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Identification of exon sequences and an exon binding protein involved in alternative RNA splicing of calcitonin/CGRP

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ABSTRACT

Transcripts derived from the 6 exon CALC I gene are differentially processed in a tissue-specific fashion to include or exclude a calcitonin-specific exon 4. All cell types which transcribe a second calcitonin/CGRP gene, CALC II, exclude exon 4. Substitution of the first 30 nucleotides of CALC I exon 4 with analogous CALC II sequence was sufficient to prevent recognition of exon 4 in *in vitro* or *in vivo* RNA splicing systems. UV crosslinking detected a ~66 kDa RNA-binding protein in HeLa nuclear extract which interacted with CALC I proximal exon sequence, but not CALC II or mutant sequences. UV crosslinking of this protein was inhibited by addition of nuclear extract from a cell type which normally causes exclusion of exon 4. These results identify an important regulatory element within exon 4 and support a model in which calcitonin production requires protein interaction with this sequence to facilitate exon recognition.

INTRODUCTION

Alternative processing of eukaryotic mRNA precursors allows the production of multiple mRNAs from a single transcript (1,2). While the advantages of alternative RNA processing are clear, the regulatory mechanisms that permit such diversity are not. The CT/CGRP gene was one of the first described examples of tissue-specific alternative RNA processing (3,4). The human gene family contains at least 3 distinct members: CALC I, CALC II, and CALC III (5). The CALC I and CALC II genes are structurally similar (6 exons) but are processed differently (Figure 1). The primary RNA transcript for the CALC I gene is differentially processed in a cell-specific manner to include exon 4 in the thyroidal C-cells and exclude this exon in neuronal cells. However, mRNAs containing the CALC II exon 4 have never been observed in tissues or cell lines expressing this gene (5,6). The CALC III gene lacks an exon 4 region. Therefore, while all three of these genes maintain the capacity to produce CGRP, only the CALC I gene is capable of CT production (5). It has been suggested that the CALC I gene is evolutionarily derived from the other two genes, and that alternative processing of exon 4 is a recent occurrence (5).

Previous studies have proposed that CT-specific splicing is a default pathway, and that CGRP-specific splicing requires a dominant neuronal trans-acting factor (7,8). We agree there is a dominant factor produced by neuronal cells which results in exon 4 exclusion (9), but believe the inclusion of exon 4 is more complicated than previously suggested (7,8). There is considerable evidence the 3′ splice site signals preceding exon 4 are suboptimal (10,11). The characteristic features include a non-canonical branch point acceptor and a polypyrimidine tract interspersed with purines. These features are also found in the *Drosophilia doublesex* gene, where recognition of the included/excluded exon requires interaction between exon sequences and a developmentally-regulated RNA binding protein, *transformer-2* (12–14).

In the present study, we have further analyzed the sequences within exon 4 required for recognition of the exon. An element located within the first 30 nucleotides of exon 4 appears to be required for exon 4 inclusion, both *in vivo* and *in vitro*. This element binds a ~66 kDa protein as detected by UV crosslinking. Binding of the protein correlated with recognition of exon 4 during splicing. Mutations in exon 4 that cause exon skipping inhibit crosslinking of the 66 kDa protein. Binding of this protein is also competed by nuclear extract from a CGRP-producing cell. Therefore, we propose that exon 4 recognition during CT production is facilitated by an exon binding protein whose interaction is inhibited in neuronal cells to produce CGRP.

MATERIALS AND METHODS

Cell culture

Stock HeLa cell cultures were maintained on 100 mm plates in DMEM supplemented with 10% fetal calf serum as previously described (15,16). F9 mouse teratocarinoma cell lines were maintained on gelatin coated 100 mm plates in DMEM supplemented with 15% fetal calf serum (9).

Plasmids

Chimeric constructs were produced by standard cloning methodology. Construction of parent *in vivo* pCTG-6 and *in vitro* IVPX/CT-7 plasmids was previously described (9,10). Mutations were introduced by either insertion of synthetic DNA (pCTG-7 and IVPX/CT-19) or by PCR-mediated amplification using an oligonucleotide primer containing the appropriate mutations (pCTG-8 and IVPX/CT-21). Internal *Eco RI/Pst I* deletions of IVPX/CT-7, IVPX/CT-19, IVPX/CT-21 were performed to provide templates for production of UV crosslinking probes. Detailed description of the construction of all clones is available on request.

