Both U2 snRNA and U12 snRNA are required for accurate splicing of exon 5 of the rat *calcitonin/CGRP* gene

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ABSTRACT

Two classes of spliceosome are present in eukaryotic cells. Most introns in nuclear pre-mRNAs are removed by a spliceosome that requires U1, U2, U4, U5, and U6 small nuclear ribonucleoprotein particles (snRNPs). A minor class of introns are removed by a spliceosome containing U11, U12, U5, U4atac, and U6 atac snRNPs. We describe experiments that demonstrate that splicing of exon 5 of the rat *calcitonin/CGRP* gene requires both U2 snRNA and U12 snRNA. In vitro, splicing to *calcitonin/CGRP* exon 5 RNA was dependent on U2 snRNA, as preincubation of nuclear extract with an oligonucleotide complementary to U2 snRNA abolished exon 5 splicing. Addition of an oligonucleotide complementary to U12 snRNA increased splicing at a cryptic splice site in exon 5 from <5% to 50% of total spliced RNA. Point mutations in a candidate U12 branch sequence in *calcitonin/CGRP* intron 4, predicted to decrease U12-pre-mRNA base-pairing, also significantly increased cryptic splicing in vitro. *Calcitonin/CGRP* genes containing base changes disrupting the U12 branch sequence expressed significantly decreased *CGRP* mRNA levels when expressed in cultured cells. Coexpression of U12 snRNAs containing base changes predicted to restore U12-pre-mRNA base pairing increased *CGRP* mRNA synthesis to the level of the wild-type gene. These observations indicate that accurate, efficient splicing of *calcitonin/CGRP* exon 5 is dependent upon both U2 and U12 snRNAs.

Keywords: RNA; alternative splicing; U12 snRNA; *calcitonin*

INTRODUCTION

Alternative mRNA splicing plays an important role in the regulation of eukaryotic gene expression, allowing the production of functionally distinct protein products from a single gene. A large, and continually increasing number of genes have now been shown to be subject to differential mRNA splicing, and it is estimated that >35% of human genes undergo differential mRNA splicing (Sorek and Amatai 2001; Modrek and Lee 2002). In higher eukaryotes, alternative splicing has been shown to modulate gene expression in a developmental, sex-specific, and tissue-specific manner.

Two classes of introns are found in nuclear pre-mRNA. Splicing of the major, or U2-dependent type of intron requires U1, U2, U4, U5, and U6 snRNPs and other protein factors, whereas removal of the rare U12-dependent introns is carried out by spliceosomes containing U11, U12, U5, U4atac, and U6atac (Tarn and Steitz 1996a,b). All U12-dependent introns discovered thus far are found in pre-mRNAs that also contain several U2-dependent introns (Dietrich et al. 2001a). This suggests that recognition of U2-dependent and U12-dependent introns, in pre-mRNAs containing both classes of intron, requires cooperation between components of the major and minor type spliceosome.

The removal of U12-dependent introns is similar in many ways to the removal of U2-dependent introns. Splicing of both types of intron is carried out by two sequential nucleophilic substitution reactions (Tarn and Steitz 1996a,b). U5 snRNP is common to both types of spliceosome and U1, U2, U4, and U6 snRNPs all have functional homologs in U12-dependent splicing. In addition, U1 and U11, as well as U2 and U12 snRNPs contain several proteins in common (Will et al. 1999). One difference between U2- and U12-dependent splicing is that unlike U2-dependent introns, no U12-dependent intron has been shown to be spliced in an alternative fashion (Dietrich et al. 2001a). The potential for U12-dependent introns to undergo alternative mRNA splicing is suggested by the observation that purine-rich splice enhancers promote splicing of U12-dependent

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Abbreviations: snRNP, small nuclear ribonucleoprotein particle; snRNA, small nuclear RNA; *CGRP, calcitonin* gene-related peptide; pre-mRNA, pre-messenger RNA; PCR, polymerase chain reaction.

Article and publication are at http://www.rnajournal.org/cgi/doi/10.1261/rna.5210404.

introns (Wu and Krainer 1998; Hastings and Krainer 2001) and artificial genes containing U12-dependent introns can be alternatively spliced in vivo (Dietrich et al. 2001a).

The mammalian *calcitonin/CGRP* gene has six exons and is alternatively processed in a tissue-specific fashion (Fig. 1). Splicing together the first four exons generates *calcitonin* mRNA, which encodes the serum calcium-regulating hormone calcitonin. In thyroid C cells, >98% of the mature mRNA derived from the *calcitonin/CGRP* gene encodes calcitonin (Sabate et al. 1985). In neurons, 99% of the transcripts from the *calcitonin/CGRP* gene are processed into an mRNA containing exons 1–3, 5, and 6. This mRNA is translated into calcitonin gene-related peptide (*CGRP*), a neuropeptide (Amara et al. 1982). Processing of the *calcitonin/CGRP* pre-mRNA has been shown to be regulated at the level of both polyadenylation and splicing (Leff et al. 1987; Roesser et al. 1993; van Oers et al. 1994; Lou et al. 1995, 1998, 1999).

Here, we demonstrate that the *CGRP*-specific exon 5 of the rat *calcitonin/CGRP* gene requires both U2 snRNA and U12 snRNA for accurate inclusion into mRNA. In vitro, spliceosome recognition of exon 5 requires U2 snRNA, and accurate splicing of exon 5 is decreased greatly in the absence of free U12 snRNA. In vivo, base changes at a potential U12 interaction site in intron 4 (*calcitonin/CGRP BPSM* or *calcitonin/CGRPmut5*) greatly decreased *CGRP* mRNA production without increasing *calcitonin* mRNA levels. Coexpression of U12 snRNAs with compensatory base changes predicted to restore base pairing with *calcitonin/CGRP* BPSM pre-mRNA or *calcitonin/CGRP* mut 5 pre-mRNA increased *CGRP* mRNA production to wild-type levels.

