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Nucleotide substitutions within the cardiac troponin T alternative exon disrupt pre-mRNA alternative splicing

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ABSTRACT

The cardiac troponin T (cTNT) pre-mRNA contains a single alternative exon (exon 5) which is either included or excluded from the processed mRNA. Using transient transfection of cTNT minigenes, we have previously localized pre-mRNA cis elements required for exon 5 alternative splicing to three small regions of the pre-mRNA which include exons 4, 5, and 6. In the present study, nucleotide substitutions were introduced into the region containing exon 5 to begin to define specific nucleotides required for exon 5 alternative splicing. A mutation within the 5' splice site flanking the cTNT alternative exon that increases its homology to the consensus sequence improves splicing efficiency and leads to increased levels of mRNAs that include the alternative exon. Surprisingly, substitution of as few as four nucleotides within the alternative exon disrupts cTNT pre-mRNA alternative splicing and prevents recognition of exon 5 as a bona fide exon. These results establish that the cTNT alternative exon contains information in cis that is required for its recognition by the splicing machinery.

INTRODUCTION

Pre-mRNA alternative splicing is a fundamental regulatory mechanism of differential gene expression in eukaryotes. In contrast to constitutive splicing in which exons are spliced contiguously at invariant splice sites to produce a single mRNA, alternative splicing results from the variable use of specific alternative splice sites to produce multiple mRNAs. In addition, alternative splice site selection is generally regulated with regard to cell and/or developmental stage specificity (1, 2). Thus, the discontinuous nature of eukaryotic genes provides the basis for increased protein coding capacity and the tightly regulated expression of diverse protein isoforms via post-transcriptional mechanisms.

Alternatively spliced pre-mRNAs differ widely in the number of alternative splice sites and in the complexity of alternative splicing patterns (2). One of the simplest and most common alternative splicing patterns involves the inclusion or exclusion of a single exon. In one splicing pathway, the alternative exon is included into the mRNA like a constitutive exon while in a second pathway, the exon is "skipped" by the splicing machinery and is removed as part of the intron between two distal exons. The mechanisms which allow the splicing machinery to distinguish between alternative and constitutive exons remain unknown but presumably both cis- and trans-acting components are required. In general, pre-mRNA cis elements specifically required for alternative splicing cannot be identified by nucleotide sequence analysis. For example, the cis

elements known to be required for splicing, which includes consensus sequences at the 5' splice site, 3' splice site, and the lariat branchpoint (3-5), do not appear to differ between alternative and constitutive exons (2). Mutations that disrupt alternative splicing have been identified in a number of viral and nuclear pre-mRNAs (6-14), however, the location and role of specific cis-acting sequences have been difficult to identify. One potential difficulty is that many of these mutations represent insertions or deletions which can introduce potential spacing effects (13-16). In addition, alternative splice site selection of different and complex splicing patterns may differ mechanistically and may employ disparate cis elements.

The avian cardiac troponin T (cTNT) pre-mRNA provides a straightforward system to investigate the cis- and trans-acting elements required for the developmentally regulated alternative splicing of a single exon. The cTNT gene is specifically expressed in striated muscle and transcribes a single 9200 nucleotide pre-mRNA containing 17 constitutive exons and a single 30 nucleotide alternative exon (exon 5). Exon 5 is either included or excluded during pre-mRNA splicing (17). In addition, the alternative splicing of cTNT exon 5 is under strict developmental regulation: mRNAs which include exon 5 predominate in the early embryonic heart while those which exclude exon 5 predominate in the adult (17).

We have previously described a transient transfection system used to localize the cis elements required to define cTNT exon 5 as an alternative exon (18). cTNT minigenes transiently transfected into either muscle or non-muscle cultures produce abundant levels of both mRNAs that include and exclude exon 5. Thus, the cis elements which specifically define exon 5 as an alternative exon function within the minigene pre-mRNA and are recognized by both muscle and non-muscle cells. By systematic deletion and replacement analysis, we have previously localized sequences required for alternative splicing of exon 5 to three small regions of the pre-mRNA containing exons 4, 5, and 6 (18). To perform finer mapping of the critical nucleotides within the region containing exon 5, oligonucleotide-directed mutagenesis has been used to introduce nucleotide substitutions into the 5' and 3' splice sites flanking the alternative exon as well as within the alternative exon itself.

