of exon inclusion were relative to the total band intensities of the spliced two products. The PCR primers for 33SB-derived splicing reporters: Insulin1, 5'-GATCGGCT-TCCTGCCCTCGT-3'; Insulin2a, 5'-ACCCAGCTCCAGTTG-TGC-3'; for cTNT\textbeta derived splicing reporters: cTNT1, 5'-AGGAGATGTGGAAGAAG-3'; cTNT2, 5’-GTCATCCTG-AGTGTGGTTGGCAAG-3'. Primers for endogenous exon STREX: RbSlo1, 5'-AGTGCCTCTGTTGCTGTTCT-3', QRA59, 5’-CACATTGGAGTCTGTGTTGC-3'; for CaMKK2 exon 16: 93437s, 5'-GATCCTAGTGAAAGCAGATGAC-3', 93437a, 5'-CCACCTTTCCAAACAGACAC-3'; for LKAP exon 7: r73944s, 5’-AGCTGATCAGATTGGAAG-3’, r73944a, 5’-GCTCTAGCTGCCCTTGTC-3’.

Western Blot Analysis—To test the kinase activity of Flag-PKA and its mutant, overnight HEK293T cultures in 12-well plates were transfected with 0.6 \mu g of Myc-PTB plasmid, or cotransfected with 0.6 \mu g of pcDNA3.1(+), Flag-PKA, or Flag-PKA mutant plasmid. Cells were harvested about 1.5 days after transfection and sonication-lysed in Buffer E (20 mM HEPES KOH, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol) (31), containing 1 mM Na\textsubscript{3}VO\textsubscript{4}, 10 mM Na\textsubscript{2}PO\textsubscript{4}, and 10 mM NaF. A fraction of the cell lysates cotransfected with Myc-PTB plus Flag-PKA or PKA mutant plasmid was treated with calf intestinal alkaline phosphatase (CIAP) in 1× dephosphorylation buffer (Invitrogen) at 37 °C for about 30 min. For Western blot analysis, 5-\mu g cell lysates of each non-PKA cotransfected sample and 2.5 \mu g of each PKA-cotransfected sample (including those treated with CIAP) were resolved in Novex® 4–20% Tris-Glycine gels (Invitrogen) and transferred onto polyvinylidene difluoride membranes (Millipore), followed by blocking in TBS (500 mM NaCl, 20 mM Tris-Cl) containing 5% (w/v) dried skim milk. Total Myc-PTB and phospho-PTB were then probed by c-Myc monoclonal antibody (Clontech) and anti-phospho-Ser\textsuperscript{166}-PTB antibody (27). After three washes in TBS, membranes were incubated with the appropriate secondary antibody for about 1 h, followed by three more washes in TBS. Proteins were then visualized by using Western blotting detection reagent (GE Healthcare) and exposing to x-ray films. For detection of PKA and PKA mutant protein, membranes were washed in TBS and reprobed with a monoclonal ANTI-FLAG\textsuperscript{M2} antibody (Sigma-Aldrich Co).

Selection for CaMKIV- or PKA-responsive RNA Elements—To create a library of splicing reporters, a synthesized randomly random sequence of 13 nt in a primer was substituted for the 13-nt replacement cassette in 33SB in PCR reactions. The PCR products were digested with Apal and BglIII, and ligated with Apal- and BamHI-digested pl53In. The resulting plasmid pool contained ~1.16 × 10\textsuperscript{5} reporter clones as estimated from transformation of Escherichia coli cells with an aliquot of the plasmids. The complexity of the sequence pool was verified by randomly sequencing 17 clones, with no identical sequences obtained. The pool was cotransfected with 10 \mu g of Flag-PKA or Flag-CaMKIV-dCT plasmid and 5 \mu g of an EGFP plasmid using Lipofectamine 2000 into HEK293T cells in a 10-cm dish (~7 × 10\textsuperscript{6} cells). The transfection efficiency was estimated 24 h later to be at least 90% by counting green cells under a fluorescence microscope. Total RNA was harvested using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Al drich, Inc.) followed by digestion with RQ1 RNase-free DNase (Promega) to remove contaminating plasmid DNA, which was...
confirmed by the absence of PCR products using an RT sample without reverse transcriptase. RT-PCR was then performed to selectively amplify middle exon-included mRNA using primers targeted to splice junctions (primer SXenh-J1, 5′-GGAGGACCACAAAGGTTG-3′ and primer SXenh-J2, 5′-CCATTTGCACCTGAGG-3′). The PCR products were cloned as Sall-BamHI 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 (as estimated by transformation in E. coli) were cotransfected with 1.5 μg of Flag-PKA (or CaMKIV-dCT) and 1 μg of the EGFP plasmid into about 1 × 10^6 HEK293T cells.