Transfections

Plasmids were introduced into cell lines by DEAE-Dextran transfection as previously described (17). Cells were plated at ~25% confluence on 100 mm plates. Each plate was transfected with ~16 μg at a final DEAE-Dextran concentration of 400 μg/ml. Total RNA was isolated 48 hrs post-transfection by RNAzol™ (Cinna/Biotecx Laboratories, Houston, TX) extraction according to the manufacturer.

Polymerase chain reactions

RT-PCR reactions used total RNA isolated from transfected cells. Reactions were performed in a single tube using a modification of the method described by Kawasaki (18). Primer annealing was performed in a 10 μl reaction volume (reaction conditions: 1×PCR buffer (Perkin Elmer/Cetus, Emeryville, CA), 200 units RNasin (Promega Madison WI), 5 μg RNA, and ~0.5 μg primer) for 30 minutes at 55°C with CT and CGRP-specific primers. Annealing reactions were diluted to 30 μl to perform reverse transcriptions (reaction conditions: 1×PCR buffer (Perkin Elmer/Cetus, Emeryville, CA), 10 mM dithiothreitol, 0.4 mM dNTP's, 200 units RNasin (Promega Madison WI), 200 units MLV transcriptase (BRL, Bethesda, MD), 1 hr at 37°C). Finally, PCR amplification was performed by dilution of reverse transcription reactions to 100 μl (reaction conditions: 1×PCR buffer (Perkin Elmer/Cetus, Emeryville, CA), 10 μg RNase A, 0.4 mM dNTP's, ~0.5 μg primers, 2.5 units Amplitaq™ (Perkin Elmer/Cetus, Emeryville, CA), 30 cycles: 1 minute 94°C, 1 minute 55°C, 2 minutes 72°C). Products of the PCR reactions were analyzed by polyacrylamide electrophoresis. C-T-specific primer set A: GCGAGGGCCAGCTGTTGGGC (upstream), GAGTTTCGTTGGCATTCTGG (downstream). CGRP-specific primer set B: GCATCGCTGTCTGCGAGGG-CCAGCT (upstream), CTGCTCAGGCTTGAAGGTCC (downstream).

In vitro splicing

HeLa and F9 cell nuclear extracts were prepared as previously described (9). DNA templates were truncated at a *Dra I* site located 82 nucleotides downstream of exon 5. *In vitro* transcription of these DNA templates was performed using 1–2 μg of template with SP6 polymerase (Promega, Madison, WI)