MATERIALS AND METHODS

Recombinant DNA

Recombinant DNA methods were performed according to Maniatis et al. (19). Restriction endonucleases, S1 nuclease, DNA Polymerase I (Klenow fragment), terminal transferase, and calf intestine alkaline phosphatase were obtained from Boehringer Mannheim; T4 DNA ligase was obtained from New England Biolabs. T4 polynucleotide kinase was obtained from Pharmacia. T4 DNA polymerase I was obtained from B. Alberts, UCSF. The reaction conditions for all enzymes were as suggested by the manufacturer. Sequencing was performed on plasmid and M13 DNA using the Sequenase kit (US Biochemical).

Oligonucleotide-directed Mutagenesis

Mutants E5-1, E5-2, E5-3, I4-31, and I5-51 (Figure 1): Construction of the cTNT minigene (SM-1) and the modified minigene containing the Bgl II linker inserted into a FnuDII site 50 nucleotides downstream from exon 5 have been previously described (18). Oligonucleotide-directed mutagenesis was carried out within the plasmid to avoid the necessity of recloning to generate a single stranded intermediate (see references 20 and 21). Following linearization at the Bgl II site, the 3' exonuclease activity of T4 DNA polymerase was used to digest one strand of the plasmid. The extent of the digestions at various time points was monitored by labeling the recessed 3' end with ^{32}P-dideoxyadenosine triphosphate (ddATP) using terminal transferase followed by digestion at upstream and downstream restriction sites. The resulting fragments were separated on a denaturing acrylamide gel. Typically, digestions were performed to remove 80-140 nucleotides upstream (and downstream) of the Bgl II site thus making the region containing exon 5 single stranded. Digested DNA was extracted with phenol:chloroform:isoamyl alcohol (50:49:1), extracted with chloroform, then ethanol precipitated. Phosphorylated mutagenic oligonucleotides were annealed to the single-stranded region as described by Zoller and Smith (22). Single-stranded gaps were filled in using either T4 DNA polymerase or the Klenow fragment of DNA polymerase I in combination with T4 DNA ligase. Following transformation, mutants were selected by colony hybridization using the ^{32}P labeled mutant oligonucleotide as a probe (22). The 5' overhang of the 8 nucleotide Bgl II linker was filled in during this procedure adding a total of 12 additional nucleotides to intron 5. Mutations were confirmed by sequencing.

RSVβ7D7 wild type and E5-1 (Figure 3): A cDNA representing the 5' half of the cTNT mRNA containing exons 1-13 (17) was cloned into the Bam HI/Hind III sites of M13 mp19. Mutagenesis was carried out according to Zoller and Smith (22). Mutated and wild type cDNAs were cloned into a plasmid containing an unmodified cTNT genomic segment including exons 13-18 with 900 base pairs of 3' flanking genomic sequence. The cTNT last exon and 3' flanking genomic DNA is sufficient for correct mRNA 3' end formation (18). cDNA and genomic segments were fused at the natural Hind III site in exon 13. Because the RSV cassette used to drive SM-1 transcription did not yield bacterial clones when inserted upstream of the cDNA fragment, the promoter and initiation site of the chicken β-actin gene (23) was used. To enhance transcription, the RSV enhancer (24) was inserted upstream of the β-actin gene promoter.

SM-3'-2 E5-1 (Figure 4): A M13 single-stranded intermediate was used to introduce the E5-1 mutation into exon 5 (22). A Cla I linker was inserted into a Pvu II site within intron 4 that is 276 nucleotides upstream of exon 5. A Xho I linker was similarly introduced into a Sma I site in intron 5 located 340 nucleotides downstream of exon 5. The insertion of these two linkers has little or no effect on the alternative splicing of exon 5 as compared to SM-1 in all cell types tested (data not shown). The Cla I-Xho I insert containing exon 5 was cloned into M13 mp18 for oligonucleotide directed mutagenesis (22). The mutation was confirmed by sequencing. The

mutated Cla I-Xho I insert was then cloned into SM-3'-2 (18).

Cell Culture, Transfection, RNA Analysis

Media and media components were obtained from Cell Culture Facility at the University of Calif., San Francisco or from Gibco Laboratories. The preparation and maintenance of chick embryo fibroblast (CEF) cultures was as previously described (18). Preparation of primary skeletal muscle cultures from day 11 embryos was performed as described by Mar et al. (25).

Transfection was done according to Gorman et al (26) for both muscle and non muscle cells. Cells were plated at 8 x 10^5 cells (CEF) and 1.5 x 10^6 (skeletal muscle) per 60 mm plate. Twenty-four hours after plating, media was changed and three hours later, cells were transfected with DNA-calcium precipitates using 10 μg DNA per 60 mm plate. DNA-calcium phosphate precipitates remained on the cells for 18 hours; this was removed, the cells washed with PBS, and fresh media added. Cells were harvested the next day. Mock transfected cultures were transfected with a SM-1 construct lacking promoter and enhancer sequences.

