Cis requirements for alternative splicing of the cardiac troponin T pre-mRNA

Thomas A.Cooper*, Michael H.Cardone and Charles P.Ordahl

Department of Anatomy, University of California, San Francisco, CA 94143, USA

Received May 19, 1988; Revised and Accepted August 2, 1988

ABSTRACT

The cardiac troponin T (cTNT) pre-mRNA splices 17 exons contiguously but alternatively splices (includes or excludes) the fifth exon. Because both alternative splice products are processed from the same pre-mRNA species, the cTNT pre-mRNA must contain cis-acting sequences which specify exon 5 as an alternative exon. A cTNT minigene (SM-1) transfected into cultured cells produces mRNAs both including and excluding exon 5. The junctions of exons 4-5-6 and 4-6 in the cTNT minigene mRNAs are identical to those of endogenous cTNT mRNAs and no other exons are alternatively spliced. Thus, the SM-1 pre-mRNA is correctly alternatively spliced in transfected cells. To circumscribe the pre-mRNA regions which are required for the alternative nature of exon 5, we have constructed a systematic series of deletion mutants of SM-1. Transfection of this series demonstrates that a 1200 nt pre-mRNA region containing exons 4, 5, and 6 is sufficient to direct alternative splicing of exon 5. Within this region are two relatively large inverted repeats which potentially sequester the alternative exon via intramolecular base-pairing. Such sequestration of an alternative exon is consistent with models which propose pre-mRNA conformation as being determinative for alternative splicing of some pre-mRNAs. However, deletion mutants which remove the majority of each of the inverted repeats retain the ability to alternatively splice exon 5 demonstrating that neither is required for cTNT alternative splice site selection. Taken together, deletion analysis has limited cis elements required for alternative splicing to three small regions of the pre-mRNA containing exons 4, 5, and 6. In addition, the cTNT minigene pre-mRNA expresses both alternative splice products in a wide variety of cultured non-muscle cells as well as in cultured striated muscle cells, although expression of the cTNT pre-mRNA is normally restricted to striated muscle. This indicates that cis elements involved in defining the cTNT exon 5 as an alternative exon do not require muscle-specific factors in trans to function.

INTRODUCTION

The processing of most higher eukaryotic primary transcripts (pre-mRNAs) requires the removal of introns and the splicing of exons to form functional mRNAs. Generally, all of the exons within a pre-mRNA are contiguously spliced to produce a single mRNA. However, the pre-mRNAs of some genes are unusual in that they are processed using alternative splice sites, giving rise to multiple mRNAs. Alternative splicing appears to be an economical means for the cell to generate protein diversity (1, 2). Recently described examples of alternatively spliced genes include those encoding proteins with a wide range of cellular functions such as cytoskeletal and contractile proteins (3-6), oncogenes and proto-oncogenes (7-10), nerve growth factor (11) Drosophila EGF receptor homolog (12), T-cell receptor (13), N-CAM (14), and clathrin light chains (15).

Several studies on different genes have identified cis-acting roles for pre-mRNA sequences in the identification of alternative of splice sites, such as the polyadenylation signal (16), intron spacing (17), exon sequences (18, 19) or various intron sequences (20,21). Pre-mRNA hairpin structures have also been postulated to play a role in determining the alternative nature of splice sites (20, 22-24). However, a cohesive picture of the mechanism by which a splice site can be used alternatively has yet to emerge.

We have used the chicken cardiac troponin T (cTNT) gene to localize cis-acting sequences which define an exon for alternative splicing. The cTNT gene expresses a single 9200 nucleotide pre-mRNA containing 18 exons that is processed along either of two splicing pathways (see the top of Figure 1). In one pathway, all 18 exons are included into the mature mRNA. In the second pathway, only 17 exons are included; exon 5 is completely excluded from the mature mRNA and exons 4 and 6 are spliced together (25). In vivo, cTNT alternative splicing is under strict developmental regulation; the majority of cTNT mRNAs from early embryonic heart include exon 5 while the vast majority of cTNT mRNAs from adult heart exclude this exon. At intermediate developmental stages, both mRNAs are detected at levels that reflect the progressive transition from one cTNT mRNA isoform to the other (25). During this developmental transition, exon 5 is the only exon within the cTNT pre-mRNA which is alternatively spliced. These observations suggest that regulation of cTNT alternative splicing occurs on at least two levels. On the first level, exon 5 must be distinguished from the other 17 constitutive exons as the sole alternative exon in the cTNT pre-mRNA. On the second level, the frequency with which exon 5 is included (or excluded) must be modulated according to developmental stage. These two levels of regulation may employ shared or separate cis and trans-acting factors.

In this paper, we have focussed our attention upon the first level of regulation: Specifically, what components of the cTNT pre-mRNA are required for exon 5 to function as an alternative exon? Such components may reside close to, or far from exon 5 itself. Nucleotide sequence analysis does not distinguish exon 5 from constitutively spliced exons because flanking 5' and 3' splice sites as well as potential lariat branchpoints fit well to the consensus sequences (26-30). Moreover, distal sequences have been implicated in the alternative splicing of some pre-mRNAs (16). Therefore, we have performed a systematic deletion and transient transfection analysis of a cTNT minigene to localize the sequences required for alternative splicing of cTNT exon 5. Using this system we have eliminated several potential models for cTNT alternative splicing and have determined that all of the cis-requirements for this process are limited to a relatively small region of the pre-mRNA local to exons 4, 5 and 6.

MATERIALS AND METHODS

Construction of Recombinant Plasmids

Recombinant DNA methods were performed according to Maniatis et al. (31). Restriction endonucleases and calf intestine alkaline phosphatase were obtained from Boehringer Mannheim;

T4 DNA ligase and Bal 31 exonuclease from New England Biolabs. S1 nuclease was obtained from Miles Laboratories.

SM-1 (Figure 1): *AvaII partial digests were performed on a cTNT genomic clone; digests at* the site in exon 1 were made blunt ended and HindIII linkers were added. This site was fused with the HindIII site at the 3' end of a Rous sarcoma virus long terminal repeat (RSVLTR) cassette (32). The RSV enhancer, promoter, and transcription initiation site is within this cassette. A 3.0 kb genomic fragment containing exons 10-17 was removed by fusing blunt-ended HindIII and BamHI sites as indicated in Figure 1.

5' End Deletion Mutants (Figure 4): SM-5'-1; Sau3AI partial restriction digests were used to cut a site 39 nt upstream of exon 3. SM-5'-2; Oligonucleotide directed mutagenesis (33) was used to introduce a BamHI site into exon 4. For both constructions, the Sau3AI and BamHI sites were made blunt ended and fused with the HindIII site of the RSVLTR following addition of HindIII linkers.

3' End Deletion Mutants (Figure 5): SM-3'-1; cTNT sequence 3' to the BglII site was removed and replaced by fusion with an XhoI site at position 1766 of the skeletal α-actin gene (34) which is 140 bp upstream from the last actin exon (exon 7). This construction contains about 2.0 kb of 3' flanking genomic sequence from skeletal α-actin. SM-3'-2; the BglII site in intron 17 was filled in and fused with a HaeIII site located 34 bp 3' to exon 6. SM-3'-3; The AccI to ClaI fragment was removed from SM-3'-1 and replaced with an AccI to HindIII cTNT cDNA fragment. This fusion restores exon 6 and contains cDNA sequence to the HindIII site in exon 13 (see ref. 25).

Deletions within the inverted repeat, IR1 (Figure 6): A BglII fragment containing cTNT exons 2-9 was cloned into the BamHI site of plasmid 327ACRHMP32#3 (35). Partial FnudII digestions were performed on the resulting plasmid. Following BglII linker addition to the selected site, the cTNT insert was excised from the plasmid with ClaI. The SM-1 plasmid had been cut with BglII and collapsed following filling in. This generates a ClaI site which accepts the mutated BglII cTNT fragment and restores the gene. Bal 31 deletions were performed from the unique BglII site at FnuDII. XhoI linkers were added following the deletion reaction and the plasmids were recircularized by ligation fusing the 5' deletions with the XhoI site giving all mutants identical 3' deletion endpoints.

Deletion within the inverted repeat, IR2 (Figure 7): Following partial digestions, a ClaI linker was added to a PvuII site located within intron 4. Bal 31 deletions were performed, followed by addition of ClaI linkers and recircularization. Deletion endpoints were determined by nucleotide sequence analysis.

Cell Culture, Transfection, RNA Extraction

Media and media components were obtained from Cell Culture Facility at the University of Calif., San Francisco, CA. or from Gibco Laboratories. Primary cultures of chick embryo fibroblasts (CEF) were prepared from day 11 chick embryos after removing heads, viscera, and

limbs (36). Tissue was disscociated by trypsin and cells grown in M199 medium supplemented with 10% tryptose phosphate, 10% fetal calf serum (HyClone Laboratories), and 2% chicken serum. Fibroblast cultures were passaged at least three times prior to transfection. Skin fibroblast cultures were prepared from day 11 chicken embryos in which the skin was carefully separated from underlying tissue taking care to obtain muscle-free tissue. Following treatment with trypsin, these cultures were treated the same as CEF cultures. Quail fibroblast cell line QT-35 was a gift of M. Linial. These cells were maintained as above.

