Nucleic Acids Research, Vol. 19, No. 13 ***3601–3606***

Validation of an *in vitro* RNA processing system for CT/CGRP precursor mRNA

Gilbert J.Cote, Irene N.Nguyen, Cornelis J.M.Lips[2], Susan M.Berget[1] and Robert F.Gagel*
Department of Medicine, Baylor College of Medicine and Veterans Affairs Medical Center, Houston, TX 77030, [1]Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030, USA and [2]Department of Internal Medicine, University Hospital, State University of Utrecht, 3584 CX, Utrecht, The Netherlands

Received March 25, 1991; Revised and Accepted June 7, 1991

ABSTRACT

The pre-mRNA encoding calcitonin (CT) and calcitonin gene-related peptide (CGRP) is differentially processed in a tissue-specific fashion to include or exclude the calcitonin-specific exon 4. A minigene containing a viral first exon and exons 4, 5, and 6 from the human C-T/CGRP gene was correctly processed in transfected HeLa or F9 teratocarcinoma cells to produce mRNA that included or excluded exon 4, respectively. This processing decision could be reproduced *in vitro* using nuclear extracts from these two cell lines and an RNA precursor from a similar minigene. Supplementation of extract from HeLa cells with extract from F9 cells resulted in the F9 splicing pattern in which exon 4 was excluded. This model system may be useful for the purification of splicing factors important in the regulation of this splice choice.

INTRODUCTION

Alternative processing of eukaryotic mRNA precursors is a common and important event allowing the production of multiple mRNAs from a single transcript (1,2). Despite its relevance there has been slow progress toward an understanding of the mechanisms by which alternative processing decisions are regulated. The calcitonin gene provides an example of a tissue-specific alternative RNA processing event (3,4). The inclusion or exclusion of exon 4 (which encodes for calcitonin) is the primary processing decision (Figure 1). Exon inclusion occurs in thyroid C-cells, while exon exclusion is observed in certain neuronal cell types.

Several approaches have been used to study this processing event. Each has its advantages and disadvantages. Previous studies have duplicated the processing decision *in vivo* by transfection of the rat calcitonin gene into different cell lines (5,6). Transfected HeLa or lymphoblast cell lines include exon 4 during processing to make CT mRNA, whereas mouse F9 teratocarcinoma cells exclude the exon to make CGRP mRNA. The latter finding was expected because F9 cells are known to express CGRP (5,7). Mutation, deletion and substitution of calcitonin gene sequences prior to transfection has provided great insight into relevant *cis*-elements for regulation of the RNA processing decision. However, inherent in the use of transformed cell lines as a model is the assumption that RNA processing factors and events in transformed cells are identical to those observed in normal cells. Also, it is difficult to utilize this type of model system for the identification of splicing factors, many of which function as a part of large RNA-protein complexes.

An alternative approach is the utilization of transgenic animals. This allows examination of RNA processing in normal tissues. In transgenic mice expressing a metallothionein-calcitonin fusion gene, CT is produced in most tissues (8). These results have fostered the hypothesis that CT mRNA formation is the default processing pathway and a dominant regulatory factor is required for CGRP mRNA production (8). However, the transgene methodology is impractical for screening large numbers of mutations and can not be used to identify regulatory factors.

In vitro systems have been used widely because they provide the most certain method for purification of splicing factors. While these systems continue to provide insights into the mechanisms of constitutive pre-mRNA processing, the utilization of an *in vitro* system for alternative processing creates the additional concern of accurately duplicating *in vivo* processing. *In vitro* systems tend to process RNA inefficiently. RNAs containing 'weak' processing signals (sequences deviating from consensus), long stretches of intronic sequence, or many exons, are often poorly spliced. Most vertebrate genes are large (human calcitonin is 5682 bp(9)), and alternatively utilized exons frequently have weak processing signals (10)). To circumvent these problems in the calcitonin gene, we modified the sequence by truncation and introduced a single branch point modification, to develop a system which we believe has relevance to the *in vivo* situation.

* To whom correspondence should be addressed

MATERIALS AND METHODS

Cell Culture

Stock HeLa cell cultures were maintained on 100 mm plates in DMEM supplemented with 10% fetal calf serum as previously described (11,12). F9 mouse teratocarcinoma cells lines were maintained on gelatin-coated 100 mm plates in DMEM supplemented with 15% fetal calf serum. Culture plates were coated with a 0.1% gelatin solution prepared in phosphate buffered saline for a period of 2 hrs. HeLa cells used for extract preparation were subcultured from plates into RPMI medium supplemented with 7% fetal calf serum and grown in spinner culture. F9 cells were grown in T150 flasks for extract preparation.