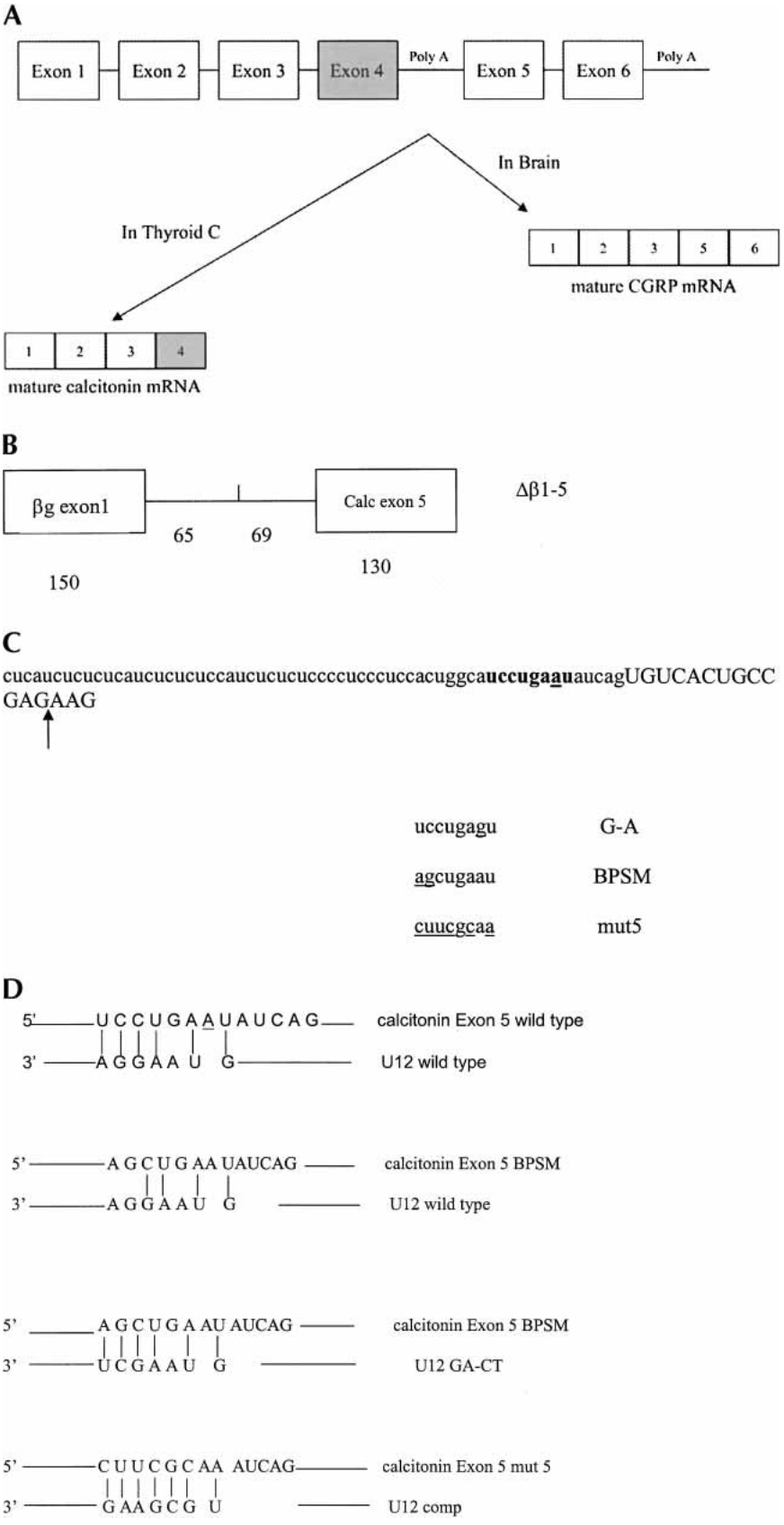

FIGURE 1. (Legend on next page)

RESULTS

Splicing of *calcitonin* exon 5 in vitro

We are interested in examining the mechanisms that control tissue-specific alternative RNA processing of the mam-

malian *calcitonin/CGRP* gene (Fig. 1A). Several elements have been identified in and around exon 4 that control exon 4 inclusion into mRNA and use of the calcitonin-specific polyadenylation site in intron 4 (Leff et al. 1987; Adema et al. 1988, 1990; Roesser et al. 1993; Yeakley et al. 1993; van Oers et al. 1994; Lou et al. 1995, 1998, 1999; Zandberg et al. 1995; Coleman and Roesser 1998). To determine whether sequences in or around exon 5 also play a role in the regulation of *calcitonin/CGRP* mRNA processing, RNA from minigenes containing exon 5 were tested in in vitro splicing reactions. Pre-RNA synthesized from constructs containing *calcitonin* exon 3 fused to *calcitonin* intron 4 and exon 5; or β *globin* exon 1 fused to the last 170 nucleotides of *calcitonin* intron 4 and exon 5 were spliced very inefficiently in vitro (data not shown). Pre-mRNA from a hybrid minigene containing human β *globin* exon 1 and 65 nt of intron 1 fused to the last 62 nt of the rat *calcitonin/CGRP* intron 4 and the first 130 nt of exon 5 (Δβ1-5, Fig. 1B) was spliced efficiently in HeLa nuclear extract (Fig. 2). Transcripts synthesized from the Δβ1-5 minigene in vitro were thus used in further experiments as a splicing substrate for study of exon 5 inclusion. The putative Δβ1-5 splice product was amplified by reverse transcription and the PCR. DNA sequence analysis of the amplified fragment demonstrated that the RNA was accurately spliced (data not shown).