Transfection was done according to Grahm and van der Eb (37) and Gorman et al (38). Cells were plated at 8 x 10^5 cells per 60mm plate. Twenty-four hours after plating, media was changed and three hours later, cells were transfected with DNA-calcium precipitates using 10 ug DNA per 60 mm plate. DNA-calcium phosphate precipitates remained on the cells for 18 hrs; this media was then removed, the cells washed with PBS, and fresh media added. Cells were harvested 24 hrs later.

Total cellular RNA was extracted using the LiCl/urea procedure (39) with the modifications below. Cells were washed once in 3 ml of PBS then lysed directly on culture dishes with 0.75 ml of lysis buffer (3M lithium chloride, 6M urea, 1mM EDTA, 20mM Tris-HCl, pH 7.5). Cells were scraped into 1.5ml Eppendorf tubes and sonicated. After 16 hours at -20° C, RNA was pelleted then purified with two phenol-chloroform extractions followed by three ethanol precipitations.

RNA Analysis

S1 nuclease analysis was performed using double stranded DNA probes as described (25). These probes were ^{32}P-5' end-labeled using polynucleotide kinase (Pharmacia) or 3'-end-labeled using T4 DNA polymerase (Bruce Alberts, UCSF) (31). The cDNA used for Probe A (Figures 3 and 5) was labeled within the first 120-150 nt of its 5' end (exons 1-3) to ensure that band intensity represents RNA levels for both mRNAs. This also prevents the detection of a protection fragment by exons 3' to exon 5 in mRNAs that exclude exon 5 (see 3'-3 lane, Figure 5). Primer extension using a single stranded primer was performed as described by McKnight et al. (40). Primer extension using double stranded cDNA fragments was performed as described (25).

RESULTS

Construction of a cTNT minigene, SM-1

Construction of the cTNT "minigene" (designated splicing minigene 1 or SM-1) used for transfection is diagrammed in Figure 1. Expression of the cTNT promoter is resticted to striated muscle (41). To express abundant levels of SM-1 pre-mRNA in diverse cell types, the cTNT gene initiation site, promoter and 5' flanking region were replaced by a Rous sarcoma virus long terminal repeat (RSVLTR) cassette which was fused to an AvaII site within cTNT exon 1 (see Methods). This cassette contains the RSV transcription initiation site in addition to RSV promoter and enhancer elements. The size of the cloned cTNT gene was reduced by one third by the deletion of a genomic segment containing exons 10-17. SM-1 contains the cTNT poly (A) addition

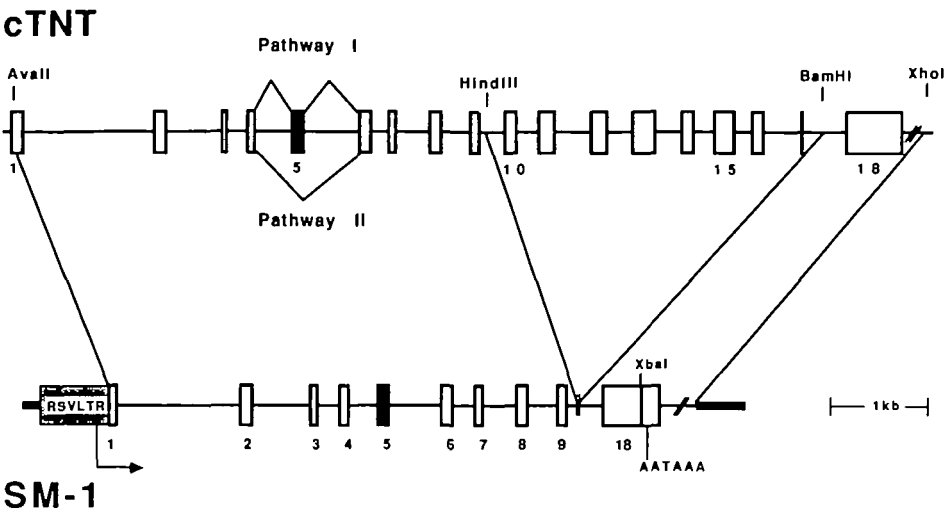


Figure 1: Alternative splicing of cTNT and construction of SM-1. Top line is a schematic illustration of the cTNT gene (exon size not precisely to scale). The two alternative splicing pathways of exon 5 are indicated as described in the text. SM-1 is shown at the bottom. Transcription initiation (arrow) is within the Rous sarcoma virus long terminal repeat (RSVLTR) (64), 36 nt upstream of the fusion site to cTNT exon 1. The restriction sites used for cloning are indicated as is the location of the poly (A) addition sequence. Heavy lines indicate vector.

site consensus sequence, poly (A) addition site, and approximately 900 bp of 3' flanking genomic sequence. Finally, an XbaI linker was inserted into an RsaI site in the 3' untranslated region 119 nt from the poly (A) addition site.

SM-1 Transcripts contain the correct 5' and 3' termini

The ability of SM-1 to direct synthesis of stable mRNA was tested by transfection into cultured skin fibroblasts (SF) and chick embryo fibroblast (CEF) cells by standard procedures (see Methods). Total cellular RNA was extracted 40-48 hours after transfection and analyzed for SM-1 mRNA. The 5' end of SM-1 RNA was determined by primer extension analysis using a 5'-end labeled oligomer that hybridizes across nucleotides at the junction of cTNT exons 1 and 2 (Figure 2A and ref. 25). RNA from CEF and SF cultures transfected with SM-1 both yield runoff products of 67 nt (Figure 2B). This result indicates that the SM-1 mRNA is correctly initiated at the expected site within the RSVLTR and that SM-1 mRNA is relatively stable in CEFs and SFs. No endogenous cTNT mRNA is detected in RNA from these cultures, as expected (compare lanes SF and CEF to lane H).

A single poly (A) addition site has been identified for the endogenous cTNT mRNA from the nucleotide sequence of several independently cloned cDNAs and the cTNT gene (25). S1 nuclease protection analysis was used to determine whether the transfected gene is able to produce SM-1

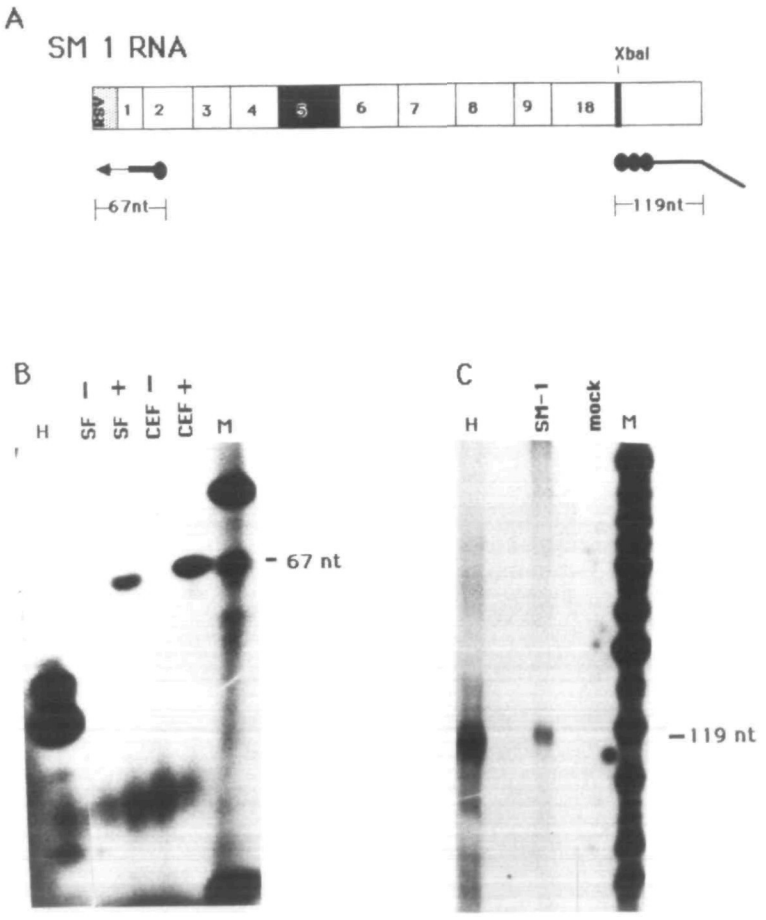


Figure 2: Determination of SM-1 RNA 5' and 3' ends. (A) diagram of SM-1 RNA showing primer extension and S1 nuclease probes and expected products. The labeled end of each probe is indicated by filled circles. The oligomer for primer extension was ^{32}P 5' end-labeled by polynucleotide kinase. The S1 nuclease probe for the 3' end was ^{32}P end-labeled from the XbaI site using T4 polymerase (see Methods). End-labeled HpaII cut pBR322 was used as marker. (B) Primer extension and, (C) S1 nuclease assays. Because both SM-1 and the S1 probe contain the artificial XbaI linker, SM-1 RNA protects a fragment 6 nt longer than does endogenous cTNT mRNA. Each lane contains either 5ug total RNA from tissue samples or 20 ug total RNA from transfected (+) or mock transfected (-) cultures. CEF cultures were assayed in (C). SF, skin fibroblast; CEF, chick embryo fibroblast; M, marker; P, probe; H, day 18 embryonic heart muscle RNA.

