REVIEW

Alternative RNA processing – its role in regulating expression of calcitonin/calcitonin gene-related peptide

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Abstract

The calcitonin/calcitonin gene-related peptide (CT/CGRP) gene is one of the earliest studied examples of alternative RNA processing. The regulatory mechanisms controlling this event are poorly understood. We have identified and characterized an intron element residing in intron 4 of the human CT/CGRP gene. This intron

element functions to enhance polyadenylation of an embedded alternative 3'-terminal exon within the CT/CGRP gene and is potentially involved in tissue-specific regulation of CT/CGRP RNA processing.

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In eukaryotic nuclei, every pre-messenger RNA (pre-mRNA) undergoes post-transcriptional processing to generate mature messenger RNA (mRNA), which is exported to the cytoplasm for translation. Post-transcriptional modification is a complex process, including capping at the 5' end, polyadenylation at the 3' end, and splicing or removal of intervening sequences (introns); splicing results in the precise ligation of coding-sequence-containing exons. Transcription and RNA processing are tightly coupled (Steinmetz 1997): there is compelling evidence that interaction of RNA processing factors with the newly transcribed RNA occurs during transcription (Daneholt 1997). Capping and polyadenylation occur concomitantly with transcription, but splicing occurs during or after transcription (Beyer & Osheim 1988).

Errors of splicing and polyadenylation can cause the production of non-functional proteins, and form the molecular basis of a large number of diseases, including endocrine diseases (Harteveld *et al.* 1994, Nakai & Sakamoto 1994). For example, in one case of hereditary vitamin D rickets, a G to C point mutation at the +5 position of the 5' donor splice site of intron 4 caused exon 4 skipping, which leads to the production of a truncated protein lacking the hormone-binding domain, through a frame-shift in translation (Hawa *et al.* 1996). In another example, in patients from several families in which familial persistent hyperinsulinemic hypoglycemia of infancy occurred, a mutation at the 3' acceptor splice site was identified to cause altered splicing with cryptic 3' splice

sites leading to a defective sulfonylurea receptor (Thomas *et al.* 1995). In a third example, several common mutations have been mapped to CYP21B in steroid 21-hydroxylase deficiency, the leading cause of impaired cortisol synthesis in congenital adrenal hyperplasia. One of the mutations, located in intron 2, causes abnormal splicing (Owerbach *et al.* 1990).

Splicing is mediated by several families of *trans*-acting factors that interact with specific RNA sequences (Moore *et al.* 1993). These include 5' and 3' (polypyrimidine-tract and branchpoint sequence) splice site sequences that define exon–intron boundaries (Fig. 1; Moore *et al.* 1993). Several new classes of exon and intron elements have now been identified that interact with the rapidly growing family of RNA binding proteins (Lou *et al.* 1995). Two prominent members of this family include small nuclear RNA (snRNA)-containing small nuclear ribonucleoprotein complexes (snRNP) and arginine–serine-rich RNA binding proteins (SR proteins). The former recognize 5' and 3' splice site signals; the latter interact with both intronic and exonic elements (Moore *et al.* 1993). A conceptual problem has been the identification of relatively short exons (<300 nucleotides) in the context of large introns. There is now compelling evidence that exons are defined by interaction of splicing factors with their cognate 3' and 5' splice sites. Communication between the factors *across* the exon results in recognition and permits precise joining of widely separated exons (Robberson *et al.* 1990).

Figure 1 Diagram showing the constitutive exon recognition of a generic internal exon and a generic terminal exon. The sequence elements involved in splicing and polyadenylation and the factors binding to these sequences are illustrated (Moore *et al.* 1993, Proudfoot 1996). Y, pyrimidine; R, purine, $n > 13$ for most of the mammalian introns.

Alternative RNA processing adds an additional level of complexity to this process. Alternative RNA processing is a ubiquitous process of fundamental importance for creating biological diversity. The inclusion or exclusion of an exon from a final mRNA can completely alter the biological behavior of a molecule, thus allowing modulation of protein functions (Leff *et al.* 1986). The differential expression of exons is often subject to developmental or tissue-specific regulations. An increasing number of genes have been shown to undergo alternative RNA processing. These include some of the important nuclear hormone receptors and ligands, for example growth hormone receptor (Dastot *et al.* 1996), luteinizing hormone receptor (Bacich *et al.* 1994), parathyroid hormone-related protein (Southby *et al.* 1996) and calcitonin/calcitonin gene-related peptide (CT/CGRP) (Amara *et al.* 1982, Rosenfeld *et al.* 1984).