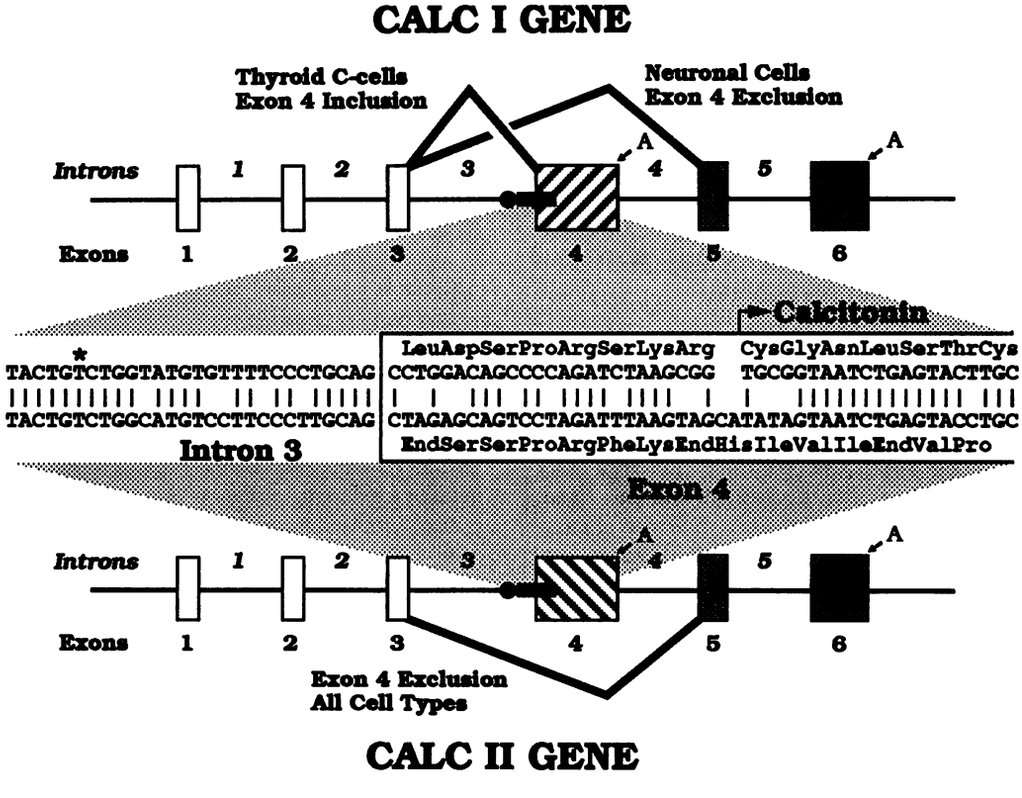

Figure 1. Alternative processing pathway of the CALC I and CALC II genes. Schematic representation of the human CALC I and CALC II genes illustrating the tissue-specific processing pathways used by both genes. Inset displays the sequence from black box in the schematic and indicates homologies between the CALC I and CALC II genes in the 3' splice site region of exon 4. The branch acceptor nucleotide is denoted by an asterisk. The cysteine residue denotes the beginning of coding sequence for the mature 32 amino acid calcitonin peptide.

in the presence of ^{32}P-UTP (800 Ci/mmole, New England Nuclear, Boston, MA) as previously described (18). Unlabeled competitor RNAs were transcribed in the presence of 1 mM ATP, UTP, CTP, diguanosine, and 0.1 mM GTP. Splicing reactions contained: 20 mM creatine phosphate; 2 mM ATP; 1.5 mM Mg_2Acetate; 1.2 mM dithiothreitol; 4% polyethylene glycol (8000 MW); 8.8% glycerol; 44 mM KCl; and 8.8 mM Tris, pH 7.9. Spliced products were analyzed by electrophoresis on denaturing polyacrylamide gels, followed by autoradiography as previously described (15, 16, 19).

UV crosslinking

UV crosslinking experiments were performed as previously described (20). Briefly, proteins were allowed to associate with ^{32}P UTP labeled RNAs in processing extract for 5 minutes at 30°C. Competition experiments were performed by mixing unlabeled RNA with the RNA probe prior to addition to nuclear extract. In mixing experiments, extracts were combined in equal amounts of total protein. Covalent crosslinking was achieved by a 10 minute exposure to a Phillips G15T8 lamp at a distance of 4 cm at 4°C. Crosslinked samples were treated for 15 minutes with RNase A (0.5 mg/ml) prior to SDS/10% PAGE electrophoresis.

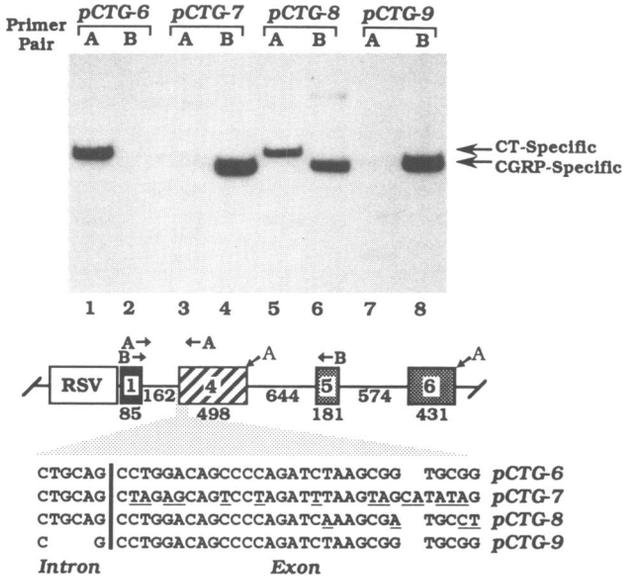

Figure 2. Mutation of calcitonin exon 4 sequence results in CGRP-specific processing in transfected HeLa cells. The diagram contains a schematic representation of the pCTG-6 minigene depicting the CALC I exon 4 sequence. Constructs pCTG-7, pCTG-8, and pCTG-9, contain the sequence alterations illustrated. Mutated nucleotides are underlined; pCTG-9 contains a 4 nucleotide deletion in the intron destroying the 3′ splice site. Numbers denote exon and intron sizes (nucleotides). Arrows indicate the relative position of PCR primers. A primers are CT-specific and B primers are CGRP-specific. HeLa cells were transfected with each of the 4 constructs. Total RNA was isolated 48 hr post-transfection. RNA was reverse transcribed using CT-specific or CGRP-specific downstream primers. The resultant cDNA was amplified by PCR in the presence of ^{32}P-dCTP. Products of the RT-PCR reactions were analyzed by polyacrylamide electrophoresis and autoradiography. Correct splicing of exon 1 to exon 4 predicts an amplification product of 222 bp, whereas an exon 1 to exon 5 splice predicts an amplification product of 195 nucleotides. In odd numbered lanes CT-specific primers were used. CGRP-specific primers were used in even lanes.