UV cross-linking and immunoprecipitation—This assay was carried out as described previously using [α-32P]CTP-labeled RNA transcripts (9). The anti-hnRNP L antibody, 4D11 (32), was purchased from Santa Cruz Biotechnology. DNA templates for RNA transcripts (9). The anti-hnRNP L antibody, 4D11 (32), was purchased from Santa Cruz Biotechnology. DNA templates for RNA transcripts (9). The anti-hnRNP L antibody, 4D11 (32), was purchased from Santa Cruz Biotechnology. DNA templates for RNA transcripts (9).

RESULTS

A Selection Strategy Based on a Context-dependent Promotion of Exon Inclusion by CaMKIV and PKA—CaMKIV inhibited splicing through the CaRRE1 element in the DUP175-based reporters (6–9); however, in reporters derived from pL53In (29), CaMKIV enhanced splicing through the same element (Fig. 1A).

In this test, CaRRE1 responses to coexpressed constitutively active Flag-CaMKIV-dCT (CaMKIV or IV) or its kinase-dead mutant (IVm) were examined in the pL53ln-based splicing reporter 33SB-CaRRE1a or b (Fig. 1A), containing exons/introns and promoters different from the previous DUP175 (6, 29, 37). In the 33SB vector transcripts, inclusion of the middle exon (63%) was essentially unchanged by CaMKIV (lanes 1 and 2). Replacing part of the middle exon with CaRRE1a or CaRRE1b sequences reduced the level of exon inclusion to 38 and 34% (lanes 3 and 5), respectively, indicating that the CaRRE1 sequences are splicing silencers. However, unlike in DUP175, the exon inclusion was increased by overexpressed CaMKIV (63 and 49% exon inclusion, lanes 4 and 6, respectively).

A similar promoting effect on exon inclusion by coexpressed Flag-PKA was also observed in the 33SB-CaRRE1 splicing reporters (Fig. 1, B and C). For this assay, we made a double mutant of PKA (PKAm, K73E, K169E) from the wild type Flag-PKA, which phosphorylates the Ser16 of the polyproline tract-binding protein (PTB, Fig. 1B) (27, 38). In the PKAm, the residues Lys73 and Lys169, essential for phosphate-binding (28), were mutated to Glu73 and Glu169, respectively. The PKAm showed no kinase activity on the PKA target Ser16 of PTB (Fig. 1B) and exhibited no effect on the exon inclusion (Fig. 1, A and C, compare lanes 1, 3, 5 in A with 1, 3, 7 in C, respectively, p = 0.25, paired Student’s t test), in comparison with the CaMKIVm that is known to be kinase-dead and have no effect on splicing (6, 8, 26). In contrast, the wild type Flag-PKA increased the exon inclusion level to 77 and 75%, respectively (Fig. 1C, lanes 4 and 6), similarly to the effect by CaMKIV (Fig. 1A). Importantly, this increase was reduced to that of the 33SB vector level by mutations in CaRRE1 (Fig. 1C, lanes 5, 6, 9, and 10). Because these clones contain the same promoter, flanking exons/introns and poly(A) signals, their differential responses to CaMKIV or PKA are properties of the insert elements themselves rather than those of the transcriptional unit.

The promoting effect of CaMKIV and PKA suggested that these protein kinases would likely enrich exons containing kinase-responsive RNA elements in a pool of random sequences during repeated selections. Moreover, because the vector already showed about 65% exon inclusion with CaMKIVm or PKAm (Fig. 1, A and C), constitutive enhancers in the random pool were expected to cause 100% exon inclusion soon in early rounds of selection and could not be further increased by the kinases during subsequent rounds of selection. Thereby this system would likely allow enrichment of silencer elements preferably targeted by the kinases through repeated selections.