Plasmids

Chimeric constructs were produced by standard cloning methodology. Constructs were derived from the adenovirus-based clone pIVPX (13), and the human calcitonin gene (14). The plasmid pCTG-6 used for transfections contains a *Sac 1/Hind 3* fragment of the human calcitonin gene, inserted 11 bp downstream of the adenovirus major late gene exon 1. Transcription was driven by a 594 bp *Bgl 2/Hind 3* fragment of the *rous sarcoma virus* long terminal repeat. IVPX/CT-7 contains sequences derived from a 1577 *Sac 1/Kpn 1* fragment of the human calcitonin gene, also inserted 11 bp downstream of the adenovirus major late gene exon 1. A *Hind 3* linker was inserted into a *Nco 1* site within intron 3, followed by an internal *Sph 1* to *Nco 1* deletion to remove the bulk of exon 4 and intron 4 sequences (15). A mutant of IVPX/CT-7 (IVPX/CT-16) altered for the branch point nucleotide was created by PCR-mediated amplification using an oligonucleotide primer containing a single nucleotide substitution. The amplified DNA fragment was subcloned as a *Sac 1/Pst 1* fragment. Construction of MINX was previously described (13). The mouse metallothionein gene exons 2 and 3 were subcloned as a *Bam H1/Hind 3* fragment into pSP65 (Promega, Madison, WI). The mouse adenosine deaminase exons 7−9 were subcloned as a *Hinc 2/Bam H1* fragment inserted into MINX at *Hind 3/Bam H1*.

Transfections

Plasmids were introduced into cell lines by DEAE-Dextran transfection as previously described (16). Cells were plated at ~25% confluency on 100 mm plates. Each plate was transfected with ~16 μg DNA at a final DEAE-Dextran concentration of 400 μg/ml. Total RNA was isolated 72 hrs post-transfection by RNAzol™ (Cinna/Biotecx Laboratories, Houston, TX) extraction according to the manufacturer.

In Vitro Splicing

HeLa nuclear extract was prepared by the method of Dignam (11). F9 nuclear extract was prepared by a slight modification of this method. Briefly, prior to extract preparation F9 cells were split and replated into 20 uncoated T150 flasks to a confluency of ~25%. F9 cells adhere poorly to uncoated flasks. Cells were harvested by centrifugation of medium containing dislodged cells (5 minutes, 3000×g, 4°C). The cellular pellet was washed 3× in phosphate buffered saline, then resuspended in 2 pellet volumes of Roeder Buffer A (11). Cells were allowed to swell 5 minutes on ice, then gently homogenized. Cell lysis was monitored microscopically. Nuclei were than harvested by centrifugation and nuclear extract preparation continued as previously described (11).

DNA templates were truncated at a *Dra 1* site located 82 nucleotides downstream of exon 5. *In vitro* transcription of these DNA templates was performed using 1−2 μg of template with SP6 polymerase (Promega, Madison, WI) in the presence of ^{32}P-UTP (800 Ci/mmole, New England Nuclear, Boston, MA) as previously described (15). All RNA substrates were purified on 5% acrylamide denaturing polyacrylamide gels prior to use. Splicing reactions contained: 1 mM creatine phosphate; 4 mM ATP; 1.5 mM Mg_2Acetate; 0.37 mM dithiothreitol; 4% polyethylene glycol (8000 MW); 8.8% glycerol; 44 mM KCl; and 8.8 mM Tris, pH 7.9. Supplementation experiments were performed with HeLa + F9 assays containing an equal amount of protein from each extract, and the HeLa alone assays containing protein equal to the total HeLa + F9. RNAs were extracted with phenol/chloroform at indicated times. Spliced products were analyzed by electrophoresis on denaturing polyacrylamide gels, followed by autoradiography as previously described (12, 13, 15).

Polymerase Chain Reactions

Spliced products were identified using oligonucleotide primers specific for human calcitonin gene exons 4 or 5 and adenovirus exon 1 in conjunction with reverse transcription and polymerase chain reaction (RT-PCR). RT-PCR reactions used either total RNA isolated from transfected cells or *in vitro* spliced RNA purified by phenol/chloroform extraction and concentrated by ethanol precipitation. RNA (15 μg total or ~5000 cpm *in vitro* spliced) was resuspended in annealing buffer and reverse transcribed with MLV transcriptase (BRL, Bethesda, MD) from the downstream PCR primer. The cDNA product was base hydrolyzed, neutralized, precipitated, and the resuspended cDNA amplified 30 cycles by Amplitaq™ PCR according to the manufacturer (Perkin Elmer/Cetus, Emeryville, CA). Primary products of the PCR reactions were analyzed by polyacrylamide electrophoresis and their identity confirmed by restriction enzyme analysis. PCR reactions were carried out under plateau conditions and therefore permit only qualitative assessment of major splicing pathways.