RESULTS

Calcitonin production requires inclusion of exon 4 in the final mRNA. Of the three human CT/CGRP genes, only the CALC I and CALC II genes possess a 4th exon. Comparison of the exon 4 sequence surrounding the 3′ splice site region of these two genes is shown in Figure 1. We noted striking sequence homology between the branch point, polypyrimidine tract and 3′ splice site region (greater than 96% sequence homology when conservative pyrimidine differences are included)(21, 22). In contrast, the first 47 nucleotides of exon 4 sequence significantly diverges in the two genes (Figure 1). The CALC II sequence contains 13 nucleotide differences and a 2 nucleotide insertion when compared to the CALC I sequence. The major sequence deviation occurs within a 5 nucleotide repeat (GCGGT). These differences introduce 2 stop codons into the NH_2-terminal peptide sequence prior to the calcitonin reading frame. To examine the possibility that the divergence of exon 4 sequence might explain the differences in the RNA processing of these two genes, we introduced mutations into CT/CGRP minigenes capable of *in vivo* and *in vitro* RNA processing (9,10).

Effect of exon 4 mutations on splicing *in vivo*

Previous studies demonstrated that the chimeric mingene pCTG-6 (Figure 2) shows cell-specific RNA processing: CT-specific processing in transfected HeLa cells and CGRP-specific splicing in F9 teratocarcinoma cells (9). Prior *in vitro* studies demonstrated that deletion of sequence between bases 18 and 45 of CT exon 4 caused exclusion of exon 4 (10). To further localize these regulatory sequences we used two strategies. First, we substituted the first 30 nucleotides of exon 4 from the CALC II gene for similar sequence in pCTG-6 (pCTG-7). Because this was done without disrupting the 3′ splice site or the remainder of the CALC I exon 4 sequence, pCTG-7 differs from pCTG-6 by only 14 nucleotides. Second, we targeted a 5 nucleotide repeat

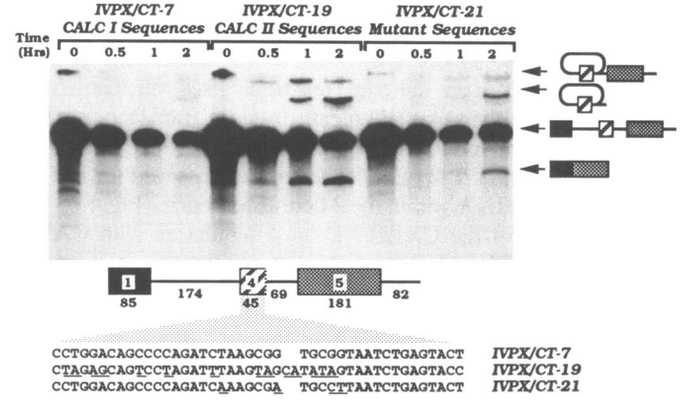

Figure 3. Mutation of calcitonin exon 4 sequence causes CGRP-specific processing in HeLa nuclear extract. The diagram contains a schematic representation of IVPX/CT-7 depicting the CALC I exon 4 sequence. Constructs IVPX/CT-19 and IVPX/CT-21, contain the exon 4 sequence alterations illustrated (mutated nucleotides are underlined). Numbers denote exon and intron sizes (nucleotides). Radiolabeled precursor RNAs made by SP6 *in vitro* transcription were assayed for splicing activity in HeLa nuclear extract. Reaction aliquots were removed at indicated times and spliced products identified by denaturing polyacrylamide gel electrophoresis and autoradiography. The position of RNA precursor, exon 1 to exon 5 spliced product and lariats are indicated. Product bands were identified by RT-PCR analysis as described in Figure 2 (not shown).