Total cellular RNA was extracted using the LiCl/urea procedure and S1 nuclease analysis was performed as described (18). The S1 probe consisted of a double stranded cDNA Hpa II fragment spanning exons 1-8 ^{32}P-5' end labeled using polynucleotide kinase (18). Primer extension was performed as described by McKnight et al. (27) using a 30 nucleotide oligomer complementary to exon 6.

RESULTS

Nucleotide substitutions within the cTNT alternative exon prevent its expression in processed mRNA

Using transient transfection of deletion and replacement mutants, we have previously localized sequences required for alternative splicing of cTNT exon 5 to three small regions of the pre-mRNA (18). One of these regions contains the 30 nucleotide alternative exon flanked by 79 nucleotides of the upstream intron and 24 nucleotides of the downstream intron. To begin an analysis of the specific nucleotides within this region that are required for exon 5 alternative splicing in vivo, cTNT minigenes containing nucleotide substitutions were constructed for transient transfection into muscle and non-muscle cells. Oligonucleotide-directed mutagenesis was used to construct the series of mutations shown in Figure 1 (see Methods). Nucleotide substitutions were introduced into three separate regions of the cTNT alternative exon by mutations E5-1, E5-2, and E5-3. In mutation E5-1, the four central pyrimidines of cTNT exon 5 are replaced by purines. Mutations E5-2 and E5-3 introduce four and five nucleotide substitutions, respectively, to create Cla I sites in the 5' and 3' regions of the exon. It should be noted that these substitutions do not alter the first or last two nucleotides of the exon which are included in the splice site consensus sequences (3). Mutations I4-31 and I5-51 introduced nucleotide substitutions into the 3' and 5' splice sites flanking exon 5, respectively. In general, mutation of the highly conserved nucleotides within the consensus sequences disrupts splicing of the adjacent exon (4, 5). Therefore, to avoid disruption of known constitutive splicing signals, the I4-31 and I5-51 mutations were designed to

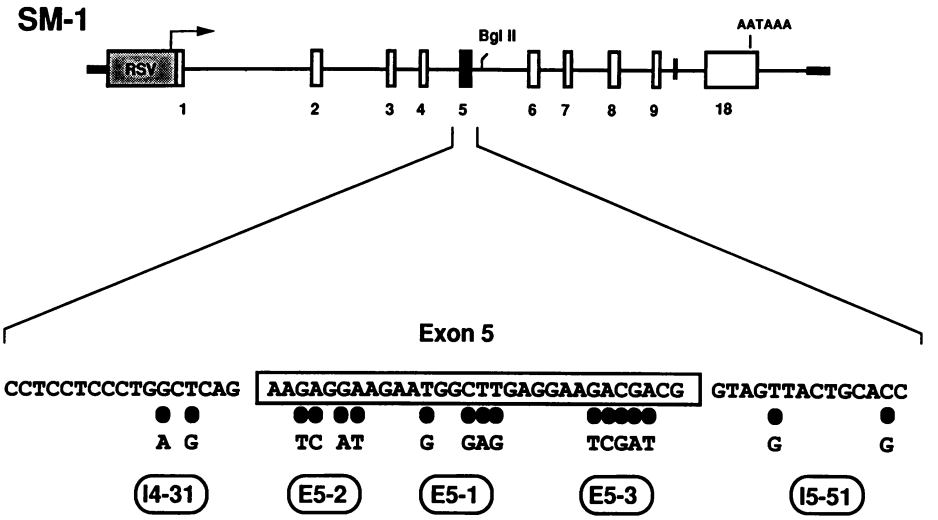

Figure 1: Five cTNT minigene mutants containing nucleotide substitutions within and immediately flanking the alternative exon. The top shows a diagram of the cTNT minigene, SM-1, used for transfection. The positions of transcription initiation within the Rous sarcoma virus (RSV) cassette and the cTNT poly (A) addition signal are indicated. Heavy lines indicate pBR322 vector. The bottom shows the nucleotide sequence of exon 5 and portions of the flanking introns. The nucleotide substitutions for each of the five separate mutations is shown.

be "neutral" or slight improvements with regard to consensus sequences. Mutation I4-31 alters the -4 and -6 position of the 3' splice site of intron 4. According to the 3' splice site consensus sequence (C/T)$_{11}$N(C/T)AG|G (3), no one nucleotide is more likely to be found at the -4 position. The 3' splice site flanking exon 5 contains a T in this position while only 2 of the 16 cTNT constitutive 3' splice sites contain pyrimidines, and G is found in 9 of these. Therefore, the T->G transition in mutant I4-31 provides the alternative exon with a 3' splice site resembling those adjacent to most cTNT constitutive exons. The G->A transition at position -6 of intron 4 provides a Hgia I site for screening.