No strong match to the U2-dependent branchpoint consensus sequence is apparent upstream of *calcitonin/CGRP* exon 5 (Fig. 1C). There is a sequence 6–13 nt 5′ of exon 5 that is a good match to the U12-dependent branchpoint sequence, allowing six of seven base-pairing interactions between the putative branch sequence and U12 snRNA (Fig. 1D). To test whether Δβ1-5 splicing is U2 or U12 dependent, 2′O-methyl oligonucleotides complementary to either U2 or U12 snRNAs (Tarn and Steitz 1996a, 1994), were added to in vitro splicing reactions containing either Δβ1-5 RNA, or β1-2 RNA. β1-2 RNA, which has the first exon, intron, and second exon of the human β *globin* gene, was used as a splice control. Pretreatment of nuclear extract with 2′O-methyl oligonucleotide U2b (2 μM), which is complementary to nt 27–49 of U2 snRNA, abolished both β1-2 and Δβ1-5 splicing (Fig. 2, lanes 3,6). Preincubation of splicing extracts with a 2′O-methyl oligonucleotide complementary to nt 11–28 of U12 snRNA had no effect on β1-2 splicing, but significantly increased cryptic splicing of

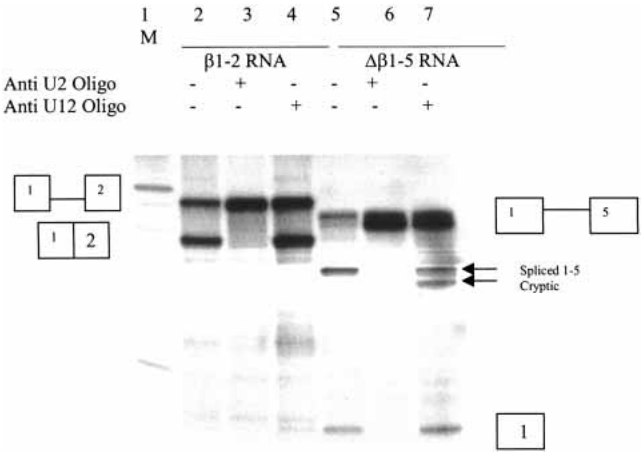

FIGURE 2. U2 and U12 dependence of β1-2 and Δβ1-5 RNA splicing. In vitro splicing reactions containing radiolabeled β1-2 (lanes *2–4*) or Δβ1-5 RNA (lanes *5–7*). Splicing reactions were carried out for 1.5 h in 50% Hela nuclear extract, and splice products were separated on 8% polyacrylamide gels containing 8 M urea, and splice products were visualized by autoradiography. Reactions in lanes *3* and *6* contained 2 μM Anti-U2 2′O-methyl oligonucleotide and 2 μM anti-U12 2′O-methyl oligonucleotide was added to lanes *4* and *7*. Oligonucleotides were added to HeLa nuclear extract and preincubated under splicng conditions for 20 min prior to addition of splicing substrates. A 100-nt DNA ladder (New England Biolabs) was 5′ radiolabeled and used as a size marker in lane *1*. The prominent band is 500 nucleotides in length.

Δβ1-5 (Fig. 2, lane 4 vs. 7). The cryptic Δβ1-5 splice product, which is barely detectable in the absence of the $U12_{11–28}$ oligonucleotide, results from usage of an AG 13 nt downstream of the authentic start of exon 5 (Fig. 1C). The cryptically spliced RNA molecule was identified both by RT–PCR, followed by DNA sequence analysis and by RNAse protection assay (data not shown). These observations indicate that splicing of both β1-2 and Δβ1-5 RNAs requires U2 snRNA and suggest that U12 snRNA affects the accuracy of Δβ1-5, but not β1-2 splicing.

To further examine the possibility that U12 snRNP is involved in calcitonin/CGRP exon 5 splicing, two mutations in the potential U12 branchpoint sequence of Δβ1-5 were constructed. One, Δβ1-5 A-G, changed the potential U12-dependent branchpoint from an A to a G. The second, ΔB1-5 BPSM, changed the branchpoint sequence from UCCUGAAU to AGCUGAAU (Fig. 1C,D). Mutation of the U12 branchpoint consensus sequence at these positions has been demonstrated to disrupt U12-dependent splicing in intron F of the human *P120* gene (Hall and Padgett 1996) and is predicted to have no effect on possible U2 snRNA interaction with the site. RNAs transcribed from the altered Δβ1-5 minigenes were used as splice substrates in HeLa nuclear extract. Changing the potential branchpoint from an A to G

FIGURE 1. The rat *calcitonin/CGRP* gene. (*A*) Structure and alternative RNA processing of the rat *calcitonin/CGRP* gene, exon sequences are denoted by boxes. (*B*) The Δβ1-5 minigene structure showing the sizes in nucleotides of the exon and intron segments. (*C*) Sequence of the rat *calcitonin/CGRP* gene at the exon 5 splice acceptor. Intronic sequence is in lowercase, and exon 5 sequence is in capitals. A candidate U12 branch sequence is in bold, and the position of cryptic splicing is indicated by the arrow. The sequence of the A-G, BPSM and mut5 base changes in the putative U12 branch sequence are also shown, with base changes underlined. (*D*) Predicted interactions between the candidate branch sequence upstream of exon 5 and U12 snRNA. The first alignment is between wild-type *calcitonin/CGRP* and wild-type U12, the second contains the *calcitonin/CGRP BPSM* base changes, the third *calcitonin/CGRP BPSM* with *U12* containing compensatory base changes, and the fourth potential base-pairing between *calcitonin/CGRP mut5* and *U12comp*.

