Volume 324, number 2, 123–126 FEBS 12541 June 1993

A distal region of the CALC-1 gene is necessary for regulated alternative splicing

D.A. Horn and D.S. Latchman

Division of Molecular Pathology, University College London Medical School, The Windeyer Building, Cleveland Street, London, W1P 6DB, UK

Received 16 March 1993; revised version received 12 April 1993

The CALC-1 gene exhibits tissue specific alternative splicing with exons 1–4 being spliced to produce the calcitonin mRNA in thyroid C cells and exons 1–3 and 5–6 being joined to produce the CGRP mRNA in neuronal cells. Previous studies have identified an element in intron 3 within the alternatively spliced region which is critical for this effect to occur. We show here that deletion of sequences downstream of the alternatively spliced region also disrupts the tissue specific pattern of alternative splicing. The manner in which these sequences act is discussed.

Alternative splicing; Calcitonin; Calcitonin gene related peptide; Gene regulation

1. INTRODUCTION

Alternative splicing is a form of post-transcriptional regulation allowing the generation of different sets of related polypeptides in different cell types. This thereby increases the coding capacity of the genome and is a means of regulating gene expression which is particularly widely used in non-dividing cells such as neurons where the reprogramming of gene transcription by altering chromatin structure is difficult (for reviews see [1,2]).

The CALC-1 gene of both rat [3] and human [4] is alternatively spliced in a tissue specific manner and has served as a model system for studying the processes regulating tissue specific patterns of alternative mRNA splicing. Thus this gene is transcribed only in thyroid C cells and in neuronal cells in the brain. In thyroid cells exons 1–4 are spliced together to produce the calcitonin mRNA whereas in neuronal cells exon 4 is omitted and splicing of the common exons 1–3 with exons 5 and 6 produces the CGRP mRNA (3, Fig. 1). Transfection of different cell types with a CALC-1 construct containing exons 1–6 and the polyadenylation sites for both mRNAs reproduced the correct in vivo splicing pattern and suggested that a specific *trans*-acting protein present in only a limited range of cell types was required to inhibit calcitonin-specific splicing and hence allow CGRP-specific splicing to occur [5,6]. Subsequent studies identified a *cis*-acting element near the 3′ splice site of intron 3 which was required for the tissue specific inhibition of calcitonin-specific splicing [7].

Based on these studies several investigations have used truncated mini gene constructs containing this region in studies of CALC-1 alternative splicing. Thus a construct containing exons 4–6 and both polyadenylation signals yielded the correct tissue-specific pattern of alternative splicing suggesting that exons 1–3 are not required for regulation of the splicing event [8]. It has also been suggested that a construct truncated within exons 3 and 5 and lacking both proximal and distal sequences as well as the CGRP-specific polyadenylation site contained all the sequences required for regulated alternative splicing [9]. This construct has therefore been used for several studies on the mechanisms mediating such splicing (see for example [10]). However, in vitro experiments with this construct led to the production of the CGRP mRNA in several cell lines which normally produce the calcitonin mRNA [9] suggesting that sequences in the distal portion of the gene may be necessary for the correct regulation of the alternative splicing event.

To resolve this problem, we have directly compared the splicing pattern produced by transfection of different cell types with two constructs containing portions of the human CALC-1 gene expressed under the control of the RSV promoter. One of these constructs (pRSV-Cal3) contains exons 3–6 with the corresponding introns and both polyadenylation signals whereas the other (pRSV-Cal 2) is truncated in exon 5 and therefore lacks part of exon 5, all of intron 5 and exon 6 as well as the CGRP polyadenylation signal which is replaced by that of SV40 (Fig. 1).

Correspondence address: D.S. Latchman, Division of Molecular Pathology, University College London Medical School, The Windeyer Building, Cleveland Street, London, W1P 6DB, UK. Fax: (44) (71) 387 3310.