(GCGGT) for mutation (Figure 2). The construct pCTG-8 contains only 4 nucleotide mutations specifically targeted to the repeat region (Figure 2). A control construct, pCTG-9, contains a 4 nucleotide deletion which destroys the 3′ splice site preceding exon 4. Previous studies indicate this deletion results in exon 4 skipping *in vitro* (10). HeLa cells were transfected with each construct as described in Methods. Total RNA was isolated 48 hrs post-transfection and analyzed for splicing pattern by RT-PCR. For the pCTG-6 construct, CT-specific splicing was the major processing pathway (recognition of exon 4) and only minor CGRP-specific splicing was observed (exclusion of exon 4) (Figure 2, lanes 1 and 2). The introduction of CALC II sequence into exon 4 (pCTG-7) or the deletion of the 3· splice site sequences (pCTG-9) resulted in a complete shift in splicing phenotype. Exon 4 sequence was consistently excluded from the final mRNA resulting in CGRP-specific 'skip' splice (pCTG-7 lanes 3 and 4, pCTG-9 lanes 7 and 8). Mutations targeted specifically to the nucleotide repeat resulted in a mixed pattern of splicing with less efficient inclusion of exon 4 than observed for pCTG-6 (lanes 3 and 4). Identity of all PCR products was confirmed by restriction enzyme analysis. Identical results were observed with alternative primer sets (data not shown).

Effect of exon 4 mutations on splicing *in vitro*

IVPX/CT-7 is a derivative of pCTG-6, in which deletions were introduced to facilitate *in vitro* processing. We have previously shown that an RNA precursor derived from this construct is inefficiently processed in a HeLa extract-based *in vitro* splicing assay, but shows CGRP-specific splicing upon addition of F9 teratocarcinoma cell nuclear extract (9). Inefficient splicing to exon 4 in HeLa extract is a consistent observation. CT-specific splicing is only observed when modifications that improve the 3′ splice site are made (e.g. substitution of an A for a U at the branch point)(9, 11). Therefore, although CT-specific splicing is not observed *in vitro*, extracts from HeLa cells faithfully reproduce the inhibition of CGRP-specific splicing observed in transfected HeLa cells.

To determine if we could extend our *in vivo* observations, mutations similar to those described in Figure 2 were introduced into the exon 4 sequences of IVPX/CT-7, our *in vitro* construct (Figure 3). *In vitro* splicing reactions used nuclear extracts prepared from the same HeLa cells used for transfection studies. The results obtained *in vitro* paralleled those observed *in vivo*. As expected, no CT-specific splicing was observed for IVPX/CT-7 precursor RNA within the 2 hour time course (Figure 3, left). Replacement of the CALC I exon 4 sequences with CALC II sequence (IVPX/CT-19) resulted in exclusion of exon 4 to produce CGRP (Figure 3, center). Mutations introduced into the repeat sequence (IVPX/CT-21) displayed a weaker skipping phenotype (Figure 3, right).

UV crosslinking of RNA-binding proteins which recognize C-ALC I exon 4 sequence

Both *in vivo* and *in vitro* results suggest that sequences within exon 4 are important for regulated recognition of this exon. UV crosslinking was performed to determine if we could identify RNA-binding proteins which interacted with the CT exon 4 sequence. To simplify analysis, short RNA probes (55 and 57 nucleotides) containing only exon 4 sequence were used (Figure 4A). UV crosslinking revealed the existence of both specific and nonspecific RNA-binding proteins. Several HeLa nuclear proteins demonstrated binding to all RNA probes tested (Figure 4B and data not shown). However, only the CALC I sequence was capable of crosslinking of a protein of ~66 kDa. RNA probes duplicating the CALC II or mutant exon 4 sequence did not crosslink the 66 kDa protein (Figure 4B, lanes 2 and 3). Therefore, crosslinking of the 66 kDa protein correlates with exon 4 recognition (CT-specific splicing) *in vivo* and *in vitro*.

To determine the specificity of this RNA/protein interaction, competition experiments were performed (Figure 4C). UV crosslinking was performed in the presence of increasing molar ratio of cold CALC I or CALC II RNA sequence. The addition of cold CALC I sequence (lanes 1−4), but not CALC II sequence (lanes 5−8) was able to compete UV crosslinking of the 66 kDa protein. Crosslinking of other proteins was not significantly decreased by either RNA sequence.