RNA with the correct 3' end. The complementary strand of an SM-1 genomic fragment which spans the putative poly (A) addition site was labeled from the artificial XbaI site. As shown in Figure 2C, both SM-1 and endogenous cTNT mRNAs protect fragments that indicate the use of

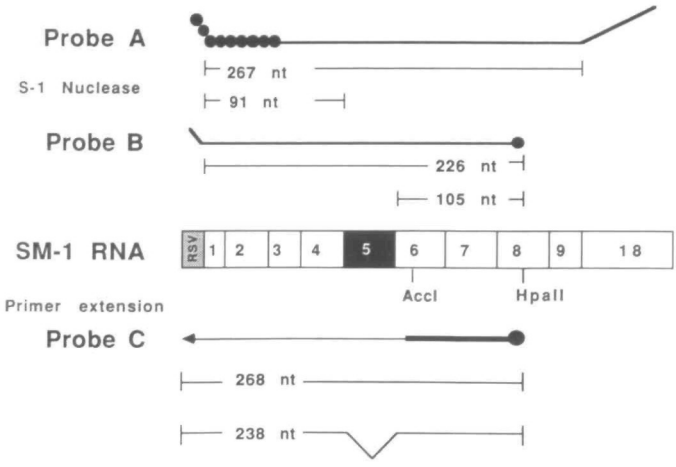
the single cTNT poly-A addition site. This result demonstrates that the unique poly (A) addition site used by the endogenous gene is also used by SM-1 following transfection into non-muscle cells. We conclude that, like the endogenous cTNT mRNA, the mRNA of the transfected SM-1 gene contains a single 5' end and 3' end and are therefore processed from a single primary transcript.

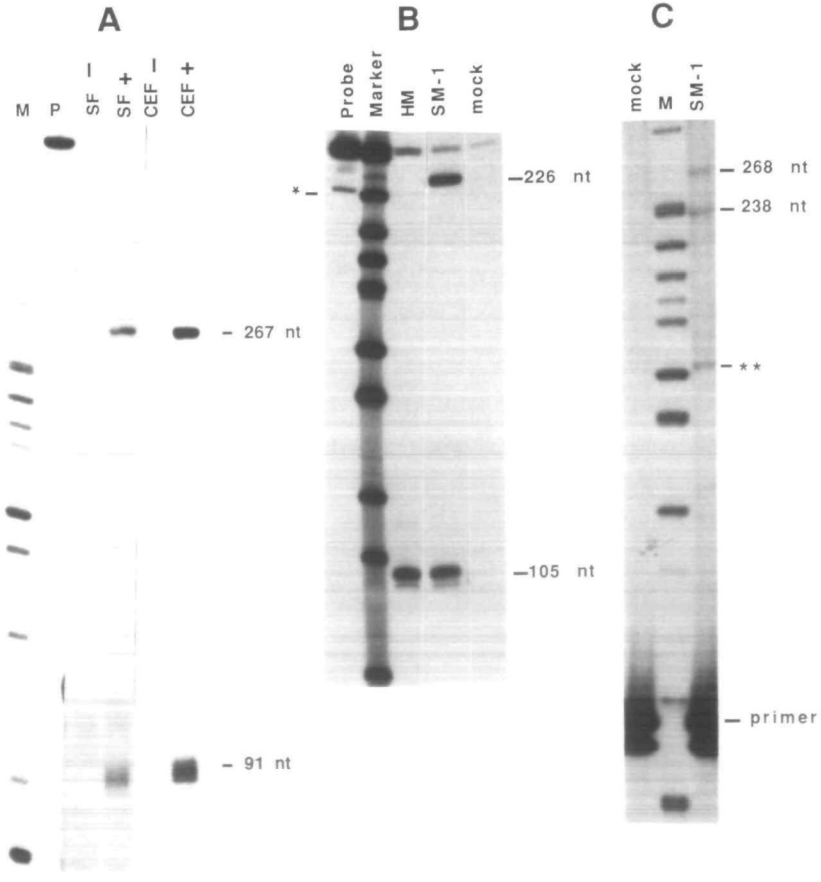
Exon 5 is alternatively spliced in SM-1 pre-mRNA

To determine whether the transfected SM-1 pre-mRNA undergoes alternative splicing, S1 nuclease analysis was performed using a cDNA probe spanning exons 1-13 (Figure 3, probe A). The complementary strand of probe A was labeled within the first three exons using T4 DNA polymerase (see Methods). RNA from the two types of fibroblast cultures transfected with SM-1 protect two predominant fragments from S1 nuclease digestion whereas RNA from mock transfected cultures do not (Figure 3A). The largest protected fragment is 267 nt in length and corresponds to an mRNA produced by splicing of SM-1 exons 1-9. SM-1 mRNA diverges from probe A at the exon 9/10 boundary due to the absence of exons 10-17 in the minigene. The smaller fragment is 91 nt in length and represents protection by accurate splicing of exons 1-4. Thus, this second fragment represents mRNAs which include exons 1 through 4 but diverge from the cDNA probe at the 3' boundary of exon 4. Both bands are similar in intensity, suggesting equivalent levels of the two RNAs, and represent by far the major protection fragments. Faint minor protection products are visible and correspond in size to RNAs that splice exons 1 through 5 but diverge from probe A at the 3' boundaries of each of exons 6-8 (Figure 3A).

To map SM-1 RNAs from the 3' side of exon 5, a 5'-end labeled Hpa II fragment from the same cDNA was used as an S1 nuclease probe (Figure 3, probe B). RNA from SM-1 transfected cultures protect only two fragments while none are detected in RNA from mock transfected cultures (Figure 3B). The fragment at 226 nt corresponds to protection of the cDNA probe by mRNA that contiguously splices exons 1-8. Because the fusion with the RSVLTR truncates exon 1, this fragment is 17 nt shorter than the the full length S1 probe (243 nt). The second band at 105 nt represents protection of the probe from exon 8 to the 5' boundary of exon 6 and is the expected protection product of mRNA that excludes exon 5.

Thus, S1 nuclease analysis detects two SM-1 RNAs. One RNA protects the cDNA probe into exon 1 while the other diverges from the cDNA probe both at the exon 4/5 boundary as well as the 5/6 boundary, consistent with alternative splicing of exon 5. However, S1 nuclease analysis does not rule out the use of cryptic splice sites and does not demonstrate accurate joining of exons 4 and 6 in those RNAs that exclude exon 5. In addition, S1 nuclease analysis does not always qualitatively or quantitatively reflect splice site usage (42; our unpublished observations). Therefore, primer extension analysis was also performed (Figure 3C). An isolated 88 bp AccI/HpaII cDNA fragment spanning exon 6 to exon 8 was 5' end labeled using polynucleotide kinase and annealed with RNA from SM-1 and mock transfected CEF cultures. As diagramed, SM-1 RNAs which include exon 5 will produce a primer extension product of 268 nt, those





excluding exon 5 will produce a runoff product of 238 nt. RNA from SM-1 transfected cultures yield both of the expected runoff products at relatively equivalent levels (Figure 3C). Therefore, the two mRNAs actually differ by the presence or absence of a 30 nt segment corresponding to exon 5. This confirms the results above, indicating the presence of two SM-1 RNA populations, one that includes and the other that excludes exon 5. In those RNAs in which exon 5 has been excluded, exons 4 and 6 are accurately spliced together. In all three assays, both bands are of similar intensity, reflecting equivalent levels of the two alternatively spliced SM-1 RNAs and indicating that the two alternative splicing pathways occur at equal frequency. In addition, in northern blot analysis, cTNT probes detect a wide band corresponding in size to spliced and polyadenylated SM-1 RNA (data not shown). No other bands were detected, even after long exposure, indicating that the SM-1 pre-mRNA is efficiently, and accurately spliced.

In addition to primary cultures of chick embryo fibroblasts and chick embryo skin fibroblasts (Figure 3), SM-1 has also been transfected into fibroblast cell lines from avian (QT-35) and mammalian (rat-2) origin as well as pure cardiac and skeletal muscle primary cultures. Interestingly, the endogenous cTNT gene in cultured cardiac and skeletal muscle cells also expresses both splice products (our unpublished observations) indicating that the developmental regulation of alternative splicing is inoperative in cultured cells (see Discussion). In all muscle and non-muscle cells types transfected thus far, the results of RNA analyses are identical to those presented in Figure 3 (data not shown). Therefore, in both cultured muscle and non-muscle cells, two SM-1 mRNA populations, including and excluding only exon 5, are expressed at equivalent levels. These results establish that cis-acting elements within the cTNT pre-mRNA that distinguish exon 5 as an alternative exon do not require muscle-specific factors in trans to function (see Discussion). In addition, these cis-elements are maintained within the SM-1 pre-mRNA and can potentially be defined by deletion analysis.