2. MATERIALS AND METHODS

2.1. *Cell culture*

ND3, HeLa and 3T3 cells were grown in DMEM (Gibco BRL with 0.11 g/l sodium pyruvate) supplemented with 10% v/v foetal calf serum (Gibco BRL) and ND7 cells were grown in L15 (Liebovitz) medium (Gibco BRL with L-glutamine) supplemented with 10% v/v foetal calf serum, 0.35% w/v sodium bicarbonate and 0.35% w/v glucose

2.2. *Transfections*

Human calcitonin/CGRP-1 minigene constructs were transfected by the calcium phosphate procedure of Gorman [11]. Cells were seeded at a density of 8×10^5/90 mm plate.

2.3. *RNA analysis*

Total RNA was isolated from transfected or untransfected tissue culture cells by a rapid guanidinium isothiocyanate method [12]. Prior to reverse transcription mRNA was typically treated with 15 units of DNase I and 20 units of RNase inhibitor (Rnasin) in 50 μl of dH_2O for 1 h at 37°C to remove traces of genomic DNA. mRNA was then extracted with phenol/chloroform and chloroform and ethanol precipitated.

Reverse transcription was carried out typically in a 20 μg volume containing 1 μg of total mRNA, 0.25 units of random hexanucleotides, 0.5 mM dATP, dCTP, dGTP and TTP (Pharmacia), 1 × Taq DNA polymerase buffer (Amersham), 12 mM DTT, 100 units of M-MLV reverse transcriptase and 20 units of Rnasin and incubated at 37°C for 1 h

PCR analysis of cDNA was carried out by the method of Kawasaki [13]. Reactions were typically in a 50 μl volume containing 30 pmol of each primer, 0.1 mM dATP, dCTP, dGTP and TTP, 1 × Taq DNA polymerase buffer (Amersham) and 2 units of Taq DNA polymerase. Following PCR amplification using a Hybaid thermal reaction, samples were extracted with 50 μl of chloroform and loaded onto agarose, ethidium bromide gels.

In preliminary experiments PCR amplifications were carried out using different cDNA dilutions and numbers of cycles to identify conditions in which the PCR product signal was linearly related to the input cDNA.

PCR amplification of calcitonin/CGRP-1 cDNA from cells transfected with pRSV-Cal2 or PRSV-Cal3 were carried out using a sense oligonucleotide derived from the RSV mRNA 5'-untranslated sequence (5'-CCATTCACCACATTGGTGTGC-3', sequence derived from [4], and the antisense oligonucleotides, h-Cal 4 (5'-TACCAGCCCAAAGAGCCACC-3') and h-CGRP5 (5'-TGGAACCCACATTGGTGGGC-3') the sequences of which were both derived from Broad et al. [15] 30 cycles were carried out at 94°C for 45 s, 64°C for 45 s and 72°C for 45 s. The resulting products were separated on 1.75% agarose/ethidium bromide gels.

2.4 *Southern blotting*

DNA was transferred onto hybond N agarose/ethidium bromide gels. Filters were prehybridised in 6 × SSC, 10% w/v dextran sulphate (Pharmacia), 0.1% w/v SDS, 5 × Denhardts and 100 μg/ml denatured herring sperm DNA (Sigma), for 2 h at 65°C. Oligo labelling of DNA fragments was carried out according to the method of Fienberg and Vogelstein [16]. Hybridisation to denatured, oligo-labelled probe was carried out in the same solution overnight at 65°C.

After hybridization filters were washed to a stringency of 0.25 × SSC and 0.1% SDS. The probe used for detection of both PCR products was derived from exon 3 of pRSV-Cal3 by *HpaI*/*Bam*HI digestion and the resulting 173-bp product was complementary with the PCR products through 67% of its length. The probe used for calcitonin PCR product detection was derived from exon 4 of pRSV-Cal3 by *Bgl*II, *Nsi*I digestion, and the resulting 237-bp product was 100% complementary with the PCR product. The probe used for CGRP PCR product detection was derived from exon 5 of pRSV-Cal3 by *Bgl*II, *Nar*I digestion and the resulting 168-bp product was complementary with the PCR product through 72% of its length.