Regulated alternative processing of the CALC I gene transcript requires that exon 4 be excluded in cell types which produce CGRP. Transfected F9 mouse teratocarcinoma cells are capable

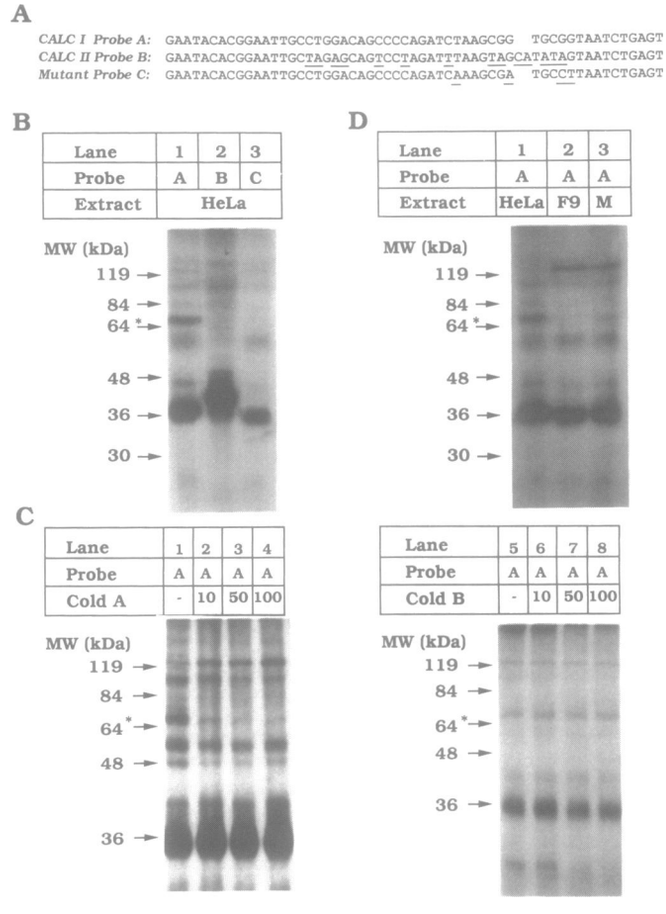

Figure 4. Identification of a 66 kDa RNA-binding protein which specifically recognizes CALC I exon 4 sequence. **(A)** Unlabeled (cold) or radiolabeled RNAs containing CALC I, CALC II, or mutated exon 4 sequence were *in vitro* transcribed and used as UV crosslinking probes as described in Methods. The *in vitro* transcribed RNA probes all utilize a common *Rsa I* site for template truncation and contain the identical 15 nucleotides of vector sequence 5′ to the start of exon 4 sequence. **(B)** UV crosslinking was performed in the presence of HeLa nuclear extract with the indicated RNA probe. **(C)** Competition experiments were performed as in (A) with the addition of the indicated molar excess of cold probe. **(D)** HeLa and F9 nuclear extracts were used alone or mixed (M, lane 3) prior to UV crosslinking. The asterisk indicates the position of 66 kDa exon binding protein. Molecular weight markers display relative protein size.

of CGRP-specific splicing (10) and nuclear extracts prepared from these cells perform CGRP-specific splicing *in vitro* (9). We did not observe significant crosslinking of the 66 kDa protein in nuclear extract prepared from F9 cells (Figure 4D, lane 2). Crosslinking experiments using a mixture of HeLa and F9 nuclear extracts also resulted in an absence of 66 kDa protein binding (Figure 4D, Lane 3). This absence of 66 kDa protein crosslinking correlates with the repression of exon 4 recognition observed in *in vitro* splicing experiments when F9 extract is mixed with HeLa extract (9). These experiments support a mechanism whereby a dominant F9 factor inhibits recognition of exon 4 by inhibiting binding of the 66 kDa polypeptide to sequences within exon 4.

DISCUSSION

Alternative RNA processing involves the decision to include or exclude specific exon sequence in the final processed RNA. *In vitro* experiments utilizing synthetic RNA substrates were the first to demonstrate a role for exon sequence in splice site selection (23). Increasing evidence suggests that exon sequences may play a role in regulating alternative RNA splicing choices. Several alternatively processed RNAs, including tropomysin (24), troponin T (25), leukocyte common antigen (26), and the *Drosophila doublesex* (12–14), rely on exon elements for proper regulation of RNA processing. Our results provide evidence to support such a role for exonic sequence within the calcitonin (C-ALC I) gene.