According to this approach, a pool of 13-nt random sequences was cloned into the middle exon of the 33SB to obtain a library of splicing reporters (containing about 1.16 × 10^7 clones). The reporters were cotransfected into HEK293T cells with the constitutively active CaMKIV or PKA plasmids. The spliced products were amplified by RT-PCR, cloned back into the vector, and cycled through five selection rounds (Fig.
Because no PKA-responsive splicing element has been reported yet and also for reasons as found out later (more net changes of exon inclusion by PKA and mostly shared elements between these two kinases as shown below), we analyzed the PKA effect in more detail in the following experiments.

**The Selected PKA- or CaMKIV-responsive RNA Elements Are Mostly SplicingSilencers**—Preliminary analyses with RT-PCR of the spliced mRNA pools from each selection round (of five rounds in total) indicated that the net changes of exon inclusion by PKA increased and peaked at round 3 (about 3-fold, compared with the starting pool). We therefore randomly picked up 48 clones from the 2nd and 3rd rounds of selection plus the 33SB vector, and analyzed their constitutive splicing and response to PKA coexpression (Fig. 2A).

The constitutive level of exon inclusion of these clones ranges from 0 to 93% (Fig. 2A, from bottom to top). A small group of clones (nos. 1–16) showed higher constitutive level of exon inclusion than the 33SB vector. Most of these clones contain motifs that are similar to the purine- or A/C-rich enhancers in the previous selection (23), as expected. For these clones, the PKA effect to increase exon inclusion is hard to assess since they already have almost 100% exon inclusion even without Flag-PKA coexpression. However, a larger group of clones (nos. 18–49) showed lower constitutive level of exon inclusion than the 33SB vector. Of the 32 clones in this group, 29 (nos. 21–49) showed at least 10% less constitutive exon inclusion than the vector, likely containing splicing silencers. PKA coexpression substantially increased the inclusion of exons in 22 clones (with
net changes at least 10% more than the vector) except 7 clones (nos. 27, 31, 44–46, and 48–49). Therefore, PKA appears to relieve the splicing repression effect to promote the inclusion of exons containing PKA-responsive silencer elements.

Analyses of 44 clones of the CaMKIV group showed similar promotion of exon inclusion through silencer elements though the net change of exon inclusion by CaMKIV is generally less than that by PKA (Fig. 2B, nos. 21–44). Besides, CaMKIV also appeared to generally repress the exon inclusion of the clones containing enhancer elements (nos. 1–13), an effect not seen for the silencers or by PKA.

Overall, the occurrence of clones responsive to PKA or CaMKIV increased from 39% or 38% in the starting pool to 57% (for both kinases) in the 3rd round, supporting that the selection indeed enriched RNA elements responsive to PKA or CaMKIV.

Consensus Motifs of PKA- or CaMKIV-responsive RNA Elements—To identify consensus motifs of the elements that are PKA-responsive, we first transferred the highly PKA-responsive elements (net changes at least 10% more than the 33SB by PKA, same as in the following texts) from selection rounds 2 and 3 to a heterologous minigene splicing reporter cTNTβ. This experiment identified 14 out of 20 elements that conferred PKA response to this heterologous gene as well (Fig. 3A, b).

These transferable elements appeared mostly CA- and A-rich. From these elements, a consensus heptamer motif CARAAHD (R: A or G; H: A, C or T; D: A, G or T, Fig. 3A) was identified using Mutiple Em Motif Elicitation (MEME) (33). In contrast, MEME analysis of the 13nt insert sequences of 17 clones from the starting random pool did not identify any consensus motif that could be found in most clones.