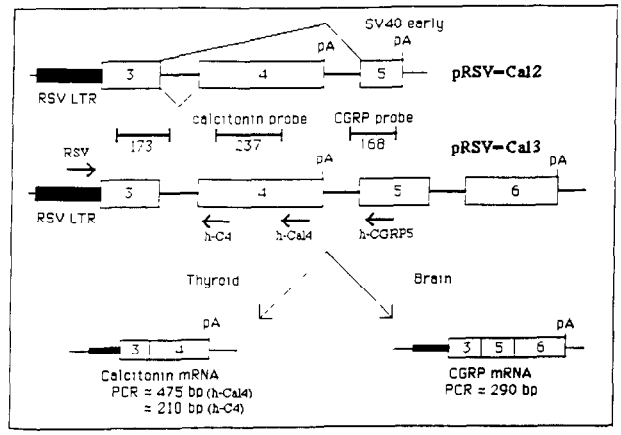

Fig 1. Schematic diagram of the pRSV Cal 2 and Cal 3 mini-gene constructs, alternative processing of the calcitonin/CGRP pre-mRNA and the nature of the PCR assay. Small arrows indicate the positions of the primers used in the PCR assays. Bars indicate the regions used to prepare probes which will hybridize to only the calcitonin-specific product (237), only the CGRP-specific product (168) or to both products (173).

3. RESULTS AND DISCUSSION

In order to test the splicing pattern produced by the pRSV Cal 2 and Cal 3 constructs they were transfected into various cell types. RNA was then prepared from the cells and used to prepare cDNA which was then amplified by the polymerase chain reaction (PCR) using a common sense oligonucleotide derived from the RSV 5'-untranslated region and anti-sense oligonucleotides specific for the calcitonin and CGRP-mRNAs (derived from exons 4 and 5, respectively). The PCR products were then electrophoresed on an agarose gel, blotted onto nitrocellulose and probed with probes specific for the calcitonin or CGRP product. The PCR products were shown to be derived from the expected regions by restriction enzyme digestion whilst the PCR amplification was shown to be quantitative relative to input cDNA in a dilution experiment (Fig. 2a).

When the ratio of calcitonin to CGRP-specific splicing was quantitated by scintillation counting of the labelled bands on the Southern blot (Fig. 2b and c), the Cal 3 construct produced the expected pattern with the neuronal cell lines ND3 and ND7 producing predominantly CGRP-specific splicing whilst the non neuronal 3T3 and HeLa cells produced predominantly calcitonin-specific splicing. In contrast transfection of the Cal 2 construct prevented the efficient production of CGRP transcripts even in cells which predominantly produces such transcripts when transfected with the Cal 3 construct.

Hence the Cal 3 construct contains all the sequences necessary for the correct splicing pattern of the Calc-1 gene with the ND3 and ND7 line producing predominantly CGRP transcripts as occurs in neurons in the