Oligonucleotide Primers

ADE primer: GCATCGCTGTCTGCGAGGGCCAGCT. 97 primer: GATCAGCACATTCAGAAGCAGGACA. DS8 primer: TTGACCATTCACCACATTGGTGTGC 96 primer: CTGTCCAGGCCCAACAGC. G41 primer: CAGCAAGCCTGCCAGCCGATGAGTCACACAGGTGGCAGTGT. GN primer: CTGCTCAGGCTTGAAGGTCC.

RESULTS

The goal of this study was to develop an *in vitro* system capable of duplicating tissue-specific RNA processing of CT/CGRP pre-mRNA. As a first step, we designed a minimal CT/CGRP gene RNA containing a first exon from the adenovirus major late transcription unit fused to the human CT/CGRP gene sequences within intron 3 (Figure 2A). This chimeric gene simplifies analysis in two ways. First, it deletes 3203 bp (exon 1 through intron 3) of sequence which we hypothesized was not required for the maintenance of alternative splicing. Second, the use of an adenovirus exon allows product identification within a background of endogenous CT and CGRP mRNAs. The chimeric gene (pCTG-6) was introduced into HeLa or F9 cells to determine if it would maintain the previously reported cell-specific RNA

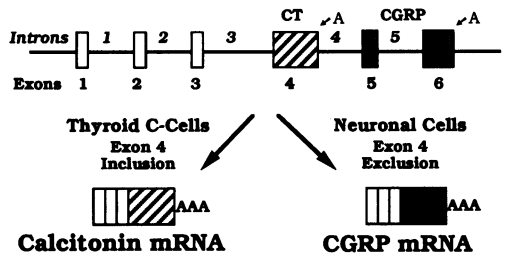
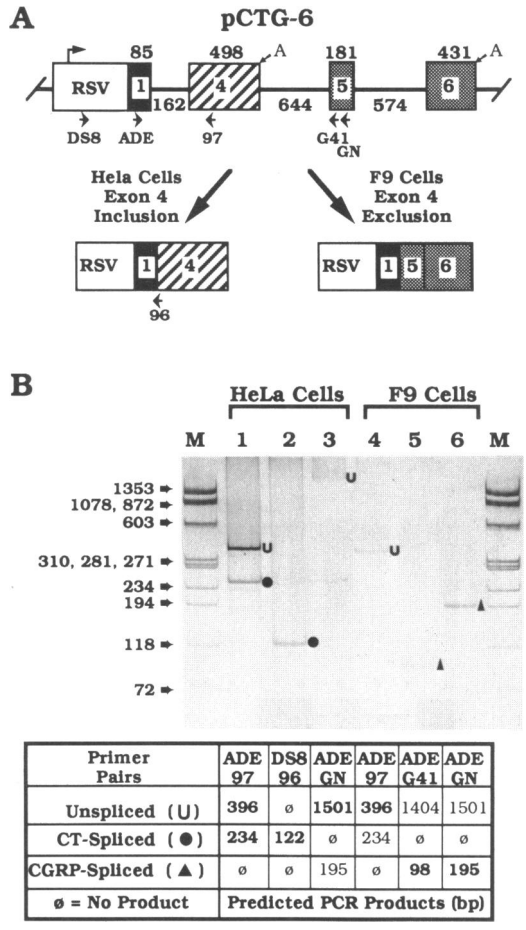


Figure 1. Alternative processing pathway of the calcitonin gene primary transcript. Schematic representation of the human CT/CGRP gene pre-mRNA illustrating the tissue-specific processing pathways.