Figure 3: Alternative splicing of SM-1 in transfected fibroblast cultures.
The upper panel shows the SM-1 mRNA and cTNT cDNA fragments used as S1 nuclease probes to characterize exon 5 splicing from the 5' side (probe A), the 3' side (probe B), as well as by primer extension analysis (probe C). Filled circles represent labeled regions of the DNA probes. Probe A was ^{32}P end labeled using T4 polymerase and has been described previously (25). This probe was labeled only as far as exon 3 so that band intensities represent relative levels for both RNAs. The first 17 nt of the cDNA used for probes A and B is upstream of the AvaII fusion site with the RSVLTR and is not protected by SM-1 RNA. For this reason, probe B is fully protected by endogenous mRNA that includes exon 5 (lane HM, panel B). Probes B and C were ^{32}P 5' end-labeled by polynucleotide kinase. The expected products from all probes are indicated. The lower panel shows the results using probes A-C. The skin fibroblast (SF) lanes in panel A are from a longer exposure of the same gel. The band identified with the asterisk in panel B is a contaminating fragment from the isolated probe. Its mobility is faster than the protection product of SM-1 (see Figure 6), and it is not protected from S1 nuclease (i.e., lanes H and mock). In panel C, the band at 170 nt (marked with **), represents a premature termination and is not detected in other primer extension experiments. Each lane contains either 5 ug total RNA from tissue samples or 20-30 ug total RNA from transfected (+) and mock-transfected (-) cultures. CEF cultures were transfected in panels B and C. SF, skin fibroblast; CEF, chick embryo fibroblast; M, marker; P, probe; H, day 18 embryoic heart muscle.

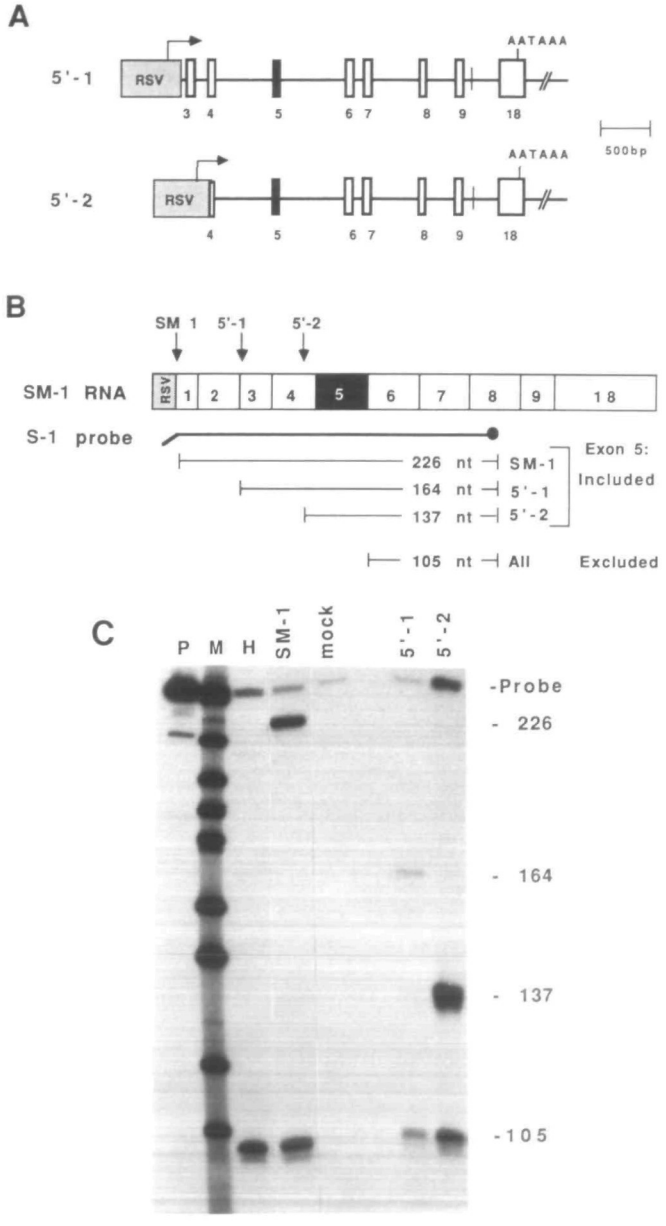


Figure 4: S1 nuclease analysis of cTNT 5' end deletion mutants. (A) 5' deletion mutants removing cTNT genomic sequence upstream of exons 3 and 4. (B) expected S1 nuclease protection products using probe B (Figure 3) and (C) S1 nuclease analysis. The first five lanes of the same film were shown in Figure 3, and are duplicated here to show positions of expected protection products. Each lane contains either 5 ug total RNA from tissue or 20 ug total RNA from transfected CEF cultures. M, marker; H, day 18 embryonic heart muscle.

As CEFs are able to recognize the cis elements which distinguish the cTNT alternative exon and provide an avian cell system, we have used transfection of SM-1 deletion mutants into these cells to assay the alternative splicing of the resultant pre-mRNAs. All results described below have been confirmed in two or more independent transfection experiments. The structure of all RNAs have been characterized by at least two of the same criteria used above; ie., either S1 nuclease from both the 5' and 3' directions and/or primer extension plus S1 mapping from one end. In many cases RNA was characterized using all three assays.

cTNT pre-mRNA sequence upstream of exon 4 is not required for alternative splicing of exon 5.

The results described above indicate that replacement of the cTNT initiation site and 5' flanking sequence with that of the RSVLTR has no effect on the ability of exon 5 to alternatively splice. To continue deletion analysis from the 5' end of the pre-mRNA, the two SM-1 deletion mutants diagrammed in Figure 4 were constructed to remove cTNT genomic sequence upstream of exons 3 and 4, respectively. All but 39 bp of cTNT genomic sequence upstream of exon 3 was deleted by fusing the RSVLTR to a natural Sau3A site (SM-5'-1, Figure 4). A BamHI site was introduced into exon 4 via oligonucleotide directed mutagenesis (33). This site was then fused to the RSVLTR to create deletion mutant SM-5'-2 (Figure 4). As in SM-1, the RSVLTR transcription initiation site is 36 bp upstream of the point of fusion with cTNT genomic sequence. Following transfection into CEF cells, RNA was extracted and assayed by S1 nuclease using probe B, Figure 3. As shown in Figure 4B and C, RNA from mutant 5'-1 protects the two fragments expected for both mRNAs which include and exclude exon 5. Similarly, 5'-2 also protects only two fragments indicating alternative splicing of exon 5. Therefore, none of the cTNT pre-mRNA sequence upstream of exon 4 is required for alternative splicing of exon 5. In the case of SM-5'-2, only the last 8 nucleotides of exon 4 remain, and three of these are altered by oligonucleotide mutagenesis; therefore, most of exon 4 is also not required for alternative splicing.

Alternative splicing of exon 5 does not require pre-mRNA 3' end formation by cTNT genomic sequence or cTNT pre-mRNA sequence downsteam of exon 6

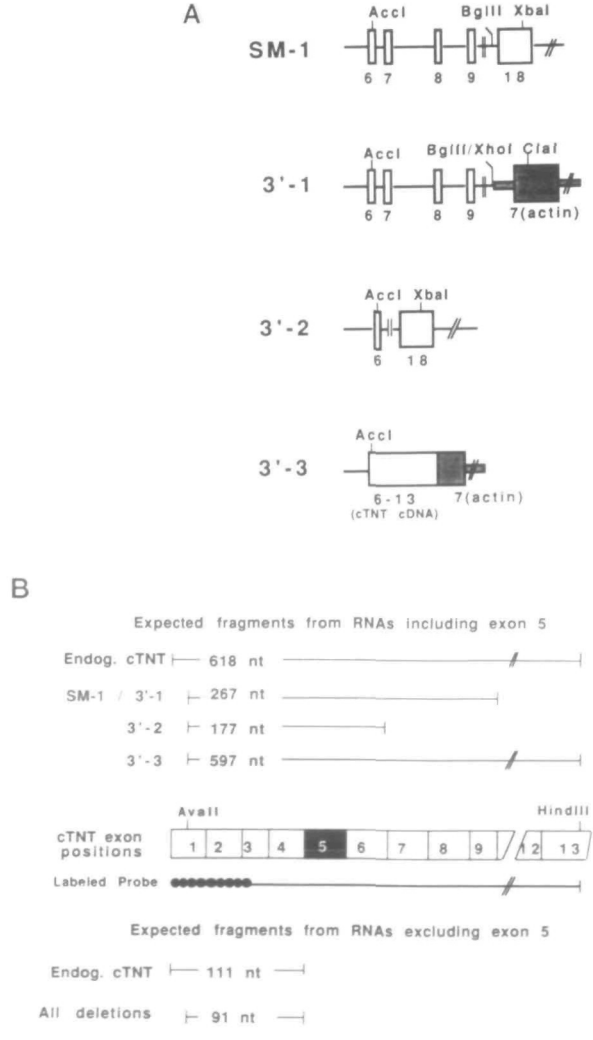
The series of deletion mutants shown in Figure 5 was constructed in order to: 1) test whether exon 5 alternative splicing was dependent upon cTNT-directed mRNA 3'-end processing; and, 2) localize a 3' boundary of cTNT sequence required for alternative splicing of exon 5,

As shown above, the 900 bp of genomic sequence 3' to the poly (A) addition site in the SM-1 minigene is sufficient for correct processing of the SM-1 mRNA 3' end. To determine the role of this region in alternative splicing, a chimeric gene was constructed which fused the last introns of both the SM-1 and the skeletal α-actin genes (34) to replace the majority of the last intron, the entire last exon, and all 3' flanking genomic sequence of cTNT with that of skeletal α–actin, a muscle-specific pre-mRNA which contains no alternative splice sites (SM-3'-1, Fig. 5A, see Methods for details). In Figure 5B, the expected protection products are diagrammed. As shown in Figure 5C, SM-3'-1 produces both RNAs which include and exclude exon 5 establishing that alternative splicing is independent of 3' end formation by cTNT 3' flanking genomic sequence and does not require cTNT pre-mRNA sequence 3' to the ninth intron.