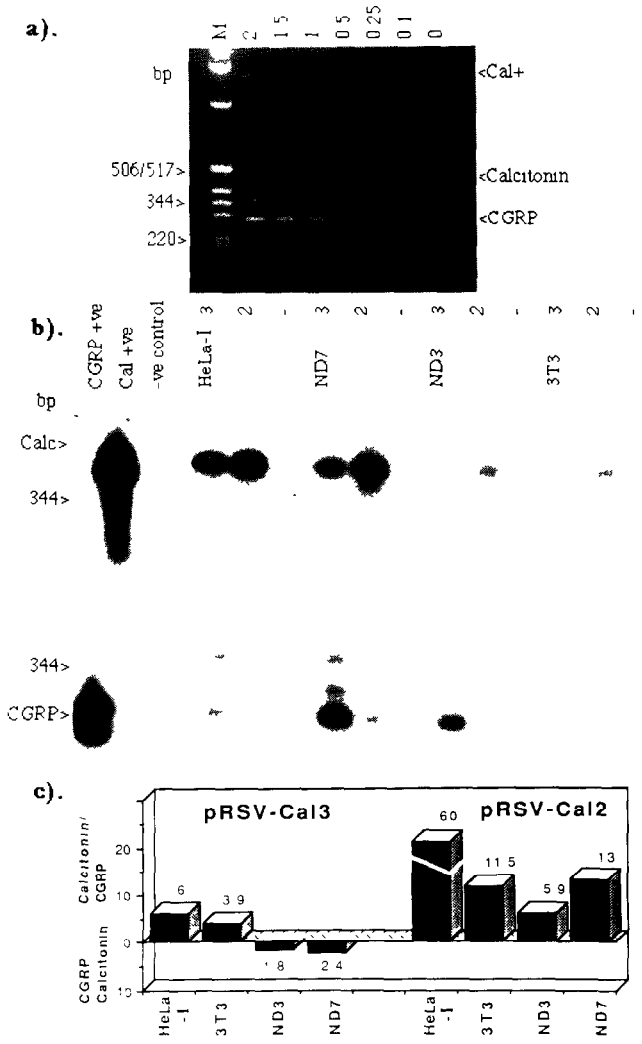

Fig. 2. (a) Agarose/ethidium bromide gel showing amplification of a dilution series of cDNA derived from ND7 cells transfected with pRSV-Cal 3. Amplification was carried out with the RSV sense primer and the h-Cal 4 and h-CGRP5 primers to detect the products of both calcitonin and CGRP-specific splicing of the RNA produced by pRSV-Cal 3. Numbers represent μl of input cDNA. 1 μl of cDNA was used in subsequent assays. The calcitonin and CGRP-specific products are indicated. Cal+ is a band amplified by the RSV and h-Cal 4 oligonucleotide primers and is derived from plasmid DNA or unspliced precursor RNA. (b) Duplicate southern blots of calcitonin and CGRP-specific PCR products probed with probes specific for each product. The calcitonin positive control is a 483 bp *Bgl*II fragment derived from exon 4 whilst the CGRP positive control is a 231 bp *Nco*I/*Pvu*II fragment derived from exon 5 Results are shown for cells transfected with either Cal 2 (2) or Cal 3 (3) or for untransfected cells (−). (c) Bar chart obtained from the data in panel a. Cell lines which carry out predominantly calcitonin-specific splicing are represented by bars above the origin whilst cells carrying out predominantly CGRP-specific splicing are represented by bars below the origin.

brain in vivo [6] whilst 3T3 fibroblasts and HeLa epithelial cells produce predominantly calcitonin transcripts as occurs in other cell types in vivo [6]. In contrast deletion of the CGRP-specific polyadenylation site together with a part of exon 5 and sequences downstream of it, disrupts this tissue specific splicing pattern and favours calcitonin-specific splicing even though intron 4 and the CGRP splice acceptor at the 5′ end of exon 5 are still present.

Although poly(A) site selection has been shown not to be the primary regulatory event controlling the tissue specific splicing of the CALC-1 transcript [5], replacement of the CGRP-specific poly(A) site with that of SV40 may favour rapid usage of the calcitonin-specific poly(A) site resulting in the removal of exon 5 prior to the onset of splicing and hence favouring calcitonin-specific splicing.

Alternatively, the deletion could act directly by affecting the splicing event itself either by interfering with the tissue specific regulation of the alternative splicing event or by reducing the utilization of the intron 4 splice sites by the general splicing machinery. Although in most cases the elements required for exon splicing are contained in the intervening intron [16] which would be present in the Cal-2 construct, there are a number of cases where exon sequences can also affect the splicing event. Thus the alternative splicing of several genes such as those encoding leukocyte common antigen [17], the *Drosophila* double sex gene [18] and the troponin T gene [19] can all be affected by alterations in the alternatively spliced exons themselves. Hence sequences in the distal part of the alternatively spliced exon 5 which is deleted in the Cal-2 construct might play a role in CALC-1 alternative splicing acting either by binding a *trans*-acting factor or by promoting the correct folding of the RNA prior to splicing.