abolished splicing of Δβ1-5 A-G RNA (Fig. 3, lane 4). Although no spliced RNA was detected, free exon 1 accumulated, suggesting that the first step of splicing was not affected, but that the second splicing step was inhibited. The UC-AG base changes led to significantly increased cryptic splicing of Δβ1-5 BPSM RNA (Fig. 3, lane 5). Splicing of Δβ1-5 BPSM RNA closely resembled Δβ1-5 RNA splicing in the presence of the 2′O-methyl oligonucleotide complementary to U12 snRNA (cf. Fig. 3, lane 5 and Fig. 2, lane 7). These observations suggest that U12 snRNA base-pairing with this potential branchpoint sequence is neccessary for accurate splicing to exon 5.

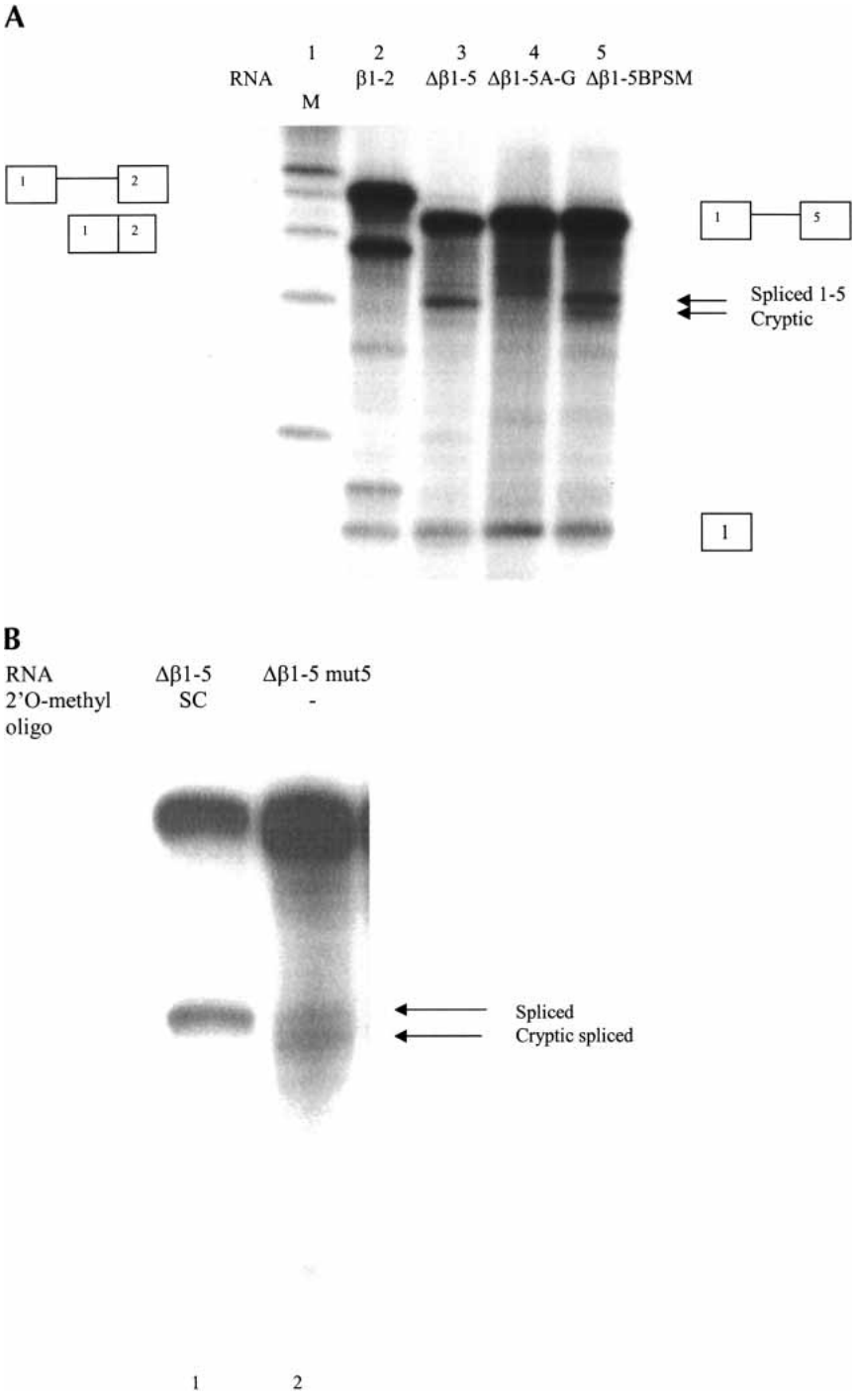

FIGURE 3. Base changes in a potential U12-dependent branchpoint affect *calcitonin/CGRP* exon 5 splicing. (*A*) Radiolabeled RNA splice substrates synthesized in vitro from β1-2, Δβ1-5, Δβ1-5 A-G, and Δβ1-5 BPSM minigenes were spliced in HeLa nuclear extract (see Fig. 1C for sequence of mutations). Splicing was carried out as in Figure 2. Lane *1* contains a radiolabeled 100-nt DNA ladder (New England Biolabs) as a size marker. (*B*) Radiolabeled Δβ1-5 RNA containing 2 μM SC oligonucleotide (lane *1*) and Δβ1-5 mut5 (lane *2*) RNA were spliced in Hela nuclear extract. Splicing was assayed as in Figure 2.