Similar examination of the 13-nt inserts of a group of highly CaMKIV-responsive clones also identified a consensus motif (C/A)AARR(C/A)A (Fig. 3B, R: purine). This motif is similar to the CA repeats of CaRRE1 (for example, the “CACAT” in clone C42). Moreover, RNA transcripts containing this motif were strongly cross-linked to a protein similar in size to the hnRNP L (Fig. 4, A and B), which binds the CaRRE1 (9). This protein was immunoprecipitated by 4D11 (Fig. 4B, right), a monoclonal anti-hnRNP L antibody (32). In contrast, barely any PTB signal
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| A | Clone | Sequence | % net change by PKA | Matrices |
|---|-------|----------|---------------------|---------|
| A3.19 | CACCG | C A R A A A T | 2.99 | 1.67 | 6.3 |
| A3.14 | AAA | A A A A A T | 2.94 | 1.4 | 5.9 |
| A3.43 | A C | G G A T A | 3.96 | 1.5 | 5.7 |
| A3.44 | A C | R A T T | 4.15 | 1.7 | 5.7 |
| A3.15 | CACGG | C A R A A A T | 3.11 | 1.3 | 6.3 |
| A2.16 | AAA | C R G C | 5.1 | 1.8 | 4.7 |
| A3.8 | A C | G C R T | 5.19 | 1.5 | 5.8 |
| A2.6 | AAA | C R G C T | 5.28 | 1.9 | 5.7 |
| A3.24 | CACGG | C A R A A A T | 4.46 | 1.6 | 6.9 |
| A3.16 | A A | G R G A | 5.32 | 1.6 | 5.7 |
| A3.5 | A A | A T R C | 5.28 | 1.4 | 6.1 |
| A3.4 | A C | A T R C | 5.31 | 1.9 | 6.1 |
| A0.9 | C A | A R | 5.46 | 1.5 | 6.8 |
| A3.9 | AAT | C A R T | 7.24 | 1.3 | 5.3 |

| B | Clone | Sequence | % net change by CaMKIV | Matrices |
|---|-------|----------|------------------------|---------|
| C1 | A A A | A A A A | 3.39 | 1.7 | 5.7 |
| C11 | A C A | C A A | 3.93 | 1.3 | 6.6 |
| C12 | A C A | A A A | 4.29 | 1.6 | 5.9 |
| C13 | C A T | T C A | 3.39 | 1.7 | 5.7 |
| C14 | A A R | R A A | 5.21 | 1.4 | 6.7 |
| C17 | A A C | A A C | 2.36 | 1.4 | 6.4 |
| C26 | A T A T | C A | 2.26 | 1.4 | 6.4 |
| C32 | A A A | A C A | 1.72 | 1.4 | 6.6 |
| C39 | C T A A A | C T | 1.94 | 1.3 | 6.3 |
| C40 | A A A A T | A A | 2.06 | 1.4 | 6.4 |
| C42 | C A R A | C A | 3.35 | 1.7 | 5.7 |
| C45 | A A C | A A C | 3.39 | 1.7 | 5.7 |
| C47 | A A T | A A T | 3.39 | 1.7 | 5.7 |
| C50 | C A T | A A T | 3.39 | 1.7 | 5.7 |
| C59 | A A A | A A C | 2.26 | 1.4 | 6.4 |
| C62 | T A A T | C A | 2.26 | 1.4 | 6.4 |
| C63 | T A A T | C A | 2.26 | 1.4 | 6.4 |

FIGURE 3. Selected highly PKA/CaMKIV-responsive RNA elements and their consensus motif. A, consensus motif shared by highly PKA-responsive elements. Upper, sequences of fourteen elements, with the nucleotides for the consensus motif aligned in the middle and sequence names to the left. Lower, frequency of each nt at each position of the heptamer motif. The heights of each nucleotide letter are proportional to its nt frequency, in decreasing order from top to bottom. The consensus sequence is shown in the graph above (R, A or G; H, A, C, or T; D, A, G, or T). The numbers in the clone/element names indicate, from left to right, selection round, number in 33SB, number in cTNTβ. B, consensus motif shared by highly CaMKIV-responsive elements. Upper, sequences of the elements from selection round 3 with CaMKIV (C) are listed with the nucleotides for the consensus motif aligned in the middle, followed by the net changes of exon inclusion by CaMKIV. Also included are 5 highly responsive sequences from the starting pool (S). Lower, consensus motif of the highly CaMKIV-responsive elements. The matrices (out of max. 10) of the motifs are indicated to the right of each panel. The CaRRE1 has a matrix score of 5.1 with the “aaccaa” motif.