The wild type 5' splice site adjacent to exon 5 contains a T in the +5 position. While not unique to the cTNT alternative exon or other alternative exons, T is found in this position in only 5% of the 139 introns surveyed by Mount (3). The T->G transition of mutant I5-51 introduces the consensus nucleotide and, consequentially, improves the complementarity of this splice site to the 5' end of U1 snRNA (28) which is required for splicing (4, 5). The C->G transition at +13 generates a Pst I site for screening.

Each of these mutations was introduced into the cTNT minigene, SM-1 (Figure 1) which has previously been shown to produce both mRNAs including and excluding exon 5 following transfection into fibroblast and skeletal muscle cultures (18). For mutagenesis, SM-1 was modified such that an 8 nucleotide Bgl II linker was inserted into a Fnud II site 50 nucleotides

downstream from the alternative exon. During the process of mutagenesis, the Bgl II site is filled in and religated adding a total of 12 additional nucleotides to intron 5 (see Methods). In one of the mutations (I5-51) a deletion occurred during mutagenesis which removed 160 nucleotides of intron 5. All of these modifications to SM-1 are in regions previously shown not to be required for alternative splicing (18). However, to determine whether the additional nucleotides in intron 5 affected alternative splicing, a control plasmid was constructed (SM-7F) that contains only the modifications introduced by the mutagenesis procedure and none of the nucleotide substitutions shown in Figure 1.

The minigenes were transiently transfected into cultures of chick embryo fibroblasts (CEFs) and primary breast muscle cultures. We have shown previously that muscle and non-muscle cells are able to both include and exclude exon 5. Because the recognition of exon 5 as an alternative exon is not cell-specific and because of the relative ease of manipulating CEFs, CEF cultures have been used for most of the transient transfection analysis. All results have been confirmed in primary muscle cultures (see below). In addition, the products of all transfections described below were characterized by S1 nuclease analysis from both the 5' and 3' directions as well as by primer extension analysis.

RNA was extracted and assayed by S1 nuclease analysis as previously described (18). The S1 nuclease probe is a 246 base pair cDNA fragment which spans exons 1-8 and includes exon 5 (Figure 2A). The complementary strand was labeled at the 5' end using polynucleotide kinase (see Methods). Because most of cTNT exon 1 within SM-1 derivatives is replaced by RSV 5'-untranslated sequence, minigene mRNAs protect fragments smaller than the full length probe. mRNAs that include exon 5 protect a 226 nucleotide fragment from S1 nuclease digestion. mRNAs in which exon 5 has been excluded and exons 4 and 6 are spliced together protect a 105 nucleotide fragment. As CEFs do not express endogenous cTNT (18), protected fragments represent RNA only from transfected minigenes.

SM-1 expresses both mRNAs that include and exclude exon 5 (Figure 2B and ref. 18). Comparison of the relative intensities of the two protected fragments at 226 and 105 nt indicate that SM-1 mRNAs including and excluding exon 5 are expressed at equivalent levels in CEF cultures. Interestingly, the addition of 12 nucleotides within intron 5 has the effect of slightly reducing the level at which exon 5 is included into the mature mRNA (compare the relative levels of the 226 and 105 nt bands protected by SM-7F to those of SM-1). This result is surprising since deletions which remove this region do not have this effect (18). However, the effects of the nucleotide substitutions shown in Figure 1 are independent of the 12 additional nucleotides within intron 5 (see below).

Analysis of RNA from all three of the mutations within the alternative exon indicate that the mRNAs that include this exon are either undetectable (E5-1 and E5-2, Figure 2B) or are expressed at levels dramatically reduced relative to mRNAs excluding the exon (E5-3, Figure 2B). On longer exposure, E5-3 shows a faint band representing very low levels of mRNAs that include the mutated exon 5 (data not shown). Clearly, all three mutations which alter the nucleotide sequence