Both the A-G and BPSM base changes introduce an AG dinucleotide and disrupt a potential polypyrimidine tract in intron 4. It is possible that placing another AG into the vicinity of the splice junction or interrupting the polypyrimidine sequence is responsible for the changes in splicing that were observed. To examine this possibility, a Δβ1-5 minigene was constructed containing six base changes in the putative U12 branchpoint predicted to abolish U12 base pairing, but not disrupt the polypyrimidine tract or introduce an AG (Δβ1-5mut5, Fig. 1C). Splicing of Δβ1-5mut5 RNA in HeLa nuclear extract was very similar to splicing of Δβ1-5BPSM. Nearly equal amounts of spliced 1-5 and cryptic splice product were produced (Fig. 3B). Preincubation of extract with a 2′O-methyl oligonucleotide with a random sequence (oligonucleotide SC), had no effect on Δβ1-5 splicing (Fig. 3B, lane 1), suggesting that increased cryptic splicing is not due to a nonspecific effect of 2′O-methyl oligonucleotide addition.

Splicing of *calcitonin* exon 5 in vivo

To test the possible involvement of U12 snRNA on exon 5 splicing in vivo, full-length *calcitonin/CGRP* genes containing the *BPSM, A-G*, and *mut5* changes were constructed and transiently transfected into either 293 cells or mouse F9 cells. Expression of the *calcitonin/CGRP* gene in 293 cells leads to the production of 50% *calcitonin* mRNA and 50% *CGRP* mRNA, whereas F9 cells produce almost exclusively *CGRP* mRNA (Roesser et al. 1993; Coleman and Roesser 1998). Whole-cell RNA was isolated from transfected cells, and RNA

was reverse transcribed into cDNA using random hexamers as primers. *Calcitonin/CGRP* splicing was assayed by competitive PCR, using a 5′ primer complementary to *calcitonin/CGRP* exon 3 and two 3′ primers, one complementary to exon 4 and one to exon 5 (Lou et al. 1995; Coleman and Roesser 1998; Tran and Roesser 2003). Significant amounts of both *CGRP* mRNA, *calcitonin* mRNA, and partially or aberrantly processed RNAs were produced from the wild-type rat *calcitonin/CGRP* gene in 293 cells (Fig. 4). The identity of the *calcitonin* and *CGRP* mRNAs was confirmed by RT–PCR of gel-excised RNAs and direct sequencing of the PCR products (data not shown). In F9 cells, *CGRP* mRNA was the predominant product, with small amounts of unprocessed RNA containing intron 3 (Fig. 4, band ~930 nt in length).

Expression of *calcitonin/CGRP* A-G led to greatly diminished levels of *CGRP* mRNA in both 293 cells or F9 cells, as compared with the wild-type gene, whereas the amount of *calcitonin* mRNA did not change (data not shown). *Calcitonin/CGRP* BPSM expression also led to a decreased CGRP:calcitonin mRNA ratio, as compared with the *CGRP:calcitonin* mRNA ratio produced from the wild-type calcitonin/CGRP gene (Fig. 4, lanes 2,6 vs. lanes 3,7). Cotransfection of the wild-type *U12 snRNA* gene, under control of the U1 promoter, with *calcitonin*/CGRP *BPSM*, increased the *CGRP:calcitonin* mRNA ratio by ~10% (Fig. 4, lanes 4,8). Coexpression of a *U12 snRNA* gene with a GA-CT change at positions 24/25 of U12, which is predicted to restore base pairing between U12 snRNA and the altered branchpoint sequence of *BPSM* (Fig. 1D), restored the *CGRP:calcitonin* mRNA ratio to 75% of that of the wild-type gene (Fig. 4, lanes 5,9).

Expression of *calcitonin/CGRP mut5* also produced very low levels of *CGRP* mRNA in both 293 and F9 cells (Fig. 5, lanes 2,7). Coexpression of wild-type U12 RNA increased the *CGRP:calcitonin* mRNA ratio by 10%, as compared with the wild-type *calcitonin/CGRP* gene (Fig. 5, lanes 3,8 vs. lanes 2,7). Coexpression of *U12comp*, which is predicted to be capable of base-pairing with *calcitonin/CGRP mut5* pre-mRNA, fully rescued *CGRP* mRNA production.