Interestingly, similar UV cross-linking immunoprecipitation with the CA-rich A3.15 and C7 RNA probes, but not a control transcript containing CG repeats, also identified hnRNP L as a prominent binding factor (Fig. 4C). In addition, a ~62-kDa protein band just below hnRNP L is clearly visible in the A3.15 sample as well (Fig. 4C, lane 2, arrow). Moreover, hnRNP L binding to C7 and C42 appeared stronger than to the A3.15 probe (compare 4B, lane 7 and 4C, lane 6 with 4C, lane 3).

Of the other two bands (75 and 35 kDa) that are specific for the enriched element probes but not the mCaRRE1 or the CG probe, the 35 kDa band showed no difference in relative intensity among the element probes (Fig. 4C), suggesting that it is not an element-specific band; however, the 75 kDa band showed a much higher relative intensity in the C42 sample.

Thus, the CA-rich motifs enriched in the PKA- and CaMKIV-responsive elements likely bind proteins differentially; one of the common protein factor is the hnRNP L. Taken the random enrichment of these elements together with the essential role of hnRNP L in CaMKIV-regulated splicing through CA repeats (9), hnRNP L is likely a preferred target of PKA and CaMKIV in regulating splicing. Together, the CA-rich content and the identification of hnRNP L as a binding factor suggest that the selection indeed identified consensus motifs that are known to be CaMKIV-responsive.

PKA and CaMKIV Specifically Share Their Responsive Elements—As shown above, both CaMKIV and PKA promote inclusion of exons containing CaRRE1 (Fig. 1, A and C), and their consensus motifs appear similar in the CA- and A-rich content. Moreover, hnRNP L was identified as one binding factor for both kinase-selected elements (Fig. 4). These together suggest that the two kinases may share similar elements.

To examine the kinase specificity of these elements, four highly PKA-responsive clones were tested with CaMKIV and PKCy (Fig. 5, A and B). These clones showed clear responses to CaMKIV (Fig. 5A), with CaMKIV-induced net changes ranging from 15 to 31%, well above that of the 33SB vector alone (4%). In contrast, three clones had no response to PKCy above the 3SB level (Fig. 5B, lanes 1–8), and one clone (A3.44) showed only 9% increase of exon inclusion over the vector (lanes 9 and 10). Thus, the PKA-responsive elements are preferably responsive to both PKA and CaMKIV but not PKCy.

The sharing of motifs between PKA and CaMKIV is also supported by that substituting the CaRRE1 core sequence with CAAAAAG, from the consensus motif of PKA-responsive elements (Fig. 3A), retained the PKA effect (Fig. 5C). Therefore, the CaRRE1 core motif is replaceable by a PKA consensus motif.

Comparisons of these kinase effects through the elements relative to that through the vector sequence indicate that the PKA and CaMKIV effects on splicing are significantly stronger than PKCy (Fig. 5D). Therefore, PKA and CaMKIV specifically share most of these tested RNA elements in controlling alternative splicing.

The Consensus Motifs of the Selected RNA Elements Are Critical for Kinase-dependent Splicing and Are Significantly Enriched in Alternative Exons—To verify that the consensus motifs are critical for kinase effects on splicing, two strongly PKA-responsive clones containing CA dinucleotides or A-rich motifs were mutated and tested in splicing reporter assays (Fig. 6A). The wild type clones showed significantly increased exon...
of alternative versus constitutive exon sequences. Relatively, all the five heptamers are present between 1.5 and 2-fold (1.8 ± 0.12, average ± S.D.) in the alternative exons over the constitutive exons (Fig. 6B). In contrast, five random heptamers all show about 1-fold (0.91 ± 0.2, average ± S.D.) in the alternative exons. Therefore, these CA-containing or A-rich motifs are significantly (p < 0.0001) enriched in alternative exons.