It is clear therefore that sequences in the distal part of the CALC-1 gene are required for correct tissue-splicing, although further work will be required to identify the mechanisms by which they act. Moreover, the existence of such distal sequences is likely to explain the anomalous behaviour of truncated constructs lacking these sequences when added to in vitro RNA processing systems. Thus in the experiments of Bovenberg et al. [9] a construct truncated within exon 5 underwent CGRP-specific splicing in HeLa cell extracts which naturally carry out calcitonin-specific splicing. In order to induce this construct to undergo calcitonin-specific splicing, it was necessary to mutate the sequence at the calcitonin-specific branch acceptor, leading to the suggestion that this motif may play a role in the regulation of alternative splicing [10]. In view of our experiments however, it is possible that this effect simply reflects the use of distally truncated constructs and would not be observed with constructs containing downstream sequences. Hence studies on the mechanism of CALC-1 alternative splicing should not be carried out with constructs truncated in this region.

Acknowledgements: We thank Dr. P. Broad for the pRSV-Cal 2 and Cal 3 constructs. DAH was supported by the British Heart Foundation.

REFERENCES

[1] Leff, S E., Rosenfeld, M.G. and Evans, R.M. (1986) Annu. Rev. Biochem. 55, 1091–1117.
[2] Latchman, D.S. (1990) The New Biologist 2, 297–303.
[3] Amara, S.G., Jonas, V., Rosenfeld, M.G., Ong, E.S. and Evans, R.M. (1982) Nature 298, 240–244.
[4] Steenbergh, P.H., Hoppener, J.W.M., Zandberg, J . Van de Veen, W.J.M., Jansz, H.S. and Lips, C.J.M. (1984) Endocrin. Metabolism 59, 358–360.
[5] Leff, S.E., Evans, R.M. and Rosenfeld, M.G. (1987) Cell 48, 517–524.
[6] Crenshaw, E.B., Russo, A.F., Swanson, L W. and Rosenfeld, M.G. (1987) Cell 49, 389–398.
[7] Emeson, R.B., Hedjran, F , Yeakley, J.M., Guise, J.W. and Rosenfeld, M.G. (1989) Nature 341, 76–80
[8] Cote, G.J., Nguyen, I.N., Lips, C.J.M , Berget, S.M. and Gagel, R F. (1991) Nucleic Acids Res. 19, 3601–3606.
[9] Bovenberg, R.A.L., Adema, G J., Jansz, J.S. and Baas, P.D. (1988) Nucleic Acids Res. 16, 7867–7883.
[10] Adema, G J., van Hulst, K.L and Baas, P.D (1990), Nucleic Acids Res. 18, 5365–5373.
[11] Gorman, C.M. (1985) In: DNA cloning a practical approach, Glover, D M. (Ed.) Vol. 2, pp. 143–190. IRL Press
[12] Wilkinson, M. (1988) Nucleic Acids Res. 16, 10394
[13] Kawasaki, E S. (1990) In: PCR protocols eds. Innis M.A., Gelfand, D.H., Sninsky, J.J. and White, J.J. (Eds.) pp. 21–27, Academic Press
[14] Yamamoto, T., Jay, G. and Pastan, I. (1980) Proc. Natl. Acad Sci. USA 77, 176–180.
[15] Broad, P.M., Symes, A.J., Thakker, R.V. and Craig, R.K. (1989) Nucleic Acids Res. 17, 6999–7011.
[16] Padgett, R.A , Grabowski, P.J., Konarska, M.M., Seiler, S. and Sharp, P A. (1986) Annu. Rev. Biochem. 55, 1119–1150.
[17] Streuli, M. and Saito, J. (1989) EMBO Journal 8, 787–796.
[18] Burtis, K.C and Baker, B S. (1989) Cell 56, 997–1010.
[19] Cooper, T A. and Ordahl, C.P. (1989) Nucleic Acids Res. 17, 7905–7921.