Regulation of Tissue-specific Splicing of the Calcitonin/Calcitonin Gene-related Peptide Gene by RNA-binding Proteins*

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Transcripts of the rat calcitonin/calcitonin gene-related peptide (CGRP) gene are alternatively spliced in a tissue-specific manner resulting in the production of calcitonin mRNA and peptide in thyroid C cells and CGRP mRNA and peptide in neurons. Transfection studies using calcitonin and chimaeric human β-globin/calcitonin exon minigene constructs showed that the splice acceptor and exon specific to calcitonin mRNA are spliced much less efficiently in CGRP-producing cells (F9 teratocarcinomas) than in cells that preferentially make calcitonin (HeLa cells). In vitro splicing of chimaeric human β-globin/calcitonin transcripts in HeLa nuclear extracts were inhibited by the addition of nuclear extract from CGRP-favoring cells or tissues such as rat brain. This inhibition was specific as splicing of human β-globin first intron transcripts was not affected by comparable amounts of rat brain extract. Fractionation of rat brain nuclear extracts allowed the partial purification of two brain-specific polypeptides of apparent molecular mass of 43 and 41 kDa which preferentially bind RNA containing the calcitonin-specific splice acceptor. Since these polypeptides cofractionate with the calcitonin mRNA-specific splicing inhibition activity, we suggest that they may mediate the inhibition of splicing observed in vitro and underlie, in part, the inefficient calcitonin mRNA production observed in CGRP-favoring cells in vivo.

In eukaryotic organisms, most precursor mRNAs (pre-mRNAs) contain intron segments that are removed to generate mature mRNAs. Such RNA splicing occurs in the nucleus in a large complex of small nuclear RNAs and proteins called the spliceosome (Padgett et al., 1986). Some pre-mRNAs can be spliced in alternative ways to produce several different mature mRNAs from a single gene. These mRNAs may then be translated into distinct polypeptides. It is now clear that the control of alternative RNA splicing is an important mechanism for regulating gene expression for a large number of genes (Smith et al., 1989; McKeeon, 1990; Maniatis, 1991). In higher eukaryotes, alternative splicing has been shown to modulate gene expression in a developmental (Nagoshi et al., 1988; Early et al., 1980), sex-specific (Baker, 1989) and tissue-specific (Helfman et al., 1986; Libri et al., 1989; Leff et al., 1987) manner.

A number of trans-acting factors that regulate alternative splicing have been identified. A 97-kDa protein from Drosophila melanogaster somatic tissue has been shown to interact specifically with sequences adjacent to a 5' splice site to inhibit removal of intron 3 of P-transposase (Laski et al., 1986; Siebel and Rio, 1990). The general mammalian splicing factor SF2 is a nonspecific RNA-binding protein that is essential for the first step of splicing. Modulation of the concentration of SF2 (Krainer and Maniatis, 1985; Krainer et al., 1990a), in vitro or alternative splicing factor in vivo can change 5' splice site usage for some genes (Krainer et al., 1990b; Ge and Manley, 1990). An activity has recently been identified from mammalian nonmuscle cells which blocks usage of a skeletal muscle specific exon of β-tropomyosin (Guo et al., 1991). Three gene products from the Drosophila sex determination pathway, Sex lethal (Sxl), transformer (tra) and transformer-2 (tra-2), have been shown to regulate alternative RNA splicing by influencing the usage of 3' splice sites (for reviews see Baker, 1989; Maniatis, 1991). However, to date, no tissue-specific factor that regulates alternative RNA splicing for specific genes has been isolated from mammalian tissue.