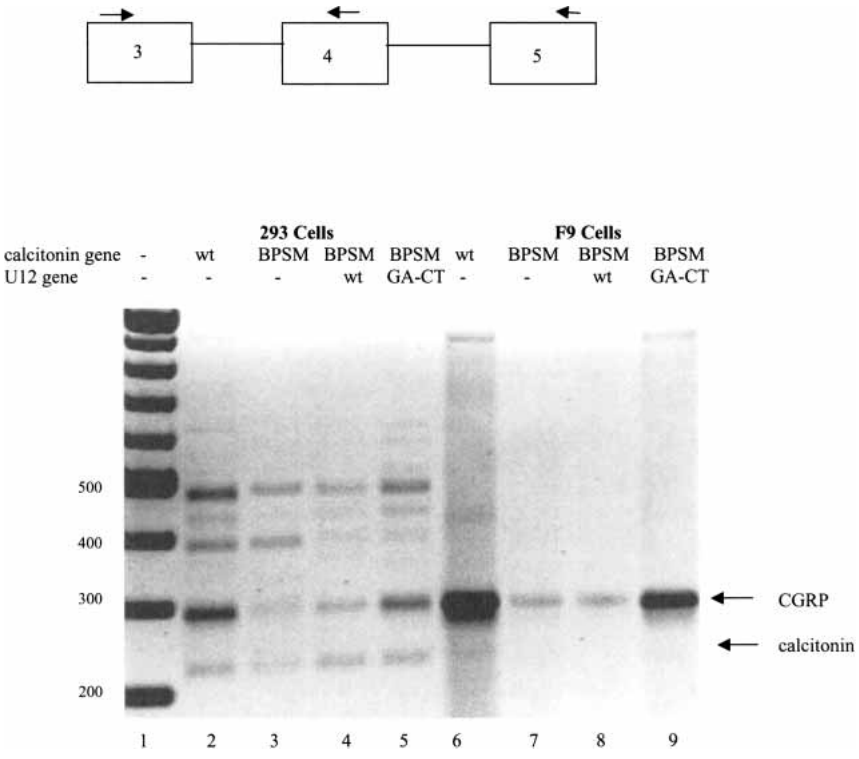

FIGURE 4. Coexpression of U12GA-CT restores *CGRP* mRNA production in cells expressing the *calcitonin/CGRP BPSM* gene. A total of 2 μg of the mammalian expression vector SRα containing the indicated *calcitonin/CGRP* gene was transfected into 293 cells (lanes *2–5*) or F9 cells (lanes *6–9*). RNA was harvested 48 h post-transfection. Wild-type (WT) *calcitonin/CGRP* (lane *2* or *6*) or *calcitonin/CGRP BPSM* (lanes *3–5,7–9*) were cotransfected with 1μg of wild-type U12 snRNA gene (lanes *4,8*) or *U12* snRNA *GA-CT*, which contains compensatory base changes (lanes *5,9*). RT–PCR analysis of the splice products was performed using primers complementary to exons 3, 4, and 5. PCR fragments were separated on 1.5% agarose gels and visualized with ethidium bromide staining under UV light. Negative image is shown. A 100-nucleotide ladder (New England Biolabs) was used as a size marker in lane *1*. Sizes are indicated in base pairs.

DISCUSSION

Tissue-specific processing of the *calcitonin/CGRP* pre-mRNA has been shown to be regulated at the level of both polyadenylation and splicing. Several sites in and around *calcitonin/CGRP* exon 4 are involved in control of *calcitonin/CGRP* pre-mRNA processing (Leff et al. 1987; Roesser et al. 1993; van Oers et al. 1994; Lou et al. 1995, 1998, 1999). Much less is known about sequences in and around exon 5 that could potentially regulate *calcitonin/CGRP* pre-mRNA processing. In vitro splicing systems using pre-mRNA substrates containing an upstream exon along with all or parts of *calcitonin/CGRP* exons 4 and 5, demonstrated that splicing to exon 5 was heavily favored over splicing to exon 4 in nuclear extracts made from either *calcitonin* or CGRP-producing cells (Bovenberg et al. 1988, 1989). Changing the noncanonical branchpoint upstream of exon 4 to the preferred A allowed usage of the exon 4 splice acceptor in nuclear extract from *calcitonin*-producing cells, but not in nuclear extract from CGRP-producing cells (Cote et al. 1991). Deletion of the exon 5 splice acceptor was shown to abolish CGRP splicing in CGRP-producing cells, but did not increase the level of *calcitonin* splicing (Yeakley et al. 1993).

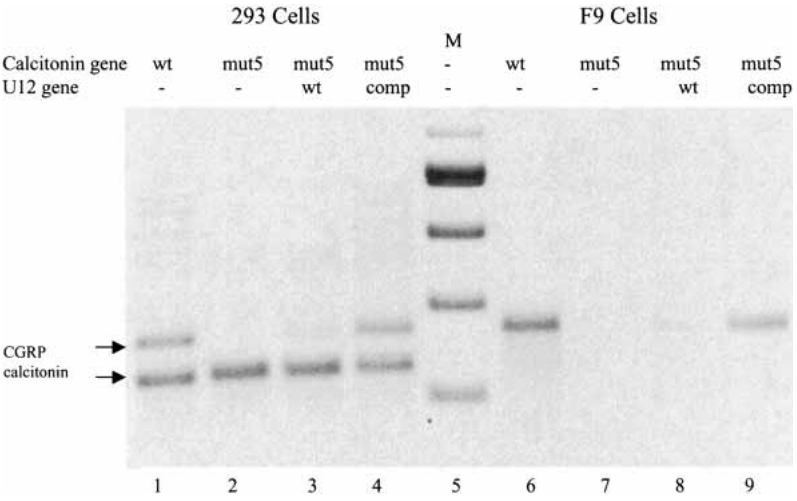

FIGURE 5. Coexpression of *U12comp* restores *CGRP* expression from the *calcitonin/CGRP-mut5* gene. RNA from cells transfected with 2 μg of wild-type (lanes *2*,*6*) or *mut5 calcitonin/CGRP* genes (lanes *2–4*,*7–9*) were analyzed by reverse transcriptase and PCR using primers complementary to calcitonin exons 3, 4, and 5 and visualized as in Figure 4. Cells were cotransfected with 1 μg of wild-type U12 (lanes *3*,*8*) or U12comp (lanes *4*,*9*). DNA size markers are in lane *5*.