processing (5,6). Total RNA was isolated 48 hrs after transfection and spliced products identified by RT-PCR. To differentiate between production of CT-specific mRNA (resulting from recognition of exon 4) and CGRP-specific mRNA (resulting from exclusion of exon 4) we used multiple downstream oligonucleotide primers specific to exon 4 or 5 (Figure 2A). To detect CT-specific splicing we used PCR primers specific to adenovirus exon 1 (ADE) and CT exon 4 (97). In transfected HeLa cells we observed products resulting from unspliced precursor (a 396 bp band) and CT-specific splicing (a 234 bp band)(Figure 2B, lane 1). In transfected F9 cells, only unspliced precursor was observed (Figure 2B, lane 4). An additional set of PCR primers was used to verify CT-specific splicing. The downstream primer (96) only recognizes the sequence created by appropriate exon 1 to 4 splicing. This primer when used with the DS8 primer gave the predicted CT-specific PCR product (Figure 2B, lane 2, 122 bp band). To detect CGRP-specific splicing we used PCR primers specific to CGRP exon 5 (G41 and GN). Both primers gave the expected CGRP-specific PCR products (98 bp for G41, 195 bp for GN, Figure 2B, lanes 5 and 6 respectively). Only unspliced precursor was observed in transfected HeLa cells with these primers (Figure 2B, lane 3). We detected no CGRP-specific mRNA. The identity of all PCR products was also confirmed by restriction enzyme analysis. No products bands were detected in untransfected HeLa or F9 cells (data not shown). We concluded from these experiments that all the sequences necessary for correct alternative processing of C-T/CGRP were present in the pCTG-6 minigene. Truncation of the gene and substitution of the adenovirus first exon did not affect cell-specific processing.

We began *in vitro* analysis using a derivative of pCTG-6 (IVPX/CT-7; Figure 3A). IVPX/CT-7 contains all known splicing regulatory sequences at the 3′ end of intron 3, the first 45 bases of the CT exon 4 and the entire sequence of exon 5 including flanking splicing signals. However, the bulk of the exon 4 (including its poly A site) and the first part of intron 4 were deleted as described in an earlier publication (15). The difference in intron size results from the insertion of a *Hind 3* linker (see Methods). While removal of these sequences prevents polyadenylation, it does not alter the splicing pattern observed *in vitro* in HeLa cell extract (15,17). *In vitro* transcription of IVPX/CT-7 produced a 634 nucleotide precursor RNA in which CT-specific splicing would remove a 172 nucleotide intron and CGRP-specific splicing would remove a 398 nucleotide intron (Figure 3A).

In vitro splicing reactions used nuclear extracts prepared from the same HeLa and F9 teratocarcinoma cells used for transfection studies. We hoped to reproduce the cell-specific *in vivo* processing



Primer Pairs	ADE 97	DS8 96	ADE GN	ADE 97	ADE G41	ADE GN
Unspliced (U)	396	ø	1501	396	1404	1501
CT-Spliced (●)	234	122	ø	234	ø	ø
CGRP-Spliced (▲)	ø	ø	195	ø	98	195
ø = No Product	Predicted PCR Products (bp)					

Figure 2. Cell-specific processing of CT/CGRP pre-mRNA. **(A)** A schematic representation of the pCTG-6 minigene and its spliced products. Numbers denote exon and intron sizes (nucleotides). Relative position of PCR primers (arrows) is illustrated. **(B)** Analysis of spliced RNA products by RT-PCR. Total RNA was isolated from HeLa (left) or F9 (right) cells 48 hrs post-transfection with pCTG-6. RNA was reverse transcribed using CT-specific primers (96 or 97) or CGRP-specific primers (G41 or GN). The resultant cDNA was amplified by PCR using promoter-specific (DS8) or exon 1-specific (ADE) primers. Products of the RT-PCR reactions were analyzed by polyacrylamide electrophoresis and silver staining. Primer pairs and the predicted PCR products are indicated below each lane. Note that unspliced precursor was rarely observed in transfected F9 cells. A *Hae 3* digest of ΦX 174 DNA served as molecular weight markers (M).

observed in these cell lines, in which the 3′ splice site in front of exon 4 would be recognized in HeLa extract, but ignored in F9 cell extract in favor of the 3′ splice site in front of exon 5. Our experiments used HeLa extract with and without supplementation with F9 extract. Extract supplementation has been successfully used to study alternative processing decisions *in vitro* (18−20). The HeLa + F9 extracts spliced the IVPX/C-T-7 precursor RNA to produce an RNA of the correct molecular weight to have resulted from the ligation of viral exon 1 to CGRP exon 5, (Figure 3B, lanes 5−8). No product was observed in the molecular weight range for a viral exon 1 to exon 4 ligation, implying a failure to recognize exon 4 in the mixed extract system. The exon-1-to-exon-5 pattern of splicing was also observed in reactions containing only F9 extract (data not shown).