The mammalian calcitonin/CGRP1 gene has six exons and is alternatively spliced in a tissue-specific fashion (Rosenfeld et al., 1983; Edbrooke et al., 1985; Leff et al., 1987). Splicing together the first four exons generates calcitonin mRNA (Fig. 1A), which encodes the serum calcium-regulating hormone calcitonin. In thyroid C cells, greater than 98% of the mature mRNA derived from the calcitonin/CGRP gene is 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 (Amaro et al. 1982). It has been demonstrated that deletion of approximately 21 nucleotides from the calcitonin/CGRP third intron, just upstream of exon 4, allows increased production of calcitonin mRNA in cells that normally make predominantly CGRP mRNA (Emeson et al. 1989). It was suggested that this deleted sequence could define a site of interaction with a negative regulator of calcitonin-specific splicing which is only expressed in CGRP-producing cells. However, the interpretation of these findings is complicated by the observation that the 21-nucleotide sequence contains part or all of the probable branch point sequences for the calcitonin-specific acceptor. Studies of the human calcitonin/CGRP gene by Cote and colleagues (Cote et al., 1990, 1991) identified a region in the 5' portion of exon 4 which is required for efficient calcitonin mRNA production in non-neuronal cells such as HeLa. Mutation of this site leads to increased...
Fig. 1. The calcitonin/CGRP gene and minigene constructs. Panel A, the structure of the rat calcitonin/CGRP gene. Thin lines and boxes represent introns and exons, respectively. Open boxes are noncoding exons (boxes 1 and 6), and stippled boxes are the coding regions common to both calcitonin and CGRP (boxes 2 and 3). The calcitonin-specific exon (box 4) is labeled calci, and the CGRP-specific coding region (box 5) is indicated. Mature mRNAs encoding calcitonin or CGRP are shown below. Panel B, minigenes constructed to examine calcitonin-specific acceptor usage. The βg1-2 construct used as a control consists of the human β-globin first exon, first intron, and the 5' 202 nucleotides of exon 2. An EcoRI site was introduced into the intron by oligonucleotide-directed mutagenesis. The cal3-4 construct contains 92 nucleotides from the 3' end of calcitonin exon 3, the entire third intron, and the 5' 148 nucleotides of the calcitonin-specific exon 4. βg1-cal4 is a hybrid minigene consisting of the human β-globin first exon, the 65 nucleotides from the 5' end of β-globin intron 1 fused to the 3' end of calcitonin third intron (243 nucleotides), and exon 4 (148 nucleotides). All constructs are under the control of the SRa promoter and have an SV40 poly(A) signal. Panel C, structure and sequence of the βg1-cal4 BP minigene. The A to C branch point change is indicated by the A at position -23.38 and 12 nucleotides of polylinker sequence flank the transcript at the 5' and 3' ends, respectively. The nucleotide sequence in the diagram depicts the 36 nucleotides of intron 3 immediately upstream of the calcitonin exon 4 splice junction. The seven-nucleotide sequence under the bar indicates the putative branch point sequence. The 21-nucleotide target sequence (underlined) has been implicated in negative regulation of the calcitonin-specific exon 4 in CGRP-producing cells (Emeson et al., 1989).
CGRP mRNA production in those cells (Cote et al., 1991) as does the addition of nuclear extracts of cell (F9) that favors CGRP mRNA production. Recent data have suggested that a 60-kDa protein found in HeLa cells may interact with this site in exon 4 (Cote et al., 1992).

We are interested in the characterization of trans-acting factors that influence tissue-specific splice site selection in the calcitonin/CGRP gene. Here we show that usage of the calcitonin-specific splice acceptor for exon 4 is very inefficient in F9 cells (which normally favor CGRP production). Inhibition of splicing at the calcitonin-specific splice junction can be reproduced in an in vitro splicing system. This in vitro assay has been used to purify partially a fraction from rat brain which contains two polypeptides that specifically bind to the calcitonin-specific splice acceptor RNA and which inhibit calcitonin-specific splicing in HeLa nuclear extracts.

MATERIALS AND METHODS

Plasmid Construction—A 915-nucleotide SstI-BamHI fragment from the rat gene encoding portions of exon 3 (see Fig. 1) was subcloned into pBS (Bluescribe, Stratagene). This fragment included 92 nucleotides of exon 3, 670 nucleotides of intron 3, and 148 nucleotides of exon 4. The SstI site in exon 3 was introduced by oligonucleotide-directed mutagenesis (Zoller and Smith, 1983). The BamHI site in exon 4 was generated by linker containing a SstI site (of a RsaI site. The $\beta$1-c4 hybrid minigene was constructed by taking the HpaI (in intron 3, 243 nucleotides upstream of the exon 4 splice junction) to BamHI fragment containing calcitonin exon 4, converting the HpaI site to EcoRI, and ligating the fragment into an EcoRI-BamHI-digested human $\beta$-globin exon 1–2 genomic fragment (Lawn et al., 1989). The EcoRI site in the first intron of human $\beta$-globin was introduced by oligonucleotide-directed mutagenesis (Zoller and Smith, 1983). The $\beta$1-c4 BP was constructed by oligonucleotide-directed mutagenesis (Zoller and Smith, 1983) of a 283-nucleotide fragment of the region flanking the exon 4 acceptor site (Cote et al., 1992). This modified acceptor 4 fragment was subcloned as an EcoRI-BamHI fragment into an EcoRI-BamHI-digested plasmid containing the original $\beta$1-c4 minigene in pBSK (Stratagene). In addition to the branch point mutation, the $\beta$1-c4 BP transcript includes 38 nucleotides of extra polylinker on its 5' end and is missing 104 nucleotides of calcitonin intron 3 sequence (–243 to –139 relative to the beginning of exon 4) that is present in $\beta$1-c4. A control minigene that was identical to $\beta$1-c4 BP, except that it contained a C at position –22, was no more efficient as an in vitro splicing substrate than $\beta$1-c4 (data not shown). For expression in F9 and HeLa cells, all minigenes were subcloned into a modified Srf vector (Takabe et al., 1988) containing a hygromycin resistance marker under control of the herpes thymidine kinase promoter. The Srf promoter is composed of the SV40 early promoter and the R segment of the US sequence of the long terminal repeat of human T cell leukemia virus type I (Takabe et al., 1988).