**Motif-containing Endogenous Exons Are Regulated by cAMP and/or Depolarization in Cell Culture**—Of these motifs, the A-rich ones in endogenous exons have not been shown to respond to cAMP and depolarization. In examination of seven A-rich consensus motif-containing exons (from ASAP1I data base), we found three exons GenBank accession no.: AB012133) gene encoding an autoantigen with RNA binding domains (39), contains 2 copies of CAAAAAA. This exon is differentially included in PC12 and GH3 cells (Fig. 7, lanes 3 and 5), suggesting that its inclusion is regulated. Depolarization of GH3 cells with KCl led to its complete inclusion (Fig. 7, lanes 3 and 4). In PC12 cells, this exon is already 100%-included before treatment and the forskolin or cAMP effect on its inclusion, if similarly promoting as the depolarization effect, cannot be assessed (lanes 5–7).

Another A-rich motif-containing exon identified is the CaMK kinase β1 (CaMKK2) exon 16 (40). This exon was regulated by both forskolin and cAMP, as well as by depolarization (Fig. 7, lanes 8–12), and was examined in more detail with hot-PCR and primer extension.

This exon, containing a conserved CAAAAAA motif at its 5′-end in mammalian genes (Fig. 8A), showed an inclusion level of about 37 and 36% in vehicle- and non-treated PC12 cells, respectively in hot-PCR (Fig. 8B, lanes 1 and 2). Upon treatment with PKA stimulus forskolin or cpt-cAMP, the exon inclusion level was reduced to about 21% (Fig. 8B, lanes 3 and 4). Therefore, the CaMKK2 exon 16 is likely controlled by the PKA pathway in PC12 cells. A similar effect was observed by treatment of GH3 cells with a depolarizing concentration of KCl (25 mm, lane 6), which controls splicing through CaMKIV (6).

To verify the role of the CAAAAAA motif in response to the kinases, we replaced the 5′-end 7 nt of the DUP175 middle exon with the motif and tested its responses to PKA or CaMKIV in HEK293T cells (Fig. 8C). The PKA cotransfection did not induce exon skipping of the DUP175 transcripts (lanes 1 and 2). Transferring the CAAAAAA motif to DUP175 promoted two cryptically spliced products in the presence of PKA mutant (lane 3), but with barely visible complete exon-skipping products (about 7%, without counting the cryptic products, same as below). Coexpressed wild type PKA led to substantial amount of exon skipping (lane 4, about 31% exon-skipping product). Importantly, mutation of the CAAAAA completely abolished the PKA effect (lane 6). Thus, the CAAAAA motif is sufficient for the PKA effect on splicing. Coexpressed wild type CaMKIV induced exon skipping as well, with an even stron-
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DISCUSSION

Several Considerations about the in Vivo Selection System and Identified RNA Elements—Previous reports selected constitutive enhancer or silencer elements in alternative splicing (23, 25). There has been no reported selection system to enrich and refine pre-mRNA elements responsive to activated protein kinases. We thus attempted to select kinase-responsive elements by modifying the method used for the constitutive enhancers (23).

The rationale of selection is based on the kinase promotion of exons containing kinase-responsive silencers in a splicing reporter already having about 65% exon inclusion (Fig. 1). As expected, most of the selected clones contain constitutive splicing silencers. The exon inclusion levels of a smaller group of enhancer-containing clones mostly either reached about 100% and not to be increased further by the protein kinase PKA or even reduced by CaMKIV (Fig. 2). Based on this observation, similar selection could be applied to enrich RNA elements responsive to other signaling protein kinases.

The selected elements appear CA- and A-rich. The consensus motif heptamers are critical for the PKA effect and are significantly enriched in alternative exons (Figs. 5 and 6), supporting their role in regulated splicing. Interestingly, CA repeats have been recently found to mediate CaMKIV-regulated splicing, with the hnRNP L as an essential component (9). Consistently, hnRNP L is identified as a factor binding to the enriched CA-rich elements as well (Fig. 4). Therefore, CA repeats or the CA-rich elements and hnRNP L are likely preferred targets of CaMKIV in controlling splicing. The consensus sequence and the matrices should be helpful for recognizing these regulatory elements in the “sea” of the genomic sequences.