To study the requirements for *calcitonin/CGRP* exon 5 usage, we constructed a hybrid minigene containing exon 1 of the human β *globin* gene and rat *calcitonin/CGRP* exon 5. RNA transcribed from Δβ1-5 was efficiently and accurately spliced in HeLa nuclear extract, although HeLa cells make predominantly calcitonin mRNA when expressing the calcitonin/CGRP gene (Roesser et al. 1993). We were unable to directly determine the site of branchpoint formation in the Δβ1-5 pre-mRNA. No RNA products were observed during splicing time courses that showed the proper kinetics or gel mobility to be potential lariat intermediates (Figs. 2, 3; data not shown). Branchpoint formation during splicing to human calcitonin/CGRP exon 5 was demonstrated to utilize an A residue 36 nt upstream of the exon 5 start at the sequence UACUCAC (Bovenberg et al. 1988). However, no candidate U2 branchpoint sequence occurs at this position in the rat intron 4 (Fig. 1C). The last 20 nt of rat intron 4 contain no obvious branchpoint sequence candidates and no pyrimidine tracts longer than 4 nt. From 21 to 164 nt upstream of rat exon 5, there are only five A residues and no Gs in the RNA. Upstream of this potential elongated polypyrimidine tract are several candidate U2 branchpoints.

Intron 4 of the rat *calcitonin/CGRP* gene does contain a potential U12-dependent branchpoint sequence UCC UGAAU, with the putative branchpoint separated from the beginning of exon 5 by 7 nt (Fig. 1C). It was previously observed that functional U12 branchpoints are found no closer than 10 nt from the 3′ splice acceptor (Dietrich et al. 2001b). Our observation that changing the A at position −7 (relative to exon 5) to a G abolishes splicing to exon 5 both in HeLa nuclear extract and 293 cells suggests that this may be the branchpoint utilized. A more likely explanation, however, is that this sequence binds U12 snRNA and acts as part of a complex to direct accurate splicing of exon 5. The U2-dependent branchpoint used may be located elsewhere.

The mechanism by which U12 snRNA might direct accurate exon 5 splicing is not clear. The β *globin* exon 1 splice donor is U1 dependent, and the sequence of the calcitonin exon 3 splice donor (AG/GUAAGG) suggests that it also is recognized by U1 (U1 donor consensus AG/GURAGU) and not U12 (consensus U12 donor/AUAUCCUU). This would seem to preclude recognition of the donor-acceptor pair by a U11-U12 di-snRNP. Glycerol gradient centrifugation suggests, however, that a fraction of U12 snRNPs are not associated with U11 snRNP (Montzka and Steitz 1988). It is possible that monomeric U12 snRNP or U12 in larger complexes not containing U11 is involved in *calitonin/CGRP* exon 5 splice recognition. In addition, it has been demonstrated that U12-dependent introns are removed more slowly than U2-dependent introns, and it has been proposed that U12-dependent introns may thus be targets for post-transcriptional regulation (Patel et al. 2002). Exon 5 dependence upon U12 may thus slow recognition of exon 5 by the splicing apparatus, allowing cell-specific regulatory mechanisms to compete. A similar situation is observed in *calcitonin/CGRP* exon 4, where the exon 4 branchpoint is a noncanonical C (in the rat gene) or U (in humans). Changing the weakly recognized branchpoint to A uncouples *calcitonin/CGRP* splicing from cell-specific regulation, allowing *calcitonin* mRNA production in all cell types (Adema et al. 1988).

Two observations suggest that U12 snRNA is involved in exon 5 splicing during the second catalytic step of splicing. First, approximately equal amounts of free exon 1, a product of the first catalytic step of splicing, were released from Δβ1-5 RNA in HeLa nuclear extract with or without pretreatment with the anti-U12 oligonucleotide (Fig. 2, lane 5 vs. 7). Second, Δβ1-5 A-G RNA, in which the U12 interaction site is disrupted, undergoes the first catalytic step of splicing in vitro, but not the second step to produce spliced RNA (Fig. 3, lane 4). These observations reinforce the idea that U12 is not acting in branchpoint selection during recognition of the splice acceptor at exon 5, but may function as a splice regulator at a later step.

Wherever the intron 4 branchpoint is located, there is strong evidence that U12 interacts with the UCCUGAAU sequence upstream of exon 5, and that this interaction is important for *CGRP* splicing. Pretreatment of nuclear extract with a 2′O-methyl oligonucleotide complementary to

U12 snRNA, shown previously to disrupt U12-dependent splicing (Tarn and Steitz 1996a) or disrupting potential base-pairing interactions between U12 and the pre-mRNA by changing the potential branch point sequence, greatly increases the level of cryptic splicing in HeLa nuclear extract. In vivo, *CGRP* mRNA production significantly decreased when rat *calcitonin/CGRP* genes containing base changes predicted to disrupt the U12 interaction site were expressed in 293 cells. Expression of *U12* genes containing compensatory base changes predicted to restore base pairing between U12 and the mutant pre-mRNA fully rescued *CGRP* mRNA production. The incomplete U12 snRNA allele specificity may be explained by the fact that U12 is being transcribed from the U1 promoter, and there may be much more U12 snRNA expressed from the transfected gene than is normally present in 293 cells. The observation that increasing the U12 gene *calcitonin/CGRP* gene ratio in cotransfections increases *CGRP* mRNA levels slightly, supports this possibility (data not shown).

The reason that base changes in the U12 interaction site have a somewhat different effect on splicing to exon 5 in vitro and in vivo is not clear. It may be that β *globin* exon 1 in the Δβ1-5 RNA used in vitro is a better splice donor than the *calcitonin/CGRP* exon 3 splice donor utilized in vivo. The stronger splice donor may allow some splicing, although much of it is to a cryptic splice acceptor site. An alternative explanation is that U12 snRNA binding is required in vivo but not essential in vitro because of splice site competition in vivo.

Taken together, our observations strongly suggest that accurate splicing to the rat *calcitonin/CGRP* exon 5 requires both U2 and U12 snRNAs. Future studies will focus on determining the makeup and mechanism of splicing complexes formed on Δβ1-5 pre-mRNA. Our data is also the first demonstration of an alternatively spliced intron that is U12 snRNA dependent. It will be important to determine the role, if any, that U12 plays in the regulation of alternative splicing of the *calcitonin/CGRP* gene.