In the HeLa cell system, IVPX/CT-7 precursor RNA was inefficiently spliced (Figure 3B, lanes 1−4). Direct examination of product RNAs revealed no bands of the correct molecular weight to be the products of splicing of exon 1 to either exon

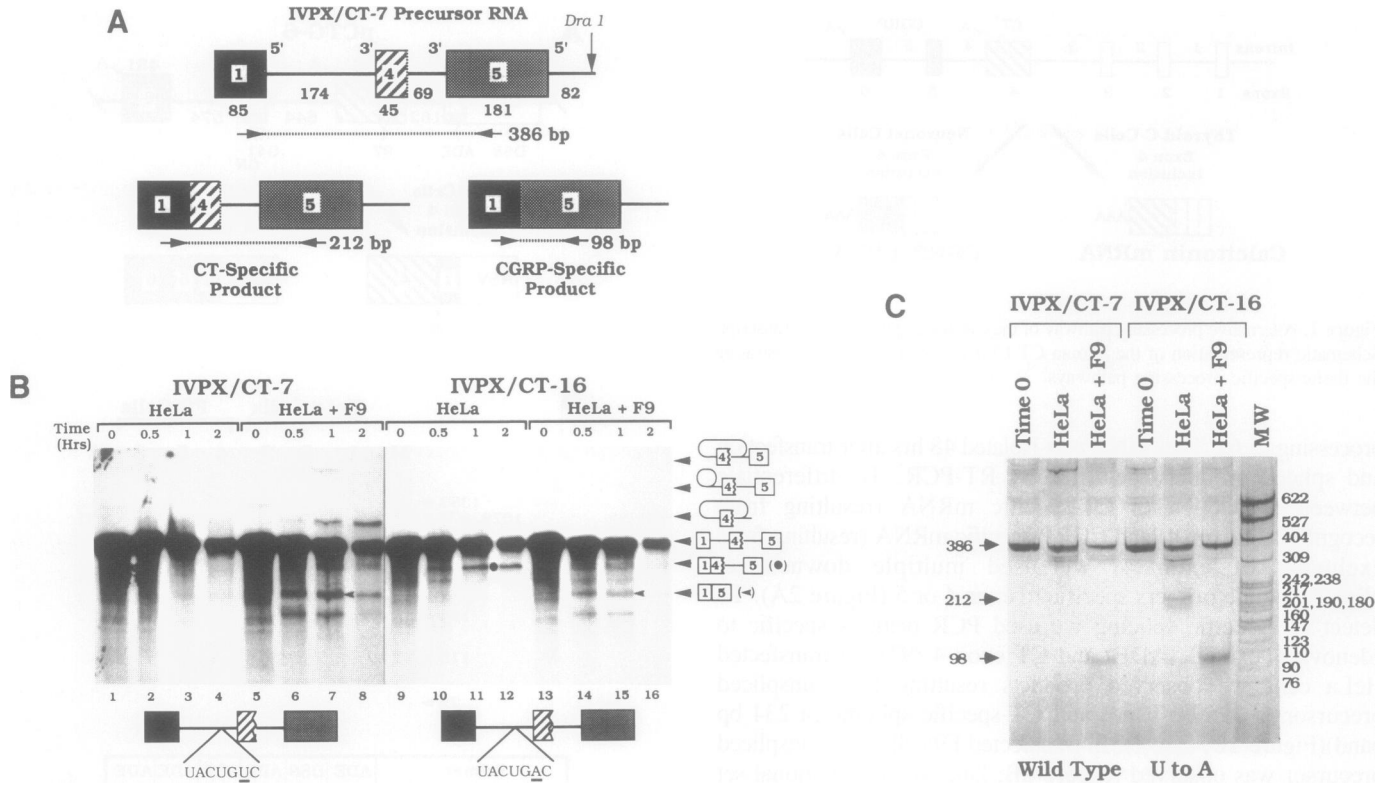


Figure 3. *In vitro* extract-specific processing of CT/CGRP pre-mRNA. **(A)** A schematic representation of IVPX/CT-7 and its spliced products. Numbers denote exon and intron sizes (nucleotides). Position of PCR primers (arrows) and predicted PCR products are illustrated. Note insertion of a *Hind 3* linker is responsible for the 12 nucleotide increase in the first intron (see Methods). **(B)** Radiolabeled precursor RNAs of IVPX/CT-7 or IVPX/CT-16 (with the indicated single nucleotide substitution, U to A, at position −23 relative to the start of exon 4) made by SP6 *in vitro* transcription were assayed for splicing activity in HeLa nuclear extract **(HeLa)** or HeLa extract supplemented with F9 nuclear extract **(HeLa + F9)**. Reaction aliquots were removed at indicated times and spliced products identified by denaturing polyacrylamide gel electrophoresis and autoradiography. The position of RNA precursor, exon-1-to-exon-4 spliced product (●), exon-1-to-exon-5 spliced product (—) and lariats are indicated. The major band located below exon-1-to-exon-5 spliced product in the HeLa + F9 lanes results from the high debranching activity present in F9 extract. Free intron 3 lariat comigrates with RNA precursor. **(C)** Analysis of *in vitro* spliced RNA products by RT-PCR. RNA precursors were subjected to *in vitro* splicing for 2 hrs in HeLa or F9-supplemented HeLa extract. Purified RNA was subjected to RT-PCR using ADE and G41 primers. Products of the RT-PCR reactions were analyzed by polyacrylamide electrophoresis and silver staining. IVPX/CT-7 pre-mRNA splicing (left) was compared to that of IVPX/CT-16 (right). DNA bands corresponding to unspliced precursor were observed in all lanes (386 bp bands). Correct splicing of exon 1 to exon 4 predicts an amplification product of 212 bp, whereas an exon-1-to-exon-5 splice predicts an amplification product of 98 nucleotides.