Deletion mutants SM-3'-2 and SM-3'-3 together remove all cTNT genomic sequence 3' to exon 6. SM-3'-2 lacks a 900 bp genomic segment containing exons 7-9. SM-3'-3 replaces all cTNT genomic sequence 3' to exon 6 with a cTNT cDNA segment. Skeletal α-actin genomic 3'-flanking DNA is responsible for 3' end formation in this construction. As shown in Fig. 5C, both deletion mutants alternatively splice exon 5. These results indicate that cTNT pre-mRNA sequence dowstream of exon 6 is not required for alternative splicing of exon 5.

Functional analysis of potential hairpin structures within SM-1 pre-mRNA

The above results have defined a 1200 nt region of the cTNT pre-mRNA from the last eight



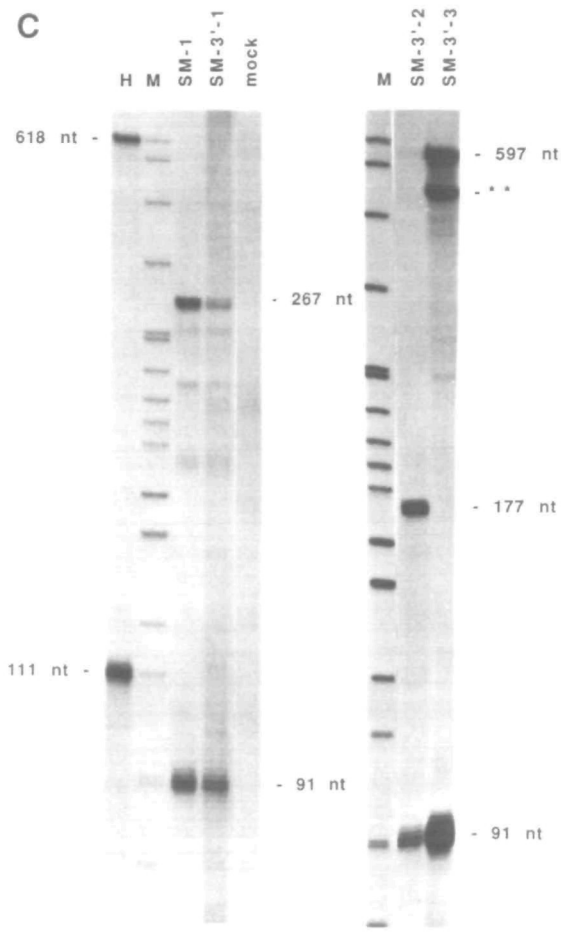


Figure 5: S1 nuclease analysis of cTNT 3' end deletion mutants. (A) constructions removing cTNT genomic regions 3' to exon 6. (B) The labeled probe (Probe A, Figure 3), cTNT exon positions and the expected S1 nuclease protection fragments are shown. (C) Results of S1 nuclease analysis. Lanes contain 5 ug total RNA from tissue samples and 20-30 ug total RNA from transfected CEF cultures. **-the T4 exonuclease digestion used to label the S1 probe for some lanes digested into exon 6 sequence, leading to ^{32}P-incorporation. This band in the SM 3'-3 lane represents protection of the probe by exons 6-13 in RNAs which exclude exon 5. M, marker; P, probe; H, day 18 embryonic heart muscle.

nucleotides of exon 4 to the 3' end of exon 6 that contains sufficient information to specifically mark exon 5 as an alternative exon. One possible role for pre-mRNA cis-acting sequences in this region is intramolecular base-pairing that would sequester splice sites and make them unavailable to the splicing machinery. In this model, higher order structure would serve as the determinative

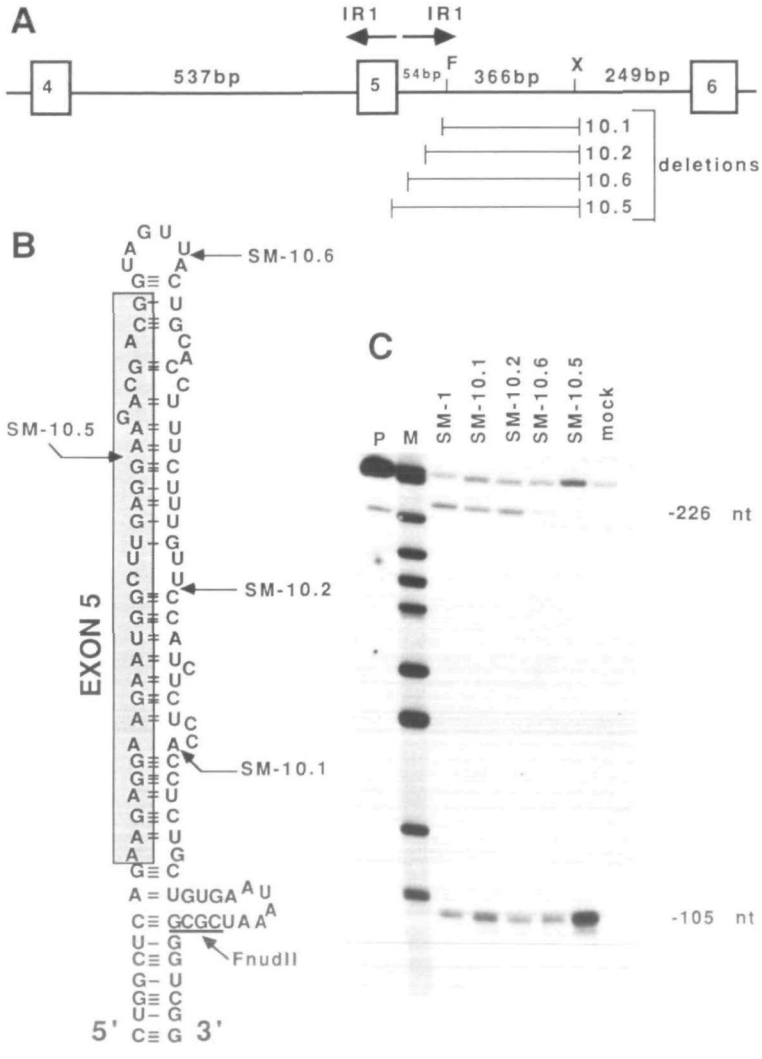


Figure 6: S1 nuclease analysis of RNA from IR1 deletion mutants. (A) cTNT pre-mRNA region containing exons 4-6. The inverted repeat IR1 is indicated by arrows. Bars indicate region deleted for each deletion mutant. (B) Nucleotide sequence and potential base-pairing of IR1. Exon 5 is outlined within a stippled box. The FnudII site is underlined. Deletion endpoints are indicated, deletions remove all sequence 3' to the arrow. (C) S-1 protection products using Probe B (Figure 3). Lanes contain 20 ug total RNA from transfected CEF cultures. F, FnudII; X, XhoI; M, marker; P, probe.

signal for alternative splicing and different pre-mRNA conformations would result in different splicing patterns (see Discussion).

In order to evaluate this model for cTNT alternative splicing, we searched for regions of potential internal base-pairing within the 1200 nt region defined above. One relatively large inverted repeat, labeled IR1, and a smaller inverted repeat, labeled IR2 were identified (Figures 6 and 7, respectively). If the complementary regions of IR1 base-pair, this inverted repeat would sequester exon 5 and its adjacent 5' and 3' splice sites within a stem-loop structure via base-pairing to a segment of downstream intron (Figure 6). The approximate free energy change of base pairing (ΔG=kcal/mole) for this structure was calculated to be -29.2 kcal/mole according to the method of Freier et al. (43), however, the likelihood that this structure actually forms is unknown. To test the role of this potential structure, complementarity of IR1 was eliminated by deleting portions of the 3' segment of the inverted repeat in intron 5. In this way, most of the IR1 base pairing could be removed without disrupting consensus splicing signals or exon sequence. Deletions were made from a unique BglII site inserted into the natural FnuDII site 54 bp 3' to exon 5 (see Figure 6A and Methods for details). The 5' endpoints of the deletions were fused to an artificial XhoI site located in intron 5 (Figure 6A). Therefore, all deletions contain identical 3' endpoints and the deletion mutants differ only in the length of sequence remaining in the 3' half of the inverted repeat. Insertion of the BglII and XhoI linkers and removal of the intervening 366 bp between these two sites had no effect on the ability to alternatively splice exon 5 (data not shown). Deletion endpoints were determined by nucleotide sequence analysis.