MATERIALS AND METHODS

Plasmid construction

The Δβ1-5 hybrid minigene was constructed by PCR amplification of a 192-nt fragment of the rat *calcitonin/CGRP* gene containing the last 62 nt of intron 4 and the first 130 nt of exon 5, using the primers 5′-ACTGAATTCCCCTCATCTCTCTCATC-3′ and 5′-ACTGGATCCAAGGCTTCAGAGCCCAC-3′ (EcoRI and BamHI sites introduced by primers are underlined). The amplified fragment was cut with BamHI and EcoRI and ligated into BamHI–EcoRI cut pBS+ β1-2, which contains an EcoRI site engineered into human β *globin* intron 1 (Roesser et al. 1993). The resulting minigene contains β *globin* exon 1, the first 65 nt of β *globin* intron 1, the last 62 nt of *calcitonin/CGRP* intron 4, and the first 130 nt of exon 5. PCR mutagenesis was utilized to construct Δβ1-5 A-G, Δβ1-5 BPSM, and Δβ1-5 mut5 using primers 5′-CACTGGCATCCTGAG*TATCAGTGTCACTGC, 5′-CACTGGCAA*G*CTGAATATCCAGTGTCACTGC, and 5′-CACTGGCAC*T*T*C*GC*AA*TATCCAGTGTCACTGC, respectively (asterisks denote base changes from the wild-type sequence). The same primers were used for PCR mutagenesis of full-length rat *calcitonin/CGRP* genes to make *calcitonin/CGRP A-G* and *calcitonin/CGRP BPSM.*

For expression in 293 cells, *calcitonin/CGRP* genes were subcloned into the HindIII–BamHI sites of HSRα as previously described (Roesser et al. 1993). Wild-type and mutant *U12GA-CT* genes were previously constructed by replacement of the U1 coding sequence in a functional *U1* gene with *U12* coding regions (Hall and Padgett 1996) and were a generous gift of Dr. Richard A. Padgett. The *U12 comp* gene was constructed by using the primers 5′-CTGAGCAGATCTATGCCTTAAACTTATGAGGCGAAGAAATAACGATTCGGGGTG and 5′-GTTAGATCGTCGACCGGGCAGATCGCAACTCC to amplify the *U12* gene by the PCR. The DNA fragment generated by PCR was digested with SalI and BglII and ligated into the U12 vector digested with the same enzymes to replace the wild-type *U12* gene with *U12 comp.*

Splicing substrates

To synthesize transcripts for in vitro splicing, minigenes were digested with BamHI to completion and gel purified. In vitro transcription was carried out in the presence of 800 Ci/mmole [α-^{32}P]UTP using T3 RNA polymerase, and all pre-mRNAs were purified on NucTrap columns (Stratagene).

In vitro splicing

Splicing in HeLa nuclear extract was carried out essentially as described (Roesser et al. 1993). Reactions were carried out at 30°C for 2 h in a total volume of 20 μL containing 50% HeLa nuclear extract. Splicing products were separated by electrophoresis on 8% polyacrylamide gels containing 8 M urea, and RNAs were visualized by autoradiography. Candidate Δβ1-5 splice products were excised from gels and eluted in 0.5 M ammonium acetate, 10 mM EDTA, 0.1% SDS at 37°C overnight. Eluted RNAs were reverse transcribed with Thermoscript reverse transcriptase (Invitrogen) using the primer 5′-AAGGCTTCAGAGCCC-3′, which is complementary to calcitonin/CGRP exon 5. The cDNAs were then amplified by PCR using the above primer and 5′-ATGGTGCACCTGACTCCTGA-3′, a primer complementary to β *globin* exon 1. The nucleotide sequence of the PCR products were determined directly. U2B 2′O-methyl oligonucleotide 5′-AUAAGAACAGAUACUACACUUGA-3′, U12 2′O-methyl oligonucleotide 5′-AUUUCCUUACUCAUAAG-3′ and scrambled primer (SC) 2′O-methyl 5′-UGCAAUCGUAGCUAAUC-3′ (Dharmacon) were added to splicing reactions to a final concentration of 2 μM.

Transfection and cell culture

The 293 cells and F9 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Cells were transfected with Lipofectamine Plus (Invitrogen) and total cellular RNA was harvested 48 h post-transfection.

RNA analysis

Total RNA was isolated using Trizol (Invitrogen) and analyzed for splice products using reverse transcriptase–PCR. cDNAs were transcribed from RNA by Thermoscript reverse transcriptase (Invitrogen) using random hexamers as primers. PCR reactions were performed using 30 amplification cycles, the PCR products were extracted, separated by electrophoresis on 1.5% agarose gels, and visualized by ethidium bromide staining and under UV light. Figures 4 and 5 are negative images of the ethidium-stained gels. The primer complementary to *calcitonin* exon 4 used for PCR amplification was 5′-CTTGTTGAGGTCTTGTGTGTA-3′, the primer complementary to exon 5 was 5′-AAGGCTTCAGAGCCCAAC-3′ and the 5′-primer complementary to exon 3 was 5′-CCTACTG GCTGCACTGGTGC-3′. These competitive PCR reactions are run in the linear range, and the ratio of *CGRP:calcitonin* found in each sample was use to quantitate the effects of mutations on *CGRP* mRNA production (Lou et al. 1995; Tran and Roesser 2003).

ACKNOWLEDGMENT

I thank Dr. Richard A. Padgett for helpful discussions and for the generous gift of the U12 snRNA expression plasmids.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

Received October 21, 2003; accepted May 10, 2004.

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