The CA-rich content also bears some similarity to the A/C-rich elements but the latter are splicing enhancers, as tested in a similar 33-nt exon derived from the β-globin gene as well (23). Moreover, the A/C-rich enhancer-binding protein YB-1 (~50
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FIGURE 6. The kinase-responsive heptamer motifs are critical for the kinase effect on splicing and are significantly enriched in alternative exons. A, splicing reporter assay with highly PKA-responsive clones and their mutants. Above the gel are the 13-nt sequences of each clone and mutated nucleotides (bold). The dotted horizontal lines in the graphs mark the baseline percentages of net changes of the vector 33SB by PKA. Below each lane are the percentages of exon inclusion and a bar graph of net changes (aligned between every two lanes) by PKA for each reporter. B, bar graph of motif abundance demonstrating the enrichment of the kinase-responsive CA-containing or A-rich motifs in alternative exons in comparison to the random sequences in the control group. Each motif (as indicated) was searched against 6125 alternative or 3775 constitutive exons of 7 to 500 nt in length, and the abundance index was taken as the result of the element occurrences per kilobases of alternative exons divided by the corresponding occurrences of constitutive exons. Therefore, an index number of 1 equals the abundance of the elements in constitutive exons. The averages of the index numbers of the selected and non-selected motif groups are shown to the right of each group. ****, p < 0.0001, two-tailed Student’s t test between the two groups.

FIGURE 7. Regulation of the motif-containing endogeneous exons by cAMP or depolarization in cell cultures. Inclusion levels of endogeneous exons STREX, LKAP exon 8, and CaMKK2 exon 16 were analyzed by RT-PCR. Motifs and exon names are above the gels. –, nontreated. C, ethanol, as the vehicle control for forskolin; FSK, forskolin (10 μM), an activator of adenylyl cyclase; cAMP, 8-cpt-cAMP (100 μg/ml), a membrane-permeable cAMP analogue. Exon-included products are indicated by black dots, and exon-excluded products by open circles. STREX-included and -excluded products are 597 and 423 nt, respectively; LKAP, 554 nt and 125 nt; and CaMKK2, 225 nt and 182 nt. The gels are representative of three experiments.

KDA is not observed as a prominent band in UV-cross-linking (Fig. 4). The A-rich contents of these elements have not been observed in PKA- or CaMKIV-regulated splicing. A known A-rich element is the AAAAAU in the exon 5 of the CD44 gene (20), but it is an enhancer. Therefore, the similarities and differences between the selected and these known elements need further investigation.

The number of clones contained in the starting pool is about one-sixth of a full coverage (6.7 × 10^3 clones) of 13-nt random sequences. The CaMKIV selection did not enrich elements similar to the G-rich CaRRE2 (8). Moreover, this system does not allow the selection and assessment of constitutive splicing enhancers in kinase-promoted exons. Therefore, besides the selected elements, the existence of other sequences that are also responsive to these kinases is not ruled out. Despite these considerations, this selection does provide the first example to enrich RNA elements that are responsive to protein kinases.

Context Dependence of Cell Signal-regulated Alternative Splicing—Context dependence of pre-mRNA elements on splicing has been observed in many cases (5, 17–19), but opposite effects of a kinase in splicing through the same element in different minigenes have not been observed. We have shown here that, through the CaRRE1, CaMKIV inhibits exon inclusion in one minigene, DUP175ST (6), but promotes exon inclusion in another minigene, 33SB-CaRRE1 (Fig. 1). The promoting effect of CaMKIV, as well as PKA, is also seen through the SELEX-enriched elements (Figs. 2, 5, and 6). Consistent with the context-dependent effect through these elements, PKA and CaMKIV also inhibit the inclusion of the motif-containing exon in DUP175 (Fig. 8).