Four deletions that removed substantial portions of the IR1 sequence are illustrated in Figure 6A and B. Deletion mutant SM-10.2 removes a particularly stable portion of the predicted duplex and should dramatically reduce the likelihood that exon 5 is sequestered within the secondary structure of this inverted repeat. For example, the calculated free energy change for the remaining hairpin structure in SM-10.2 has been reduced to -5.0 kcal/mole by the deletion. However, both SM-10.1 and 10.2 expressed equivalent levels of the two mRNAs which include or exclude exon 5, a pattern identical to that obtained with SM-1 (Fig. 6C). The third deletion mutant, SM-10.6, resulted in a reduced level of RNA which includes exon 5 compared to SM-1. However, virtually complete removal of IR1 complementarity in SM-10.6 sequence has not eliminated the exclusion of exon 5 as predicted by a model involving sequestration of exon 5 within the stem-loop structure. As indicated in Figure 6B, SM-10.6 contains only 6 nt of 3' flanking intron. The reduced level of mRNA which includes exon 5 is likely to be the result of disruption of adjacent 5' splice site sequences. Reduced splicing efficiency has been demonstrated to be the result of deletions in similar (44) and identical (45) locations within introns of other genes.

A fourth deletion mutant, SM-10.5, actually deletes into exon 5 itself, removing the 5' splice site of intron 5 as well as the 3' nine nucleotides of the exon. As expected, this deletion mutant does not correctly splice exon 5. In fact, inclusion of exon 5 has been totally eliminated (Figure

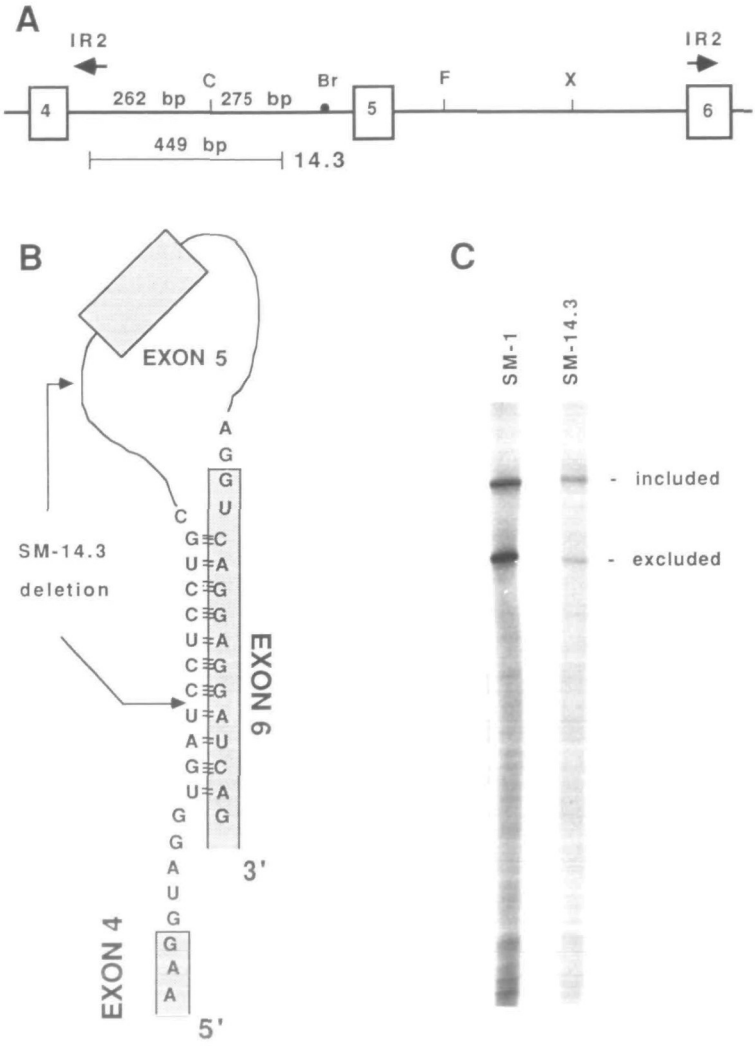


Figure 7: Primer extension of IR2 deletion mutant. (A) cTNT pre-mRNA region containing exons 4-6. Bar indicates region deleted in SM-14.3. Note that the consensus lariat branchpiont sequence of intron 4 (Br) is not affected by this deletion. (B) Potential base-pairing of IR2 and region of intron 4 removed by SM-14.3 deletion. (C) Primer extension analysis using probe C, Figure 3. Products differ by the inclusion or exclusion of exon 5. C, ClaI; F, FnudII; X, XhoI.

6C). This is consistent with several studies indicating the requirement of intact 5' splice sites for correct exon recognition (26, 46, 47).

The location and potential base-pairing of the second inverted repeat (IR2) is shown in Figure 7A and B. The 11 bp stem has an approximate free energy change of -20.0 kcal/mole. This

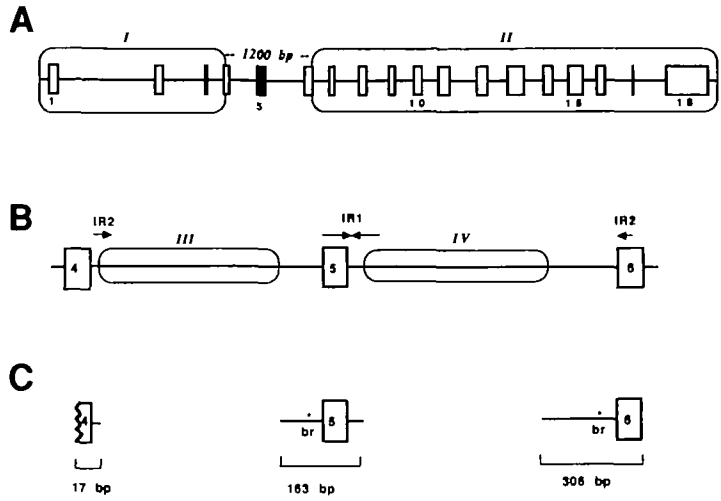


Figure 8: Summary of cTNT pre-mRNA deletion analysis. (A) Diagram of the cTNT gene. (B) Diagram of the 1200 nucleotide region containing exons 4-6. Positions of inverted repeats 1 and 2 (IR1 and IR2) are indicated in B. Shaded blocks in A and B indicate regions of the cTNT gene covered by the deletion experiments reported here. Deletion mutants correspond to shaded regions as follows (see text): Region I, SM-5'-2 (Figure 4); Region II, SM-3'-1, 3'-2, and 3'-3 (Figure 5); Region III, SM-14.3 (Figure 7), and Region IV, SM-10.2 (Figure 6). (C) Three segments of the cTNT pre-mRNA that contain sufficient information for alternative splicing of exon 5. The relative positions of the putative branchpoint sequences (br) for introns 4 and 5 are indicated.

structure is particularly attractive with regard to the alternative splicing of exon 5 because it could potentially bring the 5' splice site of intron 4 within the environs of the 3' splice site of intron 5 and increase the likelihood that exons 4 and 6 splice together. The role of this potential structure was tested with a 449 bp deletion of the 537 bp intron 4 which removes 7 of the 11 complementary nucleotides of the inverted repeat (Figure 7A and B). Primer extension analysis indicates that this deletion does not prevent the exon 4-to-exon 6 splicing pathway (Figure 7C). Therefore, neither of the potential hairpin structures identified by nucleotide sequence analysis is required for the alternative splicing of exon 5.

DISCUSSION

Alternative splicing is a ubiquitous mechanism for regulating gene expression among metazoans; however, the cis- and trans-acting factors responsible for the selection of alternative splice sites are unknown. We have used transfection of modified cTNT genes to address the mechanisms of alternative splicing by localizing pre-mRNA cis-acting elements that are required for alternative splicing of a single exon within the cTNT pre-mRNA. In this study, we have: 1) performed a systematic deletion analysis to localize cis elements that are required for alternative splicing; 2) functionally tested two natural, potential pre-mRNA stem-loop structures for

determinative roles in cTNT alternative splicing and; 3) determined whether the cis elements which define the cTNT alternative exon require interactions with tissue-specific factors in trans to function.