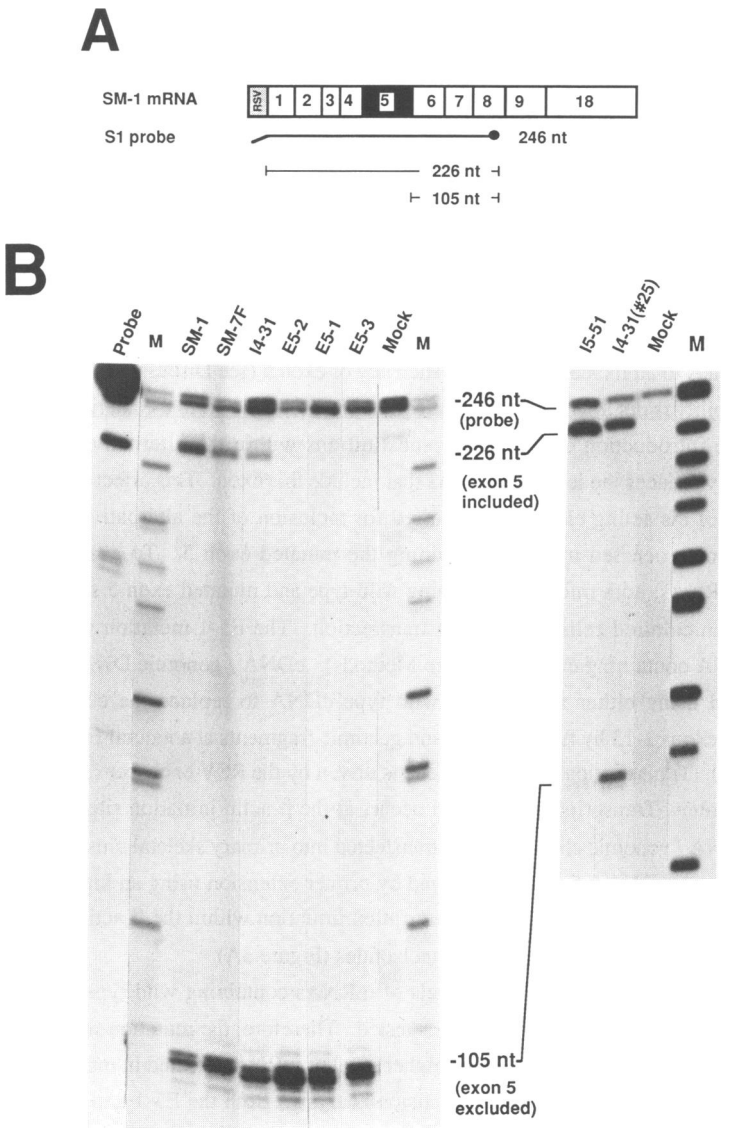

Figure 2: S1 analysis of cTNT minigene mutants transfected into chick embryo fibroblast cultures. A. Diagram of the SM-1 RNA and complementary cDNA probe showing relative positions of the exons and the expected protected fragments. The labeled end of the S1 probe is indicated by a filled circle. B. S1 nuclease analysis. Lane to lane variability in RNA levels represent different transfection efficiencies. The bands around 246 nucleotides represent reannealed probe. Mock treated CEF cultures were transfected with a plasmid containing a SM-1 construction that lacks the RSV promoter and enhancer regions. Each lane contains 20 μg of total RNA from transfected CEF cultures. Hpa II cut pBR322 was ^{32}P-labeled and used as a marker.

within the alternative exon have dramatically decreased the level of mRNAs that include this exon.

The nucleotide substitutions within the 3' splice site flanking the alternative exon have no effect on alternative splicing. The reduced inclusion of exon 5 in RNA from the I4-31 mutant (Figure 2B) is similar to that observed for SM-7F, suggesting that this effect is due to the modifications within intron 5 rather than those within the 3' splice site of intron 4. This is confirmed by a second minigene (I4-31#25, Figure 2B) which contains the I4-31 mutation but not the 12 additional nucleotides within intron 5 due to a cloning artifact. This construct produces equivalent levels of both mRNAs including and excluding exon 5, similar to SM-1 (Figure 2B).

Interestingly, the I5-51 mutation substantially increases levels of mRNAs that include the alternative exon over those which exclude the alternative exon (Figure 2B). Therefore, this mutation leads to an increased splicing efficiency of exon 5 (see Discussion).

mRNA stability is not affected by the nucleotide substitutions within the alternative exon

The introduction of nucleotide substitutions within the alternative exon eliminates or dramatically reduces the level of mRNAs that include this exon. This effect could represent either disruption of cis acting element(s) required for inclusion of the alternative exon or a selective instability of processed mRNAs containing the mutated exon 5. To evaluate the stability of mutated mRNAs, both mRNAs containing wild type and mutated exon 5 sequences were force-expressed in cultured cells by transient transfection. The E5-1 mutation was introduced into a cTNT cDNA containing exons 1-13 (see Methods). cDNA / genomic DNA chimeras were then constructed using either the E5-1 or wild type cDNA to replace the cTNT genomic region containing exons 1-13 by fusing cDNA and genomic fragments at a natural Hind III site in exon 13 (Figure 3A). Transcription of this minigene is driven by the RSV enhancer (24) and the chicken β-actin promoter. Transcription initiation occurs at the β-actin initiation site (23). Wild type and mutant cDNA / genomic chimeras were transfected into primary skeletal muscle cultures as well as CEF cultures. mRNA levels were assayed by primer extension using an kinased oligonucleotide complementary to exon 6. Correct transcription initiation within the β-actin promoter leads to a primer extension runoff product of 247 nucleotides (Figure 3A).