Transfection and Cell Culture—F9 teratocarcinoma cells and HeLa cells were transfected via calcium phosphate precipitation (Graham et al., 1973) containing a hygromycin resistance marker pBSK plasmid. All minigenes were subcloned into a modified Srf vector (Takabe et al., 1988). HeLa cells were grown in Spinster culture in Joklik media, 5% supplemented calf serum (HyClone Laboratories) for preparation of nuclear extracts for in vitro splicing. F9 cells were grown on 15-cm plates in Dulbecco's modified Eagle's medium, 10% fetal calf serum and stable HeLa transfectants selected in 400 μg/ml hygromycin (Boehringer Mannheim) and maintained in 100 μg/ml hygromycin. HeLa cells were grown in Spinster culture in Joklik media, 5% supplemented calf serum (HyClone Laboratories) for preparation of nuclear extracts for in vitro splicing. F9 cells were grown on 15-cm plates in Dulbecco's modified Eagle's medium, 10% fetal calf serum for isolation of nuclear extract.

RNA Isolation and RNase Protection Assays—Cytoplasmic RNA was isolated by hypotonic cell lysis (Ausubel et al., 1987). Whole cell RNA was prepared as described (Ausubel et al., 1987). Antisense RNA probes were prepared by in vitro transcription of the full-length 28S rRNA and calcitonin/CGRP gene containing portions of exon 3 (see Fig. 1). The 3' end of exon 3 of the EcoRI site 130 nucleotides upstream of calcitonin exon 4. These constructs were cloned into pBS (Stratagene), and in vitro transcriptions were carried out with T3 or T7 RNA polymerase. RNase protection assays were carried out as described (Ausubel et al., 1987) at a hybridization temperature of 48°C.

In Vitro Splicing Reactions and Characterization of Products—HeLa nuclear extract was isolated by the method of Dignam (Dignam et al., 1983). F9 cells and rat brain tissue were processed in the same manner. In vitro splicing reactions were carried out essentially as described (Krainer et al., 1984). Reactions were carried out at 30°C in a total volume of 20 μl containing 60% or 70% HeLa nuclear extract in Dignam buffer. D. 10% v/v F9 or rat brain nuclear extract in buffer D was added as indicated in individual figures. Splicing products were analyzed by nondenaturing PAGE. Putative spliced RNAs were excised from gels and eluted in 0.5 M EDTA, 10% SDS overnight at 37°C. $\beta$1-c4 spliced RNA was reverse transcribed using the oligonucleotide CTTGGTGGAGTCTTGTTGC, which is complementary to calcitonin exon 4, as a primer (Kawasaki, 1989). The cDNA was amplified by the polymerase chain reaction in the presence of ATGTTGGACCTGACTCTGCTGA as a β-globin exon 1 primer (Kawasaki, 1990). The amplified DNA was subcloned and its nucleotide sequence determined (Sanger et al., 1977).

RESULTS

Calcitonin-specific Splice Site Usage—Our strategy for identifying trans-acting factors that control tissue-specific splice site selection in the calcitonin/CGRP gene was first to identify the cis sequences in the gene which are sufficient to confer inhibition of calcitonin mRNA production in CGRP-favoring cells. In our initial experiments we chose to focus on the calcitonin-specific exon 4 splice acceptor (acceptor 4) in CGRP-producing cells. Previous work (Emerson et al., 1987) indicated that at least part of the differential splicing involves recognition of a sequence just upstream of the calcitonin-specific acceptor in cells that make CGRP. To determine whether the calcitonin-specific acceptor alone is the substrate for inefficient calcitonin mRNA production in neural cells, minigenes were constructed which contained the calcitonin-specific acceptor 4. One of these constructs, cal3–4, consisted of 92 nucleotides from the 3' end of calcitonin exon 3, the entire third intron, and the first 148 nucleotides of calcitonin exon 4 (see Fig. 1B). In the second minigene $\beta$1-c4, human $\beta$-globin exon 1 replaced calcitonin exon 3 and was fused to 243 nucleotides of the calcitonin third intron and the first 148 nucleotides of calcitonin exon 4. A control construct comprised of the human $\beta$-globin first exon, first intron, and second exon was also used. Each of these transcription units
was driven by the SRo promoter (Takabe et al., 1988), and they all contained the SV40 polyadenylation site at their 3′ ends.

The minigenes were transfected into HeLa cells (calcitonin-producing cell) and F9 teratocarcinoma cells (CGRP-producing). Cells stably expressing the genes were selected by growth in media containing hygromycin. Total cellular RNA was harvested from each and analyzed by RNase protection with in vitro synthesized 32P-labeled antisense RNA probes. As indicated in Fig. 2A, HeLa cells produced accurately and efficiently spliced transcripts of each of the minigenes. F9 cells, however, efficiently spliced the βgl-2 transcripts but produced very little mature cal3-4 or βgl-cal4 RNA, and no larger unprocessed precursor RNA was detected (Fig. 2B). Similar results were obtained using cells transiently transfected with these constructs (results not shown). These data do not exclude the possibility that differential RNA stability is responsible for these results; however, additional transfection and in vitro studies (see below) suggest that the differential production of exon 4-containing RNA is regulated at the level of splicing.