The majority of the cTNT pre-mRNA is not required for alternative splicing of exon 5

Recent work on several genes transcribed by RNA polymerase II indicates that RNA processing at one site may be governed by cis elements residing at distal sites. For example, correct 3' end processing for the U1 RNA gene requires specific promoter sequences in cis which are not required by most RNA polymerase II promoters (48, 49). This indicates that the assembly of a gene-specific transcription initiation complex can affect subsequent distal processing such as 3' end formation. Similarly, Sisodia et al. (50) recently demonstrated that RNA polymerase II pre-mRNAs transcribed by RNA polymerase III are neither spliced nor polyadenylated. In addition, transfection analysis of calcitonin/CGRP alternative splicing indicates that one of the two alternative splicing patterns is dependent upon an associated poly(A) addition signal (16). Based upon such observations, we anticipated sequences distal to the local environs of exons 4, 5, and 6 might be required for the splicing machinery to recognize the cTNT pre-mRNA as one carrying an alternative exon. However, our results, summarized in Figure 8, allow us to eliminate all cTNT 5'- and 3'-flanking genomic sequence as well as most of the cTNT pre-mRNA as not required for alternative splicing of exon 5. Deletion of the regions I and II in Figure 8A allows us to eliminate the following as not required for cTNT alternative splicing:

1. specific promoter sequences and thus a novel transcription initiation complex.
2. specific 3' end processing signals.
3. cTNT pre-mRNA sequence upstream of exon 4, and most of exon 4 itself.
4. cTNT pre-mRNA sequence downstream of exon 6.
5. a preferred order of intron removal outside of the region containing exons 4, 5, and 6.

These results demonstrate the independence of cTNT alternative splicing from transcription initiation, 3'-end processing, and splicing of introns not directly involved in the alternative splice.

Inverted repeats and cTNT alternative splicing

The results discussed above demonstrate that the 1200 nucleotide pre-mRNA region between the last 8 nucleotides of exon 4 and the 3' boundary of exon 6 contain sufficient information in cis to specify exon 5 as an alternative exon. Cis elements that designate exon 5 for alternative splicing may, or may not, require interaction with specialized "alternative splicing factors" in trans. In the latter case, such factors could represent supplementary or substitute components of the constitutive splicing machinery such as the developmentally regulated U1 RNA variants (51, 52). Indeed, trans-acting factors have been implicated in the recognition of alternative splice sites within viral and nuclear pre-mRNAs (17, 53, 54).

If the cis elements are functioning without specialized factors in trans, the information for exon 5 alternative splicing would be contained entirely within the pre-mRNA nucleotide sequence. It is possible that exon 5 and/or surrounding introns have unusual properties in which competition

for factors of the constitutive splicing machinery leads to both the 4-5-6 and 4-6 splicing patterns. While this is consistent with our results, the basis for such competition between these three consecutive exons is not apparent from nucleotide sequence. Both introns 4 and 5 contain good matches to the consensus sequences for lariat branchpoint sites (27-30), and 5' and 3' consensus splice sites (26). Another possibility is that the cis elements represent sequences that function through intramolecular base-pairing, generating different pre-mRNA conformations, ultimately leading to the two splicing pathways which include and exclude exon 5. The presence of inverted repeats in many alternatively processed viral and nuclear pre-mRNAs has suggested a model for alternative splicing in which intramolecular base-pairing is involved in selection of alternative splice sites by sequestering splice sites or entire exons within stem-loop structures (20, 22-24, 55). Moreover, the ability of intramolecular base-pairing to affect splice site selection in vitro and in vivo has been demonstrated experimentally (56, 57). Such a model is attractive because it could explain the apparent stochastic and intrinsic nature of cTNT alternative splicing as well as the lack of a requirement for muscle-specific factors in trans (see below).

A search for potential higher order structure in the 1200 nt pre-mRNA region defined above revealed two separate inverted repeats (IR's 1 and 2, Figures 6, 7, and 8B) that could potentially sequester exon 5 within stem-loop structures. However, deletion of regions III and IV, shown in Figure 8B, removed the intramolecular base-pairing of each of these two potential structures and did not result in the constitutive inclusion of exon 5 as would be predicted by the above model. Thus, the two natural, relatively large potential stem-loop structures identified by nucleotide sequence analysis do not appear to be required for alternative splicing of cTNT pre-mRNA. This is the first test of such a model by deletion analysis of naturally occuring inverted repeats identified in an alternatively spliced pre-mRNA. The results are consistent with a recent analysis of the effects of artificial pre-mRNA hairpins on splicing in vivo in which base-paired stems of less than 50 bp had little effect on splice site selection (58). However, our results do not rule out the possibility that unidentified short regions of base-pairing are at least partially determinative for the two splicing patterns. Indeed, relatively short regions of base-pairing are responsible for the core structure required for activity of self-splicing introns (59, 60).

Cis elments required for alternative splicing are limited to three small segments of the cTNT pre-mRNA

Deletions that removed the two inverted repeats have also removed most of the intron sequence between exons 4, 5, and 6 without disrupting alternative splicing of exon 5 (Figures 6 and 7). Therefore, taken together, the deletion analysis described above indicates that the information required in cis for cTNT alternative splicing is limited to, at most, three small segments of the pre-mRNA (Figure 8C). One segment contains the last eight bases of exon 4 and the first 9 nt of intron 4. The second segment contains exon 5 and includes 79 nt of the upstream intron and 24 nt of the downstream intron. The third segment includes the last 249 nt of intron 5 and includes all of exon 6. Both of the segments that contain exons 5 and 6 also contain the putative lariat

branchpoint sequences for introns 4 and 5, respectively. Further deletion analysis and site directed mutatgensis is in progress to define the specific sequence(s) required for alternative splicing. Preliminary evidence indicates that mutations within either of the pre-mRNA segments containing exons 5 or 6 disrupt alternative splicing exon 5 (data not shown).

Alternative splicing of cTNT exon 5 does not require muscle-specific factors in trans

cTNT expression is normally striated muscle-specific. However, using the RSV long terminal repeat to drive transcription, a cTNT minigene (SM-1) pre-mRNA has been expressed to abundant levels in cultured muscle and non-muscle cells. Chick embryo fibroblasts (CEFs) were the non-muscle cells used for most of the experiments reported here because they represent an avian cell type with a relatively high transfection efficiency. CEFs were passaged at least three times prior to transfection to select out cells of myogenic lineage as determined by loss of morphological and biochemical markers for muscle differentiation. To further rule out the possibility that SM-1 alternative splicing in transfected CEF cultures was due to a subpopulation of myogenic cells, skin fibroblast primary cultures, a quail fibroblast cell line (QT-35), and a rat fibroblast cell line (rat 2) were also transfected with SM-1. Following transfection, all three cell types produced equivalent levels of the two SM-1 splice products (Figure 3 and data not shown). In addition, stable cells lines expressing SM-1 have been made in a quail fibroblast cell line and also express both alternative splice products (our unpublished observations).

These experiments demonstrate that non-muscle cells are able to faithfully reproduce the exon specific alternative processing of a muscle-specific pre-mRNA. Interestingly, cultured striated muscle cells express the endogenous cTNT gene and, in both cardiac and skeletal muscle cultures, both endogenous cTNT splice products are expressed (our unpublished observations). Therefore, the SM-1 pre-mRNA in cultured cardiac muscle, skeletal muscle, and four types of fibroblasts reproduces the two alternative splicing pathways of the endogenous pre-mRNA in cultured striated muscle: exon 5 is specifically identified as the alternative exon and both splice products are constitutively expressed. This strongly suggests that the cis signal(s) which define exon 5 as an optional exon during processing of SM-1 pre-mRNA in fibroblasts are identical to those recognized in cTNT pre-mRNA in striated muscle. It is highly unlikely that fibroblasts, which normally do not process the cTNT pre-mRNA, would alternatively splice a single, specific exon identically to the endogenous tissue by using different cis-acting sequences. Therefore, cis elements within the cTNT pre-mRNA do not require muscle-specific factors in trans to process exon 5 as an alternative exon. However, this does not rule out the possibility that specialized factors are required in trans to define the alternative nature of exon 5 and that these factors are present in a wide variety of cell types.

Recent results indicate both tissue-specific (53) and tissue-non-specific (61-63, this paper) recognition of alternative splice sites suggesting that different pre-mRNAs may use different regulatory mechanisms for alternative splicing. The complexity of the splicing machinery may provide the basis for multiple regulatory mechanisms.

Implications for developmental regulation of cTNT alternative splicing

All cultured cells expressing either SM-1 or the endogenous cTNT gene constitutively produce both alternatively spliced mRNAs. Thus, exon 5 is recognized as an alternative exon and correctly alternatively spliced in all cells tested. However, the frequency of exon 5 inclusion/exclusion does not appear to be regulated as it is during in vivo muscle development. This indicates that the alternative splicing of the cTNT pre-mRNA may be separated into two levels of regulation which may be uncoupled from one another. One level involves regulation of the mechanics of alternative splicing; ie. discrimination of an alternative exon from constitutive exons and its alternate inclusion or exclusion during pre-mRNA processing. Our results show that this does not require the participation of cell-specific factors in trans, at least for the case of the cTNT gene. The second level of regulation is developmental, controlling the frequency with which exon 5 is included or excluded at different times during development. This second process appears to be inoperative in cultured muscle and non-muscle cells, because similar levels of both splice products are observed in most cases. The constitutive production of both splice products at similar levels suggests the possibility that first level of regulation may be inherently stochastic. If that is correct, the second level of regulation could be superimposed over the first by regulation of trans-acting factors responsible for alternative splice site recognition or which affect pre-mRNA secondary structure. Localization of cTNT pre-mRNA sequences required for exon 5 alternative splicing should lead to an understanding of the mechanism of alternative splice site selection and ultimately to characterization of the trans-regulation of this process.