The results presented here identify exon 4 sequences that are required to maintain a CT-specific pattern of splicing both *in vivo* and *in vitro*. Substitution of the first 30 nucleotides of the CT exon 4 with analogous CALC II gene sequence caused a loss of exon 4 recognition. In the absence of exon 4 recognition the default pathway was CGRP-specific splicing. The significance of this observation is that as few as 14 nucleotide changes completely disrupted normal recognition of exon 4. The precise mechanism by which CT exon 4 regulatory element functions is not clear. There is the possibility that the CALC II sequence contains a specific element inhibitory to splicing exon 4, or that we have strengthened an element required for exon skipping. We do not believe this to be the case for two reasons. First, replacement of the CALC I sequence with sequences derived from a variety of sources always resulted in exon skipping (data not shown). Second, mutations targeted to the GCGGT nucleotide repeat allow exon 4 skipping without duplicating any of the C-ALC II sequence. We have not yet determined the significance of individual nucleotide differences. Additional analysis will be required to further define the nature of the entire region.

We believe the results presented in this paper support a model whereby exon 4 sequences act to facilitate 3′ splice site recognition. Several observations suggest that CT-specific splicing involves a synergistic recognition of multiple sequence elements. The 3′ splice site sequences preceding this exon deviate significantly from consensus. There is a relatively short polypyrimidine tract and the branch point sequence contains a uridine, instead of the usual adenosine at the branch site (9,11). As a result, splicing to the CT-specific 3′ splice site is not observed under standard *in vitro* splicing conditions (9,10). Mutation of the branch acceptor to the canonical adenosine results in enhanced CT-specific splicing *in vitro* (9,11) and *in vivo* (11). Thus, the natural 3′ intron sequences preceding exon 4 functions sub-optimally. We have also observed that complete replacement of the branch point/3′ splice site region with a strong constitutive sequence eliminates the ability to exclude exon 4, even in cells which normally exclude exon 4 (unpublished observation). Therefore, the natural 3′ splice site sequences are required in addition to exon 4 sequences to ensure appropriate tissue-specific recognition of this exon.

The organization of the CALC I gene is similar to that of the *Drosophila doublesex* gene (27). This 6 exon gene also alternatively utilizes exon 4, with exon inclusion accompanied by polyadenylation. Exon 4 inclusion in *doublesex* proceeds by a pathway that involves facilitated recognition of a weak 3′ splice site (12–14). This facilitated recognition requires association of the RNA binding protein, *transformer-2*, with exon 4 sequences. Mutation of the *transformer-2* binding sequences in exon 4 results in an inability to include exon 4 in the final mRNA.

We have previously proposed that, like *Drosophila doublesex*, inclusion of the CT exon 4 requires a specific RNA binding factor interacting with sequences in exon 4 to assist recognition of the preceding 3′ splice site (10). The 66 kDa protein that crosslinks to CALC I exon 4 sequences, but not to CALC II or mutated exon 4 sequences, provides a candidate for such an exon 4 binding protein. We suggest that this factor interacts with sequences within exon 4 to facilitate recognition of the CT-specific 3′ splice site and prevent skip splicing. It is not clear whether the 66 kDa protein is a generalized exon recognition protein, but it is unlikely to be specific for calcitonin production. HeLa cells do not endogenously express the calcitonin gene, and we have observed UV crosslinking of a 66 kDa protein to other RNA substrates containing similar sequences (unpublished results).

Recognition of exon 4 is suppressed by a dominant factor found in CGRP-producing cell types (7). Crosslinking of the 66 kDa protein to the exon 4 sequence was inhibited by the addition of nuclear extract from the F9 teratocarcinoma cell line, under conditions in which we observe exclusion of exon 4 in an *in vitro*, splicing assay (9). This observation suggests that CGRP production occurs when a F9 factor inhibits association of the facilitative factor (66 kDa exon 4 binding protein) with exon 4.

Emeson *et al.* have defined another regulatory region in the CT/CGRP gene located upstream of the intron 3 branch point sequence (8). Deletion of this region results in the constitutive inclusion of exon 4 in all cell types. Although the regulatory sequences we have identified are located within exon 4, they are less than 50 nucleotides from this other regulatory sequence. Therefore, it possible that the two regulatory elements are part of a larger 3′ splice site recognition sequence. What seems clear from this and earlier investigations is that the key regulatory choice in CT/CGRP splicing occurs at or adjacent to the 3′ splice site sequences of the differentially included exon.

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