The molecular mechanisms underlying the context-dependence of a protein kinase through an element is unknown. In the case of SRp38, the splicing enhancing or repressing effects can be determined by the phosphorylation/dephosphorylation status of the protein (41). In our splicing reporter assays, the protein kinases used for the reporters DUP175ST and 33SB-CaRRE1 are the same (PKA or CaMKIV). Therefore, any changes of the phosphorylation status of a splicing factor, for example hnRNP L, are likely to be the same for both reporter systems. However, these reporters differ in their promoters, lengths/sequences of the middle exons, flanking exons/introns, and the locations of the CaRRE1 element relative to the splice sites (Fig. 1). Interestingly, the mA CaRRE1 in DUP175 and the selected elements in 33SB have several different bound factors: PTB specific for the 75-kDa protein specific for the enriched element probes (Fig. 4). Moreover, a ~62-kDa protein smaller than hnRNP L is specific for the A3.15 element (Fig. 4C, lane 2). Taking these differences together with the known combinatorial control of mammalian splicing by
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A multiple elements and influence by promoters (16, 17), one possibility may involve differential recruitment of trans-acting factors in addition to hnRNP L to the target and/or its surrounding elements/promoters by the kinases, resulting in either inhibition or promotion of splicing depending on the balance between the positive and negative regulators.

Effect of the Enriched Elements in Cells and Shared Target Elements/Exons in PKA- and CaMKIV-regulated Alternative Splicing—Similar to the previously identified depolarization-responsive elements that respond to depolarization only with multiple copies of the elements or together with other elements (6, 8, 10), a splicing reporter containing only one copy of an hnRNP L high affinity sequence, as used previously (9), was not sufficient to respond to depolarization (data not shown). It is thus likely that the CA-rich elements also require multiple copies (such as 2 copies of CAAAAAA in the LKAP exon 8, Fig. 7) or with the help of other elements in alternative exons to confer response to endogeneous signals.

The ASAPII exons containing these enriched motifs have average MaxEntScan scores of 7.5 (± 2.98) for the 3'-splice sites and 7.9 (± 2.8) for the 5'-splice sites (n = 263) as measured according to the maximum entropy model (42). These scores are not significantly lower than that of the constitutive exons (7.9 ± 3.6, 8.3 ± 3.1, for the 3’- and 5’-splice sites, respectively) (43). The relatively strong splice sites may make the exons mostly included as the LKAP exon but with the presence of the CA- or A-rich elements can still be regulated by the activation of protein kinases.

The cAMP and Ca2+ signaling pathways are known to converge at the Ser133 of the CREB protein in transcriptional control (44). Their effects on alternative splicing through common RNA elements have not been observed. Interestingly, the 33SB-derived reporters used here demonstrate preferred responses of the CA- and A-rich elements to both kinases, but not PKCy. The control of alternative splicing by PKA and CaMKIV through the same RNA elements adds
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another possible point of convergence between these two important signaling pathways.

Among the common target elements shown here however, their responses to PKA and CaMKIV overexpression are not always the same (Fig. 5D). Specific elements with variations in nucleotide sequences are preferred by each of the two kinases (Fig. 5D). Related to this variation, in the exon of a DUP17S-derived splicing reporter, a CA repeat element strongly responded to CaMKIV but barely to PKA (9), unlike in the 33SB-derived reporters (Figs. 1, 5, and 6). In this case, the reporter context appears to play an important role on the PKA effect. The shared, but differential, splicing regulation through these RNA elements by PKA and CaMKIV likely helps to refine gene expression profiles tailored for precise functions of genes and cells.

PKA is known to control the CaMKK1 (CaMKKα) through direct protein phosphorylation (45–50). The control of CaMKK2 splicing by PKA and CaMKIV through a common RNA element adds a novel way of signal cross-talk/feedback between the PKA and CaM kinase pathways. Compared with direct phosphorylation, one likely advantage of this splicing regulation is that it can be temporally longer and involved in cell functions requiring gene expression to take effect. Therefore, this regulation provides an example where the selected RNA element could play a role in regulating the important signaling networks in cells.

In summary, we used in vivo selection for PKA/CaMKIV-responsive RNA elements controlling alternative splicing and identified consensus motifs that are similar to the previously characterized CaRRE1 and CA repeats, as well as novel A-rich elements. This selection also provides an approach that could be applied to other signal pathways.

Acknowledgments—We thank Thomas Cooper for the cTNT plasmid, Doug Black for DUP splicing reporters and kinase plasmids, Harold König for the pl53In plasmid, and Christopher Lee for ASAPII database exons. We thank Mary-Lynn Duckworth and Doug Black for helpful comments.

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