As shown in Figure 3B, the levels of mRNAs containing wild type and mutated exon 5 sequence are equivalent in both cell types tested. Therefore, the mutation within exon 5 does not reduce the stability of the mRNA. The higher level of mRNA detected in muscle cultures is due to the use of more RNA in the primer extension analysis. Both the E5-1 and E5-3 mutations have also been expressed in cDNA / genomic chimeras in which cDNA exons 1-6 replace the comparable genomic segment of SM-1. For these constructions also, the levels of mRNAs containing the mutated and wild type exon 5 are equivalent (data not shown). Therefore, the E5-1 and E5-3 nucleotide substitutions within the cTNT alternative exon do not render the processed mRNA unstable. The absence of mRNAs including exon 5 in mutations E5-1, E5-2, and E5-3 is most likely to result from a disruption of alternative splicing of the minigene pre-mRNA. These results suggest that the nucleotide sequence of exon 5 itself contains cis-acting elements required for its inclusion into processed mRNA during pre-mRNA splicing (see Discussion).

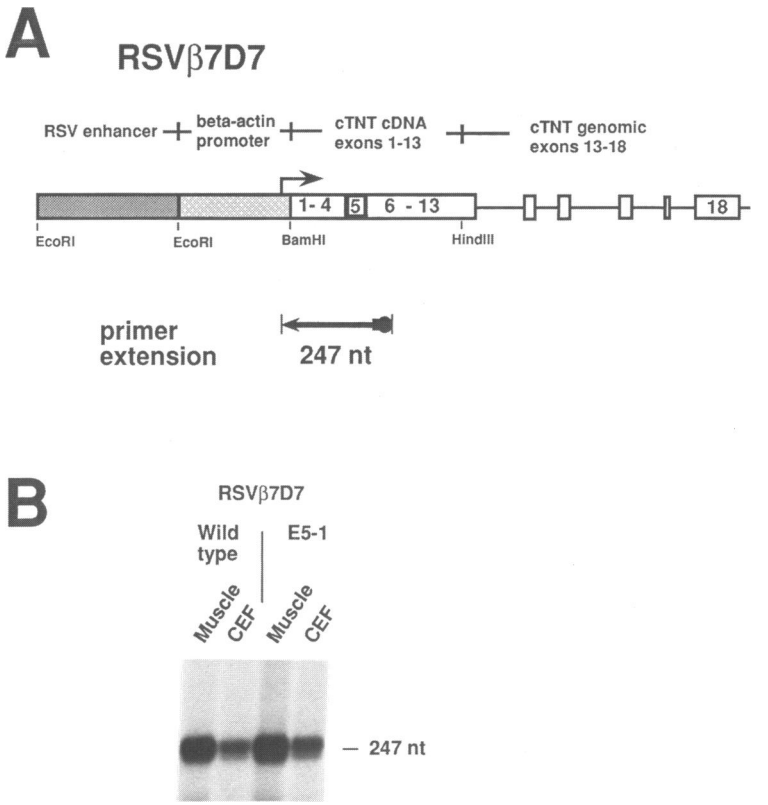

Figure 3: Forced expression of mRNAs containing the E5-1 mutation in skeletal muscle and CEF cultures. A. The construct used to drive expression of mRNAs containing wild type and E5-1 sequences is shown (see Methods and text). Primer extension was performed using a ^{32}P-5' end labeled oligomer which anneals to exon 6. The size of the expected product is 247 nucleotides. B. Primer extension products from wild type and mutated mRNAs expressed in both skeletal muscle and non-muscle cultures are of the expected size. Lanes contain 20 μg total RNA from transfected CEF cultures and 40 μg from transfected skeletal muscle cultures.