ACKNOWLEDGMENTS

We would like to thank Roy Parker and Christine Guthrie for helpful discussions and for assistance with the analysis of RNA secondary structure. This work was supported by grants from NSF and NIH to CPO. TAC was supported by a Giannini Bank of America Foundation Fellowship.

*To whom correspondence should be addressed

REFERENCES

1 Leff, S.E., Rosenfeld, M.G., Evans, R.M. (1986) Ann. Rev. Biochem. 55, 1091-1117.
2 Breitbart, R.E., Andreadis, A., and Nadal-Ginard, B. (1987) Ann. Rev. Biochem. 56, 467-495.
3 Nabeshima, Y., Fuji-Kuriyama, Y., Muramatsu, M., Ogato, K. (1984) Nature 308, 333-338.
4 Breitbart, R.E., Nguyen, H.T., Medford, R.M., Destree, A.T., Mahdavi, V., and Nadal-Ginard, B. (1985) Cell 41, 67-82.
5 Helfman, D.M., Cheley, S., Kuismanen, E., Finn, L.A., and Yamawaki-Kartaoka, Y. (1986) Mol. Cell. Biol. 6, 3582-3595.
6 Bernstein, S.I., Hansen, C.J., Becker, K.D., Wassenberg, D.R., Roche, E.S., Donaday, J.J., Emerson, C.P. (1986) Mol. Cell. Biol. 6, 2511-2519.
7 Martin-Zanca, D., Hughes, S.H., and Barbacid, M. (1986) Nature 319, 743-748.

8 Reinach, F.C., and MacLeod, A.R. (1986) Nature 322, 648-650.
9 Shtivelman, E., Lifshitz, B., Gale, R.P., Roe, B.A., and Cananni, E. (1986) Cell 47, 277-284.
10 Stanton, L.W., and Bishop, J.M. (1987) Mol. Cell Biol. 7, 4266-4272.
11 Edwards, R.H., Selby, M.J., and Rutter, W.J. (1986) Nature 319, 784-787.
12 Schejter, E.D., Segal, D., Glazer, L., and Shilo, B. (1986) Cell 46, 1091-1101.
13 Behlke, M.A., and Loh, D.Y. (1986) Nature 322, 379-382.
14 Cunningham, B.A., Hemperly, J.J., Murray, B.A., Prediger, E.A., Brackenbury, R., and Edelman, G.M. (1987) Science 236, 799-806.
15 Jackson, A.,P., Seow, H., Holmes, N., Drickamer, K., and Parham, P. (1987) Nature 326, 154-159.
16 Leff, S.E., Evans, R.M., and Rosenfeld, M.G. (1987) Cell 48, 517-524.
17 Fu, X., and Manely, J.L. (1987) Mol. Cell. Biol. 7, 738-748.
18 Somasekhar, M.B. and Mertz, J.E. (1985) Nucl. Acids Res. 13, 5591-5609.
19 Mardon, H.J., Sebastio, G., and Baralle, F.E. (1987) Nucl. Acids Res. 15, 7725-7733.
20 Khoury, G., Gruss, P., Dhar, R., and Lai, C. (1979) Cell 18, 85-92.
21 Deutscher, S.L, Bhat, B.M., Pursley, M.H., Cladaras, C., and Wold, W.S.M. (1985) Nucl. Acids Res. 13, 5771-5788.
22 Munroe, S.H. (1984) Nucl. Acids Res. 12, 8437-8455.
23 Breitbart, R.E., and Nadal-Ginard, B. (1986) J. Mol. Biol. 188, 313-324.
24 Edlind, T.D., Cooley, T.E., and Ihler, G.M. (1987) Nucl. Acids Res. 15, 8566.
25 Cooper, T.A., and Ordahl, C.P. (1985) J. Biol. Chem. 260, 11140-11148.
26 Mount, S. (1982) Nucl. Acids Res. 10, 459-472.
27 Keller, E.B., and Noon, W.A. (1984) Proc. Natl. Acad. Sci. USA 81, 7417-7420.
28 Reed, R., and Maniatis, T. (1985) Cell 41, 95-105.
29 Ruskin, B., Krainer, A.R., Maniatis, T., and Green, M.R. (1984) Cell 38, 317-331.
30 Zeitlin, S. and Efstratiadis, A. (1984) Cell 39, 589-602.
31 Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, New York; Cold Spring Harbor Laboratory).
32 Gorman, C.M., Merlaino, G.L., Willingham, M.C., Pastan, I, and Howard, B.H. (1982) Proc. Natl. Acad. Sci. USA. 79, 6777-6781.
33 Zoller, M.J., and Smith, M. (1984) DNA 3, 479-488.
34 Fornwald, J.A., Kuncio, G., Peng, I., and Ordahl, C.P. (1982) Nucl. Acids Res. 10, 3861-3876.
35 Hughes, S.H., Greenhouse, J.J., Petropoulos, C.J., and Sutrave, P. (1987) J. Virol. 61, 3004-3012.
36 Rubin, H. (1957) Virology 4, 533-562.
37 Graham, F.L., and van der Eb, A.J. (1973) Virology 52, 456-467.
38 Gorman, C.M., Moffat, L.F., and Howard, B.H. (1982) Mol. Cell Biol. 2, 1044-1051.
39 Auffray, C., and Rougeon, F. (1980) Euro. J. Biochem. 107, 303-314.
40 McKnight, S.L., Gavis, E.R., and Kingsbury, R.C. (1981) Cell 25, 385-398.
41 Cooper, T.A., and Ordahl, C.P. (1984) Science 226, 979-982.
42 Sisodia, S.S., Cleveland, D.W., and Sollner-Webb, B. (1987a) Nucl. Acids Res. 15, 1995-2011.
43 Freier, S.M., Kierzek, R., Jaeger, J.A., Sugimoto, N., Caruthers, M.H., Neilson, T., and Turner, D.H. (1986) Proc. Natl. Acad. Sci., USA 83, 9373-9377.
44 Benyajati, C., Place, A.R., Wang, N., Penz, E., and Sofer, W. (1982) Nucl. Acids Res. 10, 7261-7273.
45 Wieringa, B., Hofer, E., and Weissmann, C. (1984) Cell 37, 915-925.
46 Orkin, S.H., Kazazian, H.H., Antonarakis, S.E, Goff, S.C., Boehm, C.D., Sexton, J.P., Waber, P.G., and Giardina, P.J.V. (1982) Nature 296, 627-631.
47 Treisman, R., Orkin, S.H., and Maniatis, T. (1983) Nature 302, 591-596.
48 Hernandez, N., and Weiner, A.M. (1986) Cell 47, 249-258.
49 Neuman de Vegvar, H.E., Lund, E., and Dahlberg, J.E. (1986) Cell 47, 259-266.
50 Sisodia, S.S., Sollner-Webb, B., and Cleveland, D.W. (1987b) Mol. Cell Biol. 7, 3602-3612.

51 Forbes, D.J., Kirschner, M.W., Caput, D., Dahlberg, J.E., and Lund, E. (1984) Cell 38, 681-689.
52 Lund, E., Kahan, B., and Dahlberg, J.E. (1985) Science 229, 1271-1274.
53 Breitbart, R.E., and Nadal-Ginard, B. (1987) Cell 49, 793-803.
54 Svensson, C., and Akusjarvi, G. (1986) Proc. Natl. Acad. Sci. USA 83, 4690-4694.
55 Seiki, M., Hikikoshi, A., Taniguchi, T., Yoshida, M. (1985) Science 228, 1532-1534.
56 Solnick, D. (1985) Cell 43, 667-676.
57 Eperon, L.P., Estibeiro, J.P., and Eperon, I.C. (1986) Nature 324, 280-282.
58 Solnick, D. and Lee, S.I. (1987) Mol. Cell Biol. 7, 3194-3198.
59 Waring, R.B., and Davies, R.W. (1984) Gene 28, 277-291.
60 Cech, T.R. and Bass, B.L. (1986) Ann. Rev. Biochem. 55, 599-629.
61 Garfinkel, L.I. and Davidson, N. (1987) Mol. Cell. Biol. 7, 3826-3829.
62 Kedes, D.H. and Steitz, J.A. (1987) Proc. Natl Acad. Sci., USA 84, 7928-7932.
63 Vibe-Petersen, K., Kornblihtt, A.R., and Baralle, F.E. (1984) EMBO J. 3, 2511-2516.
64 Weiss, R., Teich, N., Varmus, H., and Coffin, J.(eds.) (1982). RNA Tumor Viruses. (Cold Spring Harbor, New York). p. 1323.