4 or exon 5 within the time frame of the experiment in Figure 3. A similar observation was reported by others for CT pre-mRNAs using the native CT gene exon 3 (21). Truncation of the RNA by removal of exon 5, utilization of the entire exon 4, or inclusion of the entire exon 4 with truncation by removal of exon 5 did not improve the efficiency of the exon-1-to-exon-4 splice (17). The observed lack of activity was not caused by use of inactive HeLa extract because this extract readily spliced other RNA precursors (Figure 4, and data not shown). Several modifications of assay conditions also failed to increase splicing activity (data not shown). The failure of the HeLa system to splice exon 1 to exon 4 could be interpreted in several ways. One possibility is that our extract preparation is absent a factor required for a C-T-specific splice. We cannot completely reject this possibility, but think it unlikely because the efficiency of this splice was not improved by several modifications of extract preparation or utilization of extracts from CT-producing cell lines (22). An alternative possibility is that splicing of exon 1 to CT exon 4 is a slow and inefficient process which is magnified by the less efficient *in vitro* assay. Studies of CT pre-mRNA processing *in vivo* indicate that the intron prior to the CT exon is the last removed during normal processing of this RNA (21), providing additional support for this viewpoint.

Examination of the splicing signals preceding exon 4 reveal that they significantly deviate from consensus (23,24) and from the signals preceding other exons within this gene. The presence of a noncanonical uridine, instead of an adenosine, at the intron 3 branch point acceptor and a shortened polypyrimine tract are the major differences. Alteration of this U to a canonical A has been reported to increase exon 4 recognition (25).

To see if we could prepare an *in vitro* splicing precursor of higher activity, we mutated the branch point acceptor of intron 3 in IVPX/CT-7 from U to A and assayed the effect of this alteration on *in vitro* splicing. This precursor spliced exon 1 to exon 4 with increased efficiency using HeLa nuclear extract as determined by the appearance of a product band of the correct molecular weight (Figure 3B, lanes 9−12). Alteration of the branch point did not appear to affect extract-specific processing; exon 4 sequences continued to be excluded in reactions in which HeLa extract was supplemented with F9 extract (Figure 3B, lanes 13−16). Therefore, the altered precursor RNA displayed an *in vitro* splicing pattern identical to the *in vivo* pattern observed with the wild type gene; exon 4 was recognized in HeLa cell extract and skipped in F9 cell extract.

To determine the authenticity of spliced products, reactions were analyzed by reverse transcription and PCR-mediated

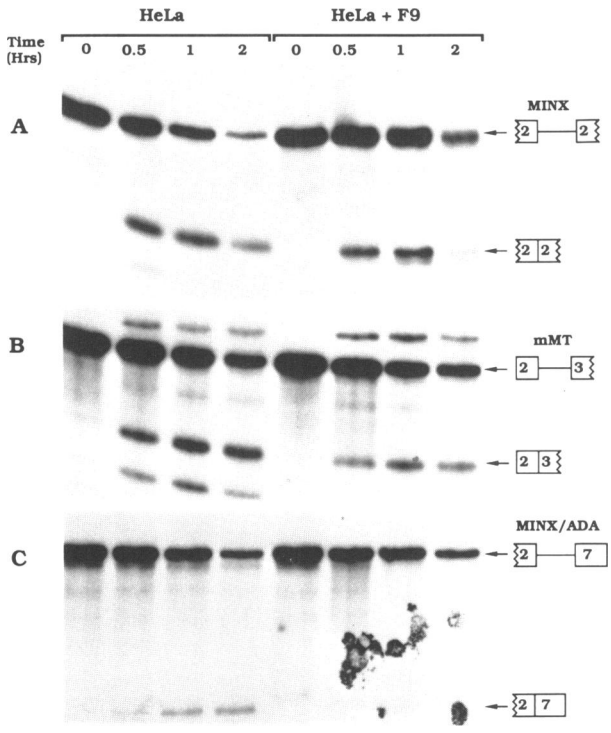


Figure 4. The effect of HeLa extract or HeLa + F9 extract on the splicing of constitutive exons. Radiolabeled precursor RNAs were assayed as described in Figure 2. Precursor RNAs contained exons from: (**A**) adenovirus (MINX) (**B**) mouse metallothionein, and (**C**) a first exon from adenovirus and a second exon from mouse adenosine deaminase. Numbers correspond to exon position in the parental gene. The position of ligated product RNAs are indicated.