Nucleotide substitutions within the alternative exon are solely responsible for disrupting alternative splicing

As described above, modifications in addition to the mutations presented in Figure 1 were introduced during mutagenesis and had the effect of reducing exon 5 inclusion (SM-7F, Figure 2B). It is possible that the exclusion of exon 5 is not due solely to nucleotide substitutions within the alternative exon but represents a summation of effects from several modifications within the SM-1 plasmid. To unequivocally demonstrate that nucleotide substitutions within the alternative exon are responsible for preventing its inclusion, a second oligonucleotide-directed mutagenesis procedure was used to introduce only the four nucleotide substitutions of E5-1 into exon 5 (see

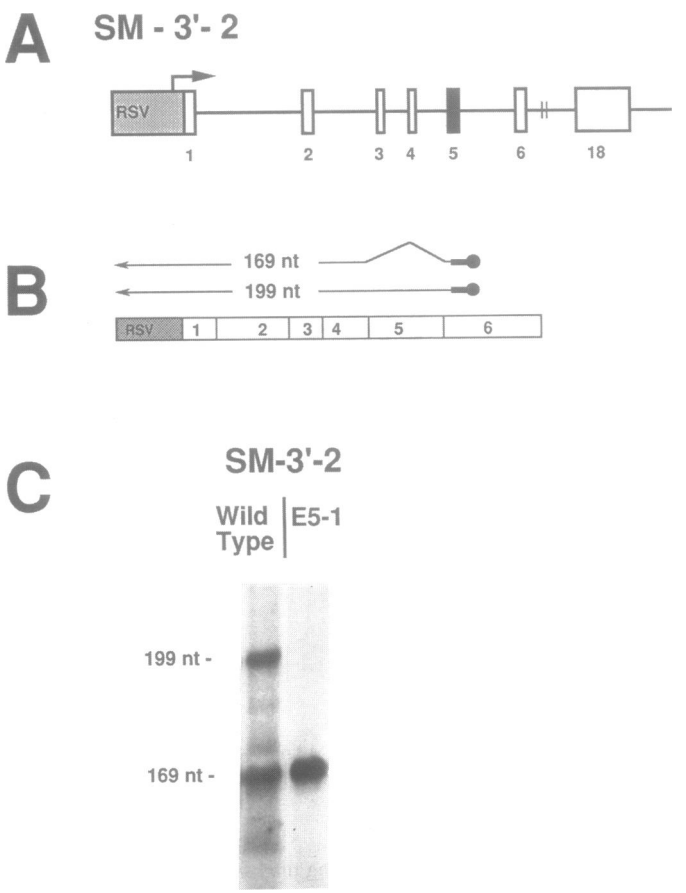

Figure 4: Primer extension analysis of the E5-1 mutation in SM-3'-2. A. Diagram of the cTNT minigene SM-3'-2. Characterization of the expression of this construct by S1 analysis has been previously described (18). B. Diagram of the expected primer extension products from SM-3'-2 mRNAs including and excluding exon 5. C. Primer extension analysis of RNA from CEF cultures transfected with SM-3'-2 minigenes containing the wild type and E5-1 sequences. Each lane contains 20 μg total RNA from transfected cultures.

Methods). The mutated exon was inserted into a second cTNT minigene containing exons 1-6, and exon 18 (SM-3-2, Figure 4A and ref. 18) which has previously been shown to express equivalent levels of the two splice products. Following transfection into CEF cultures, mRNAs were assayed by primer extension as above. As diagrammed in Figure 4B, mRNAs including exon 5 produce a extension product of 199 nucleotides; those excluding exon 5 produce an extension product of 169 nucleotides. While the parental SM-3'-2 construction expresses equivalent levels of mRNAs including and excluding exon 5, only mRNAs which exclude exon 5