To address the possibility that the absence of spliced βgl-cal4 or cal3-4 transcripts in F9 cells was because of the instability of RNAs containing exon 4 in these cells, we transfected a βgl-cal4 minigene construct that contained a mutation that was predicted to increase the efficiency of exon 4 usage in F9 cells. Studies of the human calcitonin/CGRP gene (Cote et al., 1991; Adema and Baas, 1991) have shown that a U to A mutation complementing a putative branch point sequence at a site 23 nucleotides upstream of the IVS 3/exon 4 (calcitonin-specific splice acceptor) junction greatly enhances the efficiency of exon 4 usage in HeLa nuclear extracts and in F9 cell transfection studies. Consequently, we constructed a βgl-cal4 minigene in which an analogous cytosine 23 nucleotides upstream of the calcitonin-specific exon of the rat gene was changed to an adenosine by oligonucleotide site-directed mutagenesis (the product was designated βgl-cal4 BP; Fig. 1C). This base change converts a putative calcitonin-specific acceptor branch point sequence to a perfect match with the mammalian branch point consensus sequence (Reed and Maniatis, 1988; Zhuang et al., 1989) and would be predicted to increase the efficiency of usage of the calcitonin-specific acceptor in vivo or in vitro. RNA isolated from cells stably transfected with this construct was analyzed by Northern blot and RNase protection assays (Fig. 2C). These cells were observed to produce correctly spliced βgl-cal4 RNA at levels somewhat less than observed for the βgl-2 constructs. The presence of spliced calcitonin exon-containing transcripts indicates that RNA instability cannot account for the lack of βgl-cal4 and cal3-4 spliced RNAs in the F9 cells observed in Fig. 2B. Rather, our data support the conclusion of Emeson et al. (1989), which suggests that there is negative regulation of the calcitonin-specific acceptor in F9 cells. Further, these data demonstrate that all of the information necessary for the repression of calcitonin acceptor 4 usage in neuronal cells is contained in the βgl-cal4 minigene, in the sequences immediately surrounding acceptor 4.

In Vitro Inhibition of Splicing—To identify and purify trans-acting factors from neuronal cells that influence calcitonin acceptor 4 splice site usage, we developed an in vitro assay for such factors. In this assay βgl-2, βgl-cal4, and βgl-cal4 BP RNAs synthesized in vitro with T3 RNA polymerase were incubated with HeLa nuclear extract and then examined for the formation of splicing products. Fractions of extracts from other tissues and cells could be assayed for their abilities to inhibit βgl-cal4 splicing specifically. Data 3A shows that βgl-1 and βgl-cal4 RNAs were spliced by HeLa nuclear extract. The reactions were ATP-dependent (not shown). To prove that the putative splice products were accurately spliced RNAs, they were characterized after elution from polyacrylamide gels. The candidate spliced RNAs were subjected to reverse transcription followed by amplification by the polymerase chain reaction with suitable primers (see "Materials and Methods"). The amplified products were then cloned and their nucleotide sequence determined. Lariat bands were identified on the basis of their changing mobilities on gels consisting of different percentages of polyacrylamide and by primer extension analysis (data not shown). The addition of nuclear extract from rat brain or F9 cells inhibited βgl-cal4 splicing but had no effect on βgl-1 splicing (Fig. 3A, lanes 2 and 3 versus lanes 5 and 6). Thus, the usage of calcitonin splice acceptor 4 is preferentially inhibited in vitro by the addition of nuclear extract from CGRP-favoring cells, suggesting the presence of a negative regulator of the calcitonin-specific splice acceptor in these cells. The inhibitory activity was not inactivated by preincubation of rat brain nuclear extract with micrococcal nuclease but was inactivated by incubating the rat brain extract at 90 °C for 5 min prior to the assay of splicing reactions, suggesting that the inhibition was caused by a protein or proteins and not by RNA (data not shown).

To address the sequence specificity of the inhibiting activity from rat brain nuclear extract, we sought to test inhibition against a more efficiently spliced substrate that contained the exon 4 splice acceptor. To accomplish this we tested the βgl-cal4 BP transcript described in the transfection studies in an in vitro splicing reaction. Transcripts from βgl-cal4 BP were synthesized using T3 RNA polymerase and incubated in HeLa nuclear extracts under splicing conditions. As shown in Fig. 3B, splicing of βgl-cal4 BP transcripts was nearly as efficient as the processing of βgl-1–2 RNA in this system. Although the βgl-cal4 BP transcript has a shorter intron than the parental βgl-cal4 transcript shown, the increased efficiency of splicing of βgl-cal4 BP was caused only by the C to A base change 23 nucleotides upstream of the calcitonin-specific exon. We observed no effect of shortening the intron on splicing of a βgl-cal4 transcript without this C to A change (data not shown). In the presence of added rat brain nuclear extract, splicing of βgl-cal4 BP but not βgl-1–2 transcripts was completely abolished (Fig. 3B, lane 5), providing further evidence that the inhibitory activity is specific for the calcitonin-specific acceptor. These data argue against the possibility that βgl-cal4 splicing was preferentially inhibited by CGRP-favoring cell nuclear extracts simply because the splicing of βgl-cal4 was inherently less efficient than βgl-1–2 splicing. The data also imply that splicing is blocked at an early step, as no intermediate products accumulate.