Alternative processing of rat and human CT/CGRP transcript is one of the earliest studied examples of alternative RNA processing (Amara *et al.* 1982, Rosenfeld *et al.* 1984). Two tissue types in which this gene is transcribed, the thyroidal C cell and specific neural cell types, provide clearly defined examples of the two alternative processing pathways. The pre-mRNA of the CT/CGRP gene contains six exons and can be processed in two ways (Fig. 2A). The key regulatory event in the processing choice is inclusion or exclusion of the alternative 3'-terminal exon, exon 4. In thyroidal C cells, 95% of the CT/CGRP pre-mRNA is processed to include exon 4, with usage of the exon 4 polyadenylation site; this leads to production of calcitonin peptide. In contrast, in neuronal cells, 99% of the CT/CGRP pre-mRNA is processed to exclude exon 4, resulting in inclusion of exons 5 and 6, with usage of the exon 6 polyadenylation site; this leads to the production of CGRP peptide. Therefore, through

A. Alternative RNA processing of CT/CGRP gene

B. Core sequence of the intron enhancer element

AAACTCCGCTCCTCTTCAG GTAAGACTGA

Pyrimidine-tract sequence **5' splice site**

Figure 2 A. Schematic representation of alternative RNA processing of CT/CGRP. Cell models used for this study are indicated. The black oval represents the intron element. B. Sequences of the two splicing signals in the intron element.

alternative RNA processing, two peptide hormones having completely different structures and functions are generated. Interestingly, in malignant C cells, both calcitonin and CGRP are produced in equal amounts, resulting from changes in splicing pathways (Benett & Amara 1993).

To understand the underlying mechanism that controls the regulation of CT/CGRP alternative RNA processing, several research groups have developed cell model systems to mimic the tissue-specific RNA processing pathways (Benett & Amara 1993). Our group has chosen HeLa cells for calcitonin processing (exon 4 inclusion) and T98G, a human glioblastoma cell line, for CGRP processing (exon 4 exclusion) (Fig. 2A; Lou *et al.* 1994, 1995). Because the fate of exon 4 is a key issue for this processing choice, that exon became the initial focus of these studies.

Figure 3 Interactions of splicing factors with the CT/CGRP intron element. Potential interactions between factors are indicated by thick arrows.

Exon 4 has weak RNA processing signals, in common with other examples of alternatively utilized exons. The first weak signal is the 3' splice site, which includes a non-canonical branchpoint nucleotide uridine in human or cytidine in rat, instead of the canonical branchpoint adenosine (Adema *et al.* 1988, Benett & Amara 1993) and a short, purine-interrupted polypyrimidine-tract (see Fig. 1 for consensus sequences). The second weak signal is the polyadenylation signal, including a suboptimal G/U sequence downstream of the polyadenylation cleavage site, that is required for binding of the 64 kDa CstF polyadenylation factor (Fig. 1). Terminal exons, for example exons 4 and 6 of the CT/CGRP gene, form a unique subset of exons that are defined by the 3' splice site and polyadenylation signals (Niwa *et al.* 1992). The weak signals flanking exon 4 are likely to be an important component of the alternative utilization of this exon. For example, substitution of the non-canonical branchpoint nucleotide with the consensus branchpoint results in the constitutive inclusion of exon 4 (Adema *et al.* 1988, Benett & Amara 1993, Cote *et al.* 1991). The concept is evolving in the field of alternative RNA processing that normal RNA processing factors do not recognize these weak sequences well, and additional factors are required to recognize weak exons. We hypothesized that these factors bind to exon 4 or flanking intronic sequences to enhance recognition of this exon. That these factors are ubiquitous is suggested by experiments with transgenic mice expressing rat CT/CGRP gene, which showed exon 4 inclusion in most tissues, and exclusion only in neuronal and cardiac tissues (Crenshaw *et al.* 1987).

Support for our hypothesis is provided by the identification of several sequence elements, including three exon 4 sequence motifs, a sequence upstream of the exon 4, and a sequence downstream of exon 4 (Emeson *et al.* 1989, Cote *et al.* 1992, Roesser *et al.* 1993, van Oers *et al.* 1994, Yeakley *et al.* 1993). We have focused on a sequence element located downstream of exon 4, because it has several interesting features. First, the initial 276-nucleotide intron 4 sequence, extending from the AATAAA hexonucleotide of exon 4, is 63% identical in human, mouse

and rat species (Lou *et al.* 1995). This is unusual for intron sequences, and suggests that it has important functions, as intron sequences are not normally conserved. Secondly, buried in the middle of intron 4, there are two splicing signals: a perfect 5' splice site sequence and a pyrimidine-tract sequence that immediately precedes the 5' splice site sequence (Fig. 2B; Lou *et al.* 1995). These two sequences are out of context; they are normally found at the 5' and 3' ends of an intron (Fig. 1). Particularly intriguing is the presence of a 5' splice site sequence downstream of a polyadenylation signal, because 5' splice site sequences in last exons have been shown to affect polyadenylation both positively and negatively (Furth *et al.* 1994, Niwa *et al.* 1992, Wassarman & Steitz 1993). We hypothesized that a good 5' splice site sequence located downstream of exon 4 could facilitate exon 4 polyadenylation.