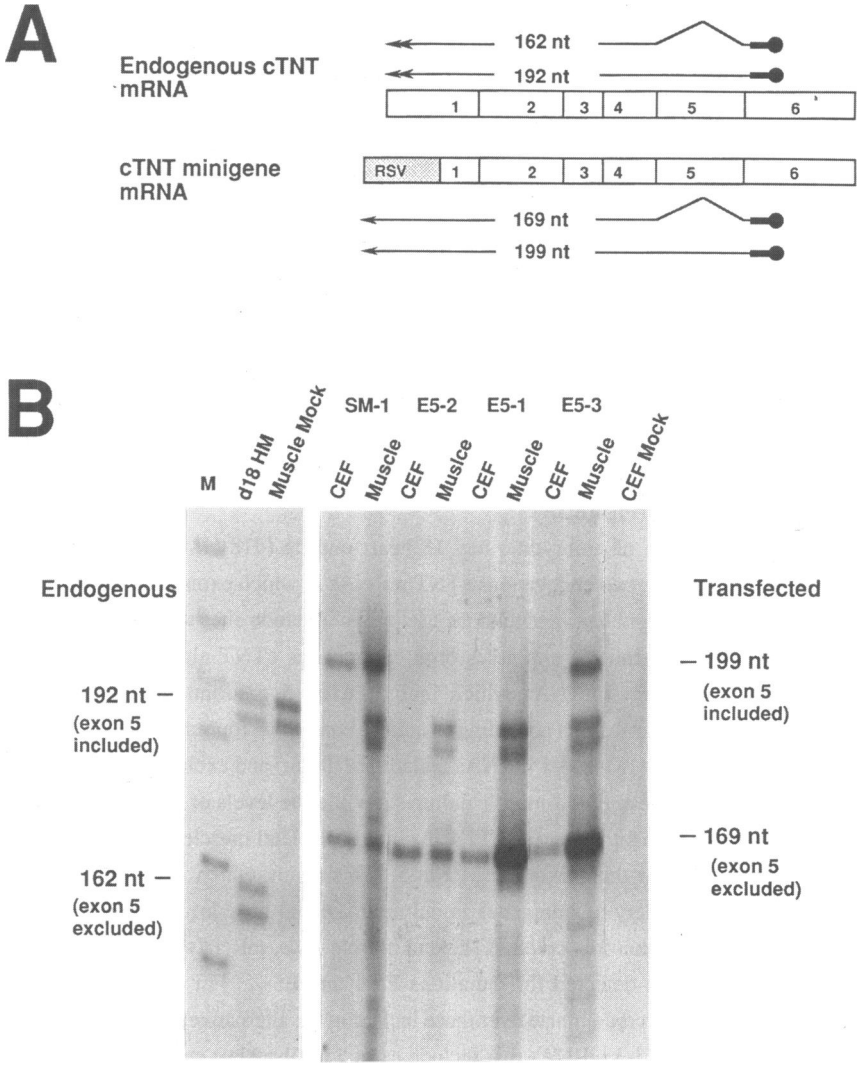

Figure 5: Primer extension analysis of exon 5 point mutants expressed in skeletal muscle and CEF cultures. A. Diagram of expected primer extension products from both the endogenous and transfected cTNT genes for mRNAs including and excluding exon 5. The mRNAs from transfected minigenes yield runoff products that are seven nucleotides longer than those from the endogenous gene. B. Primer extension analysis. The same transfection cocktail was used for parallel cultures of muscle and non-muscle cells. The positions of the primer extension products from endogenous cTNT mRNAs and the transfected cTNT minigene mRNAs are indicated. Mock treated cultures were transfected with a plasmid containing a SM-1 construction that lacks promoter and enhancer sequences. Lane d18HM contains 3.6 µg total RNA from embryonic day 18 heart muscle. 20 µg of total RNA was assayed from transfected skeletal muscle or CEF cultures.

are detectable from the construction containing the E5-1 nucleotide substitutions (Figure 4C). Therefore, in two different minigene constructions, the E5-1 mutation disrupts cTNT pre-mRNA alternative splicing. These results also demonstrate that as few as a four nucleotide substitutions within exon 5 prevent its inclusion into processed mRNA.

Recognition of the mutated exon 5 is disrupted in muscle as well as non-muscle cells

Expression of the endogenous cTNT gene is limited to cardiac and skeletal muscle (17, 29), yet pre-mRNAs from transfected minigenes are alternatively spliced in non-muscle cultures as well as in muscle cultures (Figure 2 and ref. 18). The results presented in Figure 2 indicate that fibroblasts fail to include the mutated alternative exon 5 into processed mRNA. However, it is possible that cell-specific differences between muscle and non-muscle cells could lead to differences in the recognition of the mutated cTNT exon. Mutants E5-1, E5-2, and E5-3 were transfected into primary cultures skeletal muscle and CEF cells. RNAs were assayed by primer extension as above. The primer extension products of mRNAs from the transfected minigene and the endogenous gene can be readily be distinguished as those from the transfected minigene are seven nucleotides longer (Figure 5A).

Primer extension of embryonic day 18 heart muscle (d18 HM, Figure 5B) shows the extension products of the two endogenous cTNT mRNAs in which exon 5 is included (192 nt) or excluded (162 nt). The doublets are due to the use of two initiation sites separated by 3 nucleotides (17). Cultured skeletal muscle expresses both endogenous cTNT alternative splice products (Muscle Mock), however, mRNAs which include exon 5 predominate. The ratio of the endogenous splice products varies according to culture conditions (our unpublished observations). As previously described (18), SM-1 mRNAs including (199 nt) and excluding (169 nt) exon 5 are expressed in both muscle and non-muscle cultures. While the levels of SM-1 mRNAs including and excluding exon 5 are equivalent in CEFs, in cultured skeletal muscle cells, mRNAs including exon 5 