mapping (Figure 2C). Oligonucleotide primers specific for exons 1 and 5 (ADE and G41) were used to detect all possible splicing products (Figure 2A). A CGRP-specific spliced product band (exclusion of exon 4; 98 bp) was observed for both the wild type (IVPX/CT-7) and mutant (IVPX/CT-16) precursor RNA when splicing reactions were carried out in the presence of F9 extract (Figure 3C). This analysis revealed no trace of a spliced product in which exon 4 was recognized. Therefore, the F9 extract-mediated *in vitro* splicing reproduced the fidelity of processing observed in transfected F9 cells. The CT-specific 212 bp product, indicative of recognition of exon 4 and splicing of exon 1 to exon 4 was observed for the mutant precursor RNA (IVPX/CT-16) but not for the wild type RNA (IVPX/CT-7) in the presence of HeLa extract.

We concluded from these results that modification of the branch point nucleotide improved the efficiency of the CT-type splice in HeLa extract without affecting the F9-specific 'skip' splice. We believe this is related to the improvement in branch/splice site strength, but cannot at this time completely exclude the possibility that this modification alters an important *cis*-regulatory element.

We also considered the possibility that the splicing differences were related to the quality or efficiency of the extract. To control for this possibility, several different two exon splicing constructs (human adenovirus, murine metallothionein, or murine adenosine deaminase) were assayed in both systems. Precursor RNAs containing exons from human adenovirus (Figure 4A) or from murine metallothionein (Figure 4B) spliced accurately and with equal efficiency in HeLa or F9-supplemented HeLa extracts. A precursor RNA containing a murine adenosine deaminase exon (Figure 4C) was spliced less efficiently in F9-supplemented extract than in HeLa extract. We concluded from these experiments that both extracts were active in the splicing of these constructs giving us little reason to believe that the splicing differences observed could be explained merely on the basis of extract activity. The differences in splicing observed for the MINX/ADA construct was reproducible. The relevance of this finding is not clear but may reflect the presence of cell-specific splicing factors.

DISCUSSION

The *in vitro* system described reproduces the cell-specific processing choice for the CT/CGRP gene observed in intact cells. Our results clearly show that removal of exons 1–3 of the C-T/CGRP and replacement by adenovirus exon 1 does not affect the processing decision. Further truncation of the gene by removal of intronic sequence, as described by others (6,10), also does not affect the processing choice. Processing of this minigene construct in the F9 *in vitro* system shows a correct cell-specific splice, whereas we found it necessary to modify the intron 3 branch point acceptor nucleotide from a U to an A to observe efficient CT-type splicing.

We believe this *in vitro* system possesses the minimum elements necessary for tissue-specific splicing. IVPX/CT-16 has a valid first exon and all appropriate splice sites necessary for either a CT- or CGRP-specific splice. The necessity to modify the intron 3 branch point acceptor to obtain efficient splicing of exon 1 to exon 4 is of some concern. Inefficient splicing may play an important role in the regulation of this processing decision. This would be especially true if specific regulatory factors were required to compete with normal splicing factors in order to modify the splice decision. In fact, we find additional modifications of IVPX/CT-7 which further strengthen the 3′ splice site of exon 4, act to inhibit splicing to exon 5 in F9 supplemented extract (unpublished observation).

Complementation of HeLa with F9 extract or use of F9 extract alone in the *in vitro* system results in the exclusion of exon 4 from the final spliced product. Our studies suggest this effect results from the interaction of dominant factor(s) present in F9 cell extract rather than a general increase in splicing efficiency. We believe that appropriately sized splicing intermediates and products, and the independent RT-PCR results provide reasonable evidence to authenticate the splice products. Therefore, these results and those of prior investigators (5,6) support the hypothesis that an F9-specific factor exists which causes the exclusion of exon 4 from the final splice product.