We established the importance of the two intronic splicing signals by extensive mutational analyses (Lou *et al.* 1995). Deletion or mutation of the 5' splice site sequence or the pyrimidine-tract sequence dramatically decreased recognition of exon 4 when the mutant construct was transfected into a cell type (HeLa) that normally includes exon 4 (Lou *et al.* 1995). This effect is caused, at least in part, by regulation of polyadenylation; the presence of intact 5' splice site and pyrimidine-tract sequences was necessary for polyadenylation. Mutation of these sequences resulted in decreased polyadenylation in an *in vitro* polyadenylation cleavage assay, utilizing a truncated exon 4 polyadenylation site and HeLa cell nuclear extract (Lou *et al.* 1996). This is the first example of regulation of polyadenylation by an intron element. As the 5' splice site and pyrimidine-tract sequences are general splicing signals, it is conceivable that splicing factors bind to these sequences and interact with polyadenylation factors. The 5' splice site is usually recognized by U1 snRNP through base pairing and can also bind other splicing factors, including members of the SR family of proteins (Kohtz *et al.* 1994). We demonstrated that U1 snRNA binds to the 5' splice site sequence in the calcitonin intron element, by use of RNase H protection (Fig. 3, Lou *et al.* 1996) and the psoralen cross-linking assay (a method for detecting

RNA–RNA interactions; H Lou, unpublished observation). The functional relevance of the base pairing between U1 snRNA and the intronic 5' splice site was shown by demonstrating the abolition of exon 4 *in vitro* polyadenylation cleavage activity in the absence of U1 snRNA (Lou *et al.* 1996).

The 5' splice site sequence in the calcitonin intron element also binds other splicing factors, analogous to a true 5' splice site. One of the factors involved is alternative splicing factor (ASF/SF2) (Fig. 3). The interaction of splicing factors with this element raised a question: why is this 5' splice site sequence not utilized as a 5' splice site? A clue is provided by the binding of polypyrimidine-tract binding protein (PTB) to the pyrimidine-tract sequence preceding the 5' splice site sequence (Lou *et al.* 1996; Fig. 3). PTB is generally considered to be a negative splicing factor. It has been shown to bind to the pyrimidine-tract at the 3' splice site and compete with the essential splicing factor U2 snRNP auxiliary factor (U2AF) – an interaction that is required for splicing (Zamore & Green 1991). PTB has also been implicated in the tissue-specific repression of splicing for several genes (Lin & Patton 1995, Chan & Black 1997). It is therefore possible that PTB prevents U1 snRNA from interacting with other splicing factors, thereby preventing the potential 5' splice site in the enhancer from being used as a true 5' splice site. The U1 snRNA bound at the 5' splice site sequence would then be available to interact with non-splicing components, for example polyadenylation factors, resulting in enhanced exon 4 polyadenylation. We are currently investigating the mechanism by which splicing components assembled on the calcitonin intron element interact with polyadenylation factors.

If this intron element has a role in the cell-specific regulation of CT/CGRP RNA processing, how is the regulation achieved? One simple, but not applicable, explanation might be the presence of different amounts of the factors that interact with the intron element in cells that include or exclude exon 4; however, western blot analysis showed similar levels of PTB or ASF/SF2 in HeLa and T98G cells (unpublished observation). There are several other possible explanations. First, PTB or ASF/SF2 could be modified differently in the two cell types, for example by differential phosphorylation. Secondly, additional unidentified proteins may be involved in the regulation. A third possibility, which we favor, is the presence of a factor that interferes with the interaction between U1 snRNA, ASF/SF2, PTB or other positive-acting factors and the intron element in cells that exclude exon 4. We and others are searching for such a factor. A few neuron-specific RNA binding proteins have been isolated (Koushika *et al.* 1996). It would not be surprising if one of these neuron-specific proteins were shown to be involved in the regulation of CT/CGRP RNA processing.

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