Fractionation of Rat Brain Nuclear Extracts—In vitro inhibition of calcitonin βgl-cal4 and βgl-cal4 BP splicing was used as an assay to follow the fractionation of rat brain nuclear extract. We used a three-step fractionation scheme: ammonium sulfate precipitation followed by DEAB-cellulose column chromatography and Sephadex G-150 gel filtration. Following Sephadex G-150 column chromatography, the activity inhibiting βgl-cal4 BP splicing was found to elute as a single peak in one to several fractions (Fig. 4A). The G-150-purified fraction was also shown to inhibit βgl-cal4 splicing without inhibiting βgl-1–2 splicing in vitro (data not shown).

To study further the RNA binding specificity of this inhibitory activity, we examined the effect of added unlabeled competitor RNAs on the inhibition of βgl-cal4 BP splicing by the active G-150 fraction. Because of the observations of Emeson and colleagues (Emeson et al., 1989) that a 21-nucleotide target sequence might mediate the inhibition of calcitonin splice acceptor usage in F9 cells, we tested the ability of an unlabeled RNA containing this sequence to protect βgl-cal4 BP splicing from inhibition by the active G-
FIG. 2. **Expression of minigene constructs in HeLa and F9 cells.** Total cellular RNAs were harvested from cells stably expressing the minigene constructs and analyzed by RNase protection. Full-length, radiolabeled, antisense RNAs from the individual minigenes were hybridized to the cellular RNAs. Following RNase A and T1 digestion of unhybridized RNA, protected fragments were separated by denaturing PAGE on 5% gels. Products were visualized by autoradiography. Radiolabeled HinfI-cut pBR322 fragments were run in parallel as size markers (1600, 527, 398, 346, 344, 298, 221, 154, 76, and 74 nucleotides in length). Lanes containing full-length undigested probe RNA (P), total cellular RNA from cells containing the SRα vector with no insert (mock), and total RNA transfected with the indicated minigene (TR) are shown for each. Panel A, HeLa stable cell line RNAs, 10 μg/reaction. Panel B, F9 stable cell line RNAs, 20 μg/reaction. Panel C, F9 stable cell line RNAs, 40 μg/reaction. The probes were 32P-labeled antisense RNAs from the 410 nucleotides surrounding the calcitonin acceptor 4 region.

150 rat brain fraction. The addition of a 40-fold molar excess of this RNA containing the target sequence, but not tRNA, almost completely protected βg1-cal4 BP splicing from inhibition by the G-150 fraction (Fig. 4B). The addition of a 40-fold excess of the RNA containing the target sequence, but not an RNA containing only pBluescript polylinker sequence, was also effective in protecting βg1-cal4 splicing from inhibition by the active rat brain G-150 fraction (data not shown). The ability of added target RNA, but not random RNA, to protect βg1-cal4 or βg1-cal4 BP in vitro splicing implies that
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Fig. 3. In vitro splicing reactions. Panel A, radiolabeled transcripts from the βg1–2 construct or βg1-cal4 construct were incubated for 2 h in the presence of HeLa nuclear extract and ATP. Splicing products were analyzed by denaturing PAGE and visualized by autoradiography. pBR322-HindIII markers are shown and spliced RNAs indicated. The addition of rat brain nuclear extract (RB) or F9 cell nuclear extract is indicated at the top of the figure. Panel B, radiolabeled transcripts from βg1–2 and βg1-cal4 BP were incubated in the presence of HeLa nuclear extract 90 min. Splicing products were examined as in panel A. Spliced RNAs, lariat structures, and free exon 1 RNAs are indicated. The addition of rat brain nuclear extract is indicated at the top of each lane in micrograms of total protein.

Fig. 4. Fractionation of rat brain nuclear extract. Panel A, G-150 column profile. In vitro splicing reactions with radiolabeled βg1-cal4 BP RNA were carried out as in Fig. 3A except that 5 μl of G-150 column fractions were added prior to splice incubation. Splice products were analyzed by denaturing PAGE on a 5% gel. Molecular size markers are indicated (HindIII markers), as are spliced RNA and free lariat products. The column fraction added to each reaction is indicated. Panel B, in vitro splicing of βg1-cal4 BP and βg1–2 transcripts. Radiolabeled βg1–4 BP or βg1–2 RNA was incubated with HeLa nuclear extract in either the absence or presence of rat brain G-150 fractionated nuclear extract (indicated at the top of the figure). A 40-fold excess of unlabeled 56-nucleotide RNA containing the 21-nucleotide target sequence as a competitor RNA was added to the reaction in lane 7 as indicated. RNAs were separated by denaturing PAGE, and splicing products are indicated.

the activity inhibiting splicing to the calcitonin-specific acceptor acts by binding to specific RNA sequences.

The fractions from the Sephadex G-150 column were also tested for their ability to bind directly to a 32P-labeled RNA that contains approximately 380 nucleotides surrounding the calcitonin-specific acceptor. Only those fractions that have the ability to inhibit calcitonin-specific splicing form a complex with this acceptor 4 RNA (Fig. 5A). The gel retardation assay was also used to examine the specificity of RNA binding
of the active rat brain G-150 fractions. Radiolabeled RNA transcribed from the minigenes βg1-cal4 and βg1-2 was used. As shown in Fig. 5B, the addition of fractions containing the inhibitory activity from the Sephadex G-150 column causes a mobility shift for both RNAs on a nondenaturing polyacrylamide gel, indicating that the active fractions contain protein(s) that can bind each of the RNAs. The addition of a 20-fold molar excess of unlabeled competitor RNA (either RNA containing the 21-nucleotide target sequence or RNA from the pBS poly linker) completely abolished binding of this protein to the βg1-2 transcript but had no effect on βg1-cal4 binding (compare lanes 3 and 4 with lanes 7 and 8). These data suggest that the RNA binding activity in this fraction preferentially interacts with sequences surrounding the calcitonin-specific acceptor (calcitonin acceptor 4) and that the 21-nucleotide target sequence is an inefficient competitor in this assay.

The inhibitory activity of G-150 fractions was further characterized by UV cross-linking analysis. The partially purified protein was prebound to labeled RNA containing the calcitonin acceptor 4 splice site and exposed to 254 nm radiation. Following digestion with RNase A, proteins that were covalently tagged with labeled RNA were analyzed on 10% SDS-polyacrylamide gels. As shown in Fig. 6A, labeled RNA is covalently bound to two polypeptides that migrate to an approximate molecular mass of 43 and 41 kDa in an extract-dependent fashion. The two peptides are preferentially bound to RNA containing the calcitonin-specific acceptor. Titration of the amount of various unlabeled RNAs necessary to compete away binding to labeled transcript containing the calcitonin-specific acceptor is shown in Fig. 6B. Binding of the labeled acceptor 4 RNA to these polypeptides was more effectively competed by a 4-fold molar excess of unlabeled acceptor 4 RNA than by a 400-fold excess of a nonspecific RNA made from the pBS poly linker (compare lanes 5 and 6). An RNA containing the 21-nucleotide target sequence competed more potently than nonspecific RNA but much less potently than RNA containing the entire calcitonin acceptor 4 region. A labeled RNA containing the 21-nucleotide target sequence was shown to make only a weak complex with partially purified extract, and this complex could be abolished by the addition of moderate amounts of nonspecific competitor RNA (data not shown). These data are consistent with the interpretation that the partially purified rat brain fraction binds specifically to sequences surrounding the calcitonin mRNA-specific splice acceptor and that the 21-nucleotide target sequence comprises only part of the recognition sequence.

**Tissue Specificity**—Nuclear extract from rat spleen was fractionated in parallel with nuclear extract from rat brain. Rat spleen nuclear extract did not inhibit calcitonin acceptor 4 splice site usage in vitro (data not shown). After DE52-cellulose step gradient chromatography, all of the spleen fractions were tested for RNA binding activity by gel shift analysis and UV cross-linking. All of the spleen fractions bound acceptor 4 RNA in a gel retardation assay, but they did not form defined complexes as did the rat brain fraction A-150, which contained all of the calcitonin acceptor 4 RNA binding activity (data not shown). After UV irradiation of labeled RNAs in the presence of extract, labeled RNA from calcitonin acceptor 4 was covalently attached to a polypeptide from spleen with an apparent molecular mass of approximately 65 kDa (not shown). However, binding of the spleen fractions to calcitonin acceptor 4 RNA could be completely inhibited by modest excesses of tRNA (data not shown), suggesting that binding of the spleen fractions was not specific for acceptor 4 RNA.

**DISCUSSION**

We would like to elucidate the mechanisms controlling tissue-specific alternative splicing of transcripts of the mammalian calcitonin/CGRP gene. In particular, we are interested in defining and isolating trans-acting factors that mediate the regulation of this process. It is likely that such trans-acting factors are themselves expressed or regulated in a tissue-specific manner. Moreover, it is possible that the trans-regulators of calcitonin/CGRP differential splicing are members of an important family of molecules which helps to modulate splicing in the numerous genes now known to undergo alternative splicing.

It has been proposed that splicing exon 3 to the calcitonin-specific acceptor (the 3′ splice acceptor of intron 3) is inhibited in cells that make CGRP (Emeson et al., 1989). Deletion of sequences just upstream of the calcitonin-specific exon partially relieves this negative regulation and allows greater
Fig. 6. UV cross-linking analysis. Panel A, radiolabeled βg1-cal4 transcript was incubated in the presence or absence of G-150 column fractionated rat brain nuclear extract and then exposed to ultraviolet light (254 nm), treated with RNase A, and analyzed by SDS-PAGE. Bands were visualized by autoradiography. Molecular mass standards are indicated (lane M, in kDa). Panel B, ultraviolet cross-linking of βg1-cal4 transcript to rat brain nuclear extract G-150 fraction 4. Protected RNAs were visualized following RNase treatment, SDS-PAGE, and autoradiography. Unlabeled RNAs were added as competitors during preincubation with extract. Acceptor 4 RNA contains 243 nucleotides of the 3’ end of calcitonin intron 3 and 148 nucleotides of calcitonin exon 4. pBS is a nonspecific 65-nucleotide RNA synthesized from the pBS polylinker. Target RNA is derived from the pBS polylinker containing the 21-nucleotide target sequence (Fig. 3B). The molar excess of unlabeled competitor RNA versus radiolabeled βg1-cal4 transcript is indicated.

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amounts of calcitonin mRNA production in cells that otherwise favor CGRP production. Our finding that the calcitonin-specific acceptor is recognized inefficiently as a splice junction in F9 (CGRP-favoring cells) but efficiently in HeLa (calcitonin-favoring) cells supports this conclusion. Furthermore, this inhibition of the calcitonin-specific acceptor usage can be reproduced in vitro. The calcitonin-specific splice acceptor is utilized as a splice junction in HeLa nuclear extract, but the addition of small amounts of nuclear extract from CGRP-favoring cells inhibits this splicing. These data suggest that there is a negative regulator of calcitonin-specific splicing in CGRP-producing cells which acts at or near calcitonin acceptor 4.

An RNA transcript containing approximately 290 nucleotides surrounding the calcitonin-specific acceptor appears to be a sufficient substrate for cell-specific inhibition. We are proposing a model in which binding of a putative negative regulator near the calcitonin-specific acceptor competes either with binding of components of the spliceosome or with factors in non-neuronal cells which enhance calcitonin exon usage (Cote et al., 1992). Such inhibitory activity may only operate against moderately weak splice acceptors. Indeed, it has been demonstrated that a mutation of the putative branch point of the human calcitonin-specific acceptor to make it a better match to the branch point consensus sequence (Reed and Maniatis, 1988; Zhuang et al., 1989) allows formation of higher amounts of calcitonin mRNA in CGRP-producing cells (Fig. 2; Adema et al., 1990; Adema and Baas, 1991). Transcripts from the rat calcitonin gene containing the same base change (our βg1-cal4 BP) are not spliced in vitro in the presence of rat brain extract. These results are consistent with the proposal that nucleotide changes in the 3’ splice site which promote spliceosome formation lessen the ability of the negative regulator to bind and inhibit splicing in vivo, without appreciably decreasing binding of the negative regulator in vitro. The slower kinetics of in vitro splicing may allow a negative factor to bind and inhibit spliceosome formation.

We believe that the 21-nucleotide target sequence is itself
only part of the RNA sequence recognized by the negative regulator. This idea is supported both by the fact that the RNA binding activity that was isolated consists of at least two polypeptides and that the target sequence is recognized by the binding complex several times less potently than the entire calcitonin-specific acceptor. It is also supported by the finding that splicing of the β1-cal4 BP transcript is inhibited in vitro by rat brain extract despite the fact that the C to A base change is within the target sequence. We are presently determining the minimum sequences recognized by the RNA binding activity by deletion analysis.

It is possible that the activity we have partially purified may correspond in part or in whole to an activity recently reported to be present in F9 cell extracts (Cote et al., 1991). Those authors report that this activity promotes skipping of human calcitonin exon 4 and splicing to exon 5 when splicing a chimaeric adenovirus exon 1 to calcitonin exons 4 and 5 transcript in vitro. Cote et al. (1990) suggested that the sequences mediating regulation of exon 4 skipping reside within the first 45 nucleotides of exon 4. Although our data suggest that the 21-nucleotide target sequence may correspond in part of the recognition for the regulation of exon 4 usage, our data do not exclude an involvement of the site described by Cote et al. (1991).

The ability to reproduce cell type-specific splice inhibition in vitro has allowed the partial purification from rat brain of two polypeptides that preferentially bind to the calcitonin-specific acceptor and preferentially inhibit splicing to this acceptor. The two polypeptides have approximate molecular masses of 43 and 41 kDa, inclusive of the covalently bound RNA. Of the known proteins with RNA binding activity, only C1 and C2 proteins (43 and 41 kDa, respectively; Serrafin et al., 1988) found in heterogeneous nuclear ribonucleoprotein particles (hnRNP) and the recently described HuD protein (Szabo, 1991) have similar molecular masses. The hnRNP C proteins preferentially bind to the 3′ end of introns (Swanson and Dreyfuss, 1988) and were reported to be essential for splicing in vitro systems (Choi et al., 1986). The distribution of C1 and C2 proteins has not been shown to be tissue-specific, and they bind strongly to single-stranded DNA. The RNA-binding proteins that we have identified bind single-stranded DNA very weakly (data not shown), and their distribution appears to be specific to brain. Furthermore, the hnRNP C proteins behave differently during purification by DEAE chromatography, adhering to the column at 150 mM salt, whereas the polypeptides we have identified elute from the column under these conditions. In the absence of antibodies that recognize rodent hnRNP C proteins or purified extracts of a human cell line or tissue that makes a CGRP splicing choice, we cannot completely rule out the possibility that our peptides are hnRNP C proteins. However, if they are hnRNP C proteins, then one must propose that either hnRNP C proteins are modified specifically in rat brain or that they associate with rat brain-specific factors that alter their chromatographic behavior. Barring this latter interpretation, our data suggest that the calcitonin-specific acceptor-binding polypeptides are not hnRNP C1 or C2.

The other known RNA-binding protein of approximately the same size is HuD, a neuronal antigen recognized by the sera of patients with antibody-associated paraneoplastic encephalomyelitis. This antigen has now been cloned and sequenced (Szabo et al., 1991) and shown to have homology to the Drosophila proteins Elav and Sex-lethal. Antibodies to HuD recognize a number of proteins in rat brain extracts but do not react with proteins in our partially purified fractions. Thus it is likely that neither of our peptides is HuD nor any other known RNA-binding protein.

A number of RNA-binding proteins have been shown to regulate alternative RNA splicing in other systems. In D. melanogaster the sex determination pathway is regulated by a number of such factors (for review see Baker, 1989). The female-specific product of the Sxl gene, one of the early acting genes in the pathway, inhibits splicing to default splice acceptors in both its own pre-mRNA and the nascent transcript of the tra gene. The Sxl gene product thereby directs female-specific splicing of tra and Sxl (Bell et al., 1988; Sosnoski et al., 1989; Inoue et al., 1990). Both the female-specific tra gene product and the tra-2 gene product are necessary for positive regulation of a female-specific splice acceptor in the doublesex (dxs) pre-mRNA (Nagoshi and Baker 1990; Burts and Baker, 1989; Hoshijima et al., 1991; Hedley and Maniatis, 1991; Ryner and Baker, 1991). The suppressor of white apricot (su(wa)) gene product, a Drosophila gene not involved in the sex determination pathway, inhibits removal of the first intron in its own pre-mRNA (Zachar et al., 1987). The tra-2 and Sxl proteins contain the RNP motif found in RNA-binding proteins (Amrein et al., 1988; Goralski et al., 1989; Bandzudiis et al., 1989; Query et al., 1989; Scherly et al., 1989). The tra, tra-2 and su(wa) gene products contain regions rich in serine and arginine repeats thought to be involved in RNA recognition (Amrein et al., 1988; Goralski et al., 1989; Boggs et al., 1987; Chou et al., 1987; Bingham et al., 1988). Therefore, there are examples of both positive and negative regulation of splice acceptor sites by RNA-binding proteins in Drosophila as well as an example of more than one polypeptide being required for regulation of a single splice acceptor site.

Presently there is only one known example of a purified mammalian regulator of alternative RNA splicing. SF2 is a general component of spliceosomes necessary for 5′ splice site cleavage and lariat formation (Krainer and Maniatis, 1985; Krainer et al., 1990a). SF2 contains both an RNA binding domain consensus sequence and a region rich in serine and arginine residues (Ge et al., 1991; Krainer et al., 1991). The concentration of SF2 can determine 5′ splice site choice in vitro (Krainer et al., 1990b). The concentration of alternative splicing factor, a factor identical to SF2, can alter the ratio of SV40 large T and small t antigen splicing in cultured cells (Ge and Manley, 1990). Thus, the activity described herein is one of the first identified from mammalian cells which regulates splice acceptor usage. Recently, an activity has been identified which blocks the use of a skeletal muscle-specific exon of β-tropomyosin in non-muscle cells (Guo et al., 1991). This activity appears to be a polypyrimidine tract-binding protein, and therefore it does not appear to be tissue-specific (Mulligan et al., 1992). Thus, the activity-inhibiting calcitonin acceptor 4 usage may represent the first tissue-specific regulators of alternative RNA splicing to be identified in mammalian cells.

Although fractionation of both rat brain and spleen tissues suggests that the inhibitor of calcitonin-specific splicing is present only in brain tissue, it is possible that the activity is present in spleen at much lower levels. It is also possible that only one of the two polypeptides that interact with the calcitonin-specific acceptor is tissue-specific. The question of tissue specificity of the activity can be more easily addressed following the purification of its components and/or the identification and isolation of the genes encoding the activity.

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REFERENCES