How such a factor might function is not yet clear. Earlier studies in our system identified an absolute requirement for the first 45 nucleotides of exon 4 to prevent splicing to exon 5 in HeLa nuclear extract (15). Other evidence suggests a short sequence upstream from the intron 3 branch point is necessary for exclusion of exon 4 (6). All investigators in this field agree that sequences within the intron 3 branch/splice site and proximal exon 4 are the most likely sites of interaction (6,15,25). Whether a single F9 factor causes the exclusion of exon 4 or an additional factor (as we believe) is required for exon 4 inclusion, is not at present clear.

We believe this model system or some future derivative will be useful to address several of these questions and provide a complementation-type assay for purification of the putative F9 factor.

ACKNOWLEDGMENTS

We thank Richard D.Palmiter for providing the mouse metallothionein gene, and Rodney E.Kellems for a fragment of the mouse adenine deaminase gene. These studies were supported by USPHS grants RR 05425 (G.J.C.), GM 35670 (S.M.B.) and DK 38146 (R.F.G.), and a Veterans Administration Career Development Award to R.F.G.

REFERENCES

1. Breitbart, R.E., Andreadis, A. and Nadal, G.B. (1987) *Annu Rev Biochem* **56**, 467–495.
2. Leff, S.E., Rosenfeld, M.G. and Evans, R.M. (1986) *Annu Rev Biochem* **55**, 1091–1117.
3. Rosenfeld, M.G., Amara, S.G. and Evans, R.M. *Science* **225**, 1315–1320.
4. Amara, S.G., Jonas, V., Rosenfeld, M.G., Ong, E.S. and Evans, R.M. (1984) *Nature* **298**, 240–244 (1982).
5. Leff, S.E., Evans, R.M. and Rosenfeld, M.G. (1987) *Cell* **48**, 517–524.
6. Emeson, R.B., Hedjran, F., Yeakley, J.M., Guise, J.W. and Rosenfeld, M.G. (1989) *Nature* **341**, 76–80.
7. Binet, E., Laurent, P. and Evain-Brion, D. (1985) *J. Cell Physiol.* **124**, 288–292.
8. Crenshaw, E.B., Russo, A.F., Swanson, L.W. and Rosenfeld, M.G. (1987) *Cell* **49**, 389–398.
9. Broad, P.M., Symes, A.J. , Thakker, R.V. and Craig, R.K. (1989) *Nucleic Acids Res.* **17**, 6999–7011.
10. Bovenberg, R.A., Adema, G.J., Jansz, H.S. and Baas, P.D. (1988) *Nucleic Acids Res* . **16**, 7867–7883.
11. Dignam, J.E., Lebovitz, R.M. and Roeder, R.G. (1983) *Nucleic Acids Res.* **11**, 1475–1489.
12. Berget, S.M. and Robberson, B.L. (1986) *Cell* **46**, 691–696.
13. Zillmann, M., Zapp, M.L. and Berget, S.M. (1988) *Mol Cell Biol* **8**, 814–821.
14. Cote, G.J., Abruzzese, R.V., Lips, C.J.M. and Gagel, R.F. (1990) *J Bone Min Res.* **5**, 165–171.
15. Cote, G.J., Nguyen, I.N., Berget, S.M. and Gagel, R.F. (1990) *Mol. Endo.* **4**, 1744–1749.
16. Lopata, M.A., Cleveland, D.W., and Sollner-Webb, B. (1984) *Nucl. Acids Res.* **12**, 5707–5717.
17. Cote, G.J., Berget, S.M., Nguyen, I.N. and Gagel, R.F. (1990) In Cohn, D.V., Glorieux, F.H. and Martin, T.J. (eds.), Calcium Regulation and Bone Metabolism, Basic and Clinical Aspects. Excerpta Medica, Amersterdam Vol. 10, pp. 64–68.
18. Ge, H. and Manley, J.L. (1990) *Cell* **62**, 25–34.
19. Krainer, A.R., Conway, G.C. and Kozak, D. (1990) *Cell* **62**, 35–42.
20. Siebel, C.W. and Rio, D.C. (1990) *Science* **248**, 1200–1207.
21. Bovenberg, R.A., Meerendonk W.P., P.D. Baas, P.H. Steenbergh, C.J. Lips and Jansz, H.S. (1986) *Nucleic Acids Res.* **14**, 8785–8803.
22. Cote, G.J. and Gagel R.F. (1986) *J. Biol. Chem.* **261**, 15524–15528
23. Reed, R. and Maniatis, T. (1988) Genes Devel. **2**, 1268–1276.
24. Adema, G.J., Bovenberg, R.A., Jansz, H.S. and Baas, P.D. (1988) *Nucleic Acids Res* **16**, 9513–9526.
25. Adema, G.J., van Hulst, K.L. and Baas, P.D. (1990) *Nucl. Acids Res.* **18**, 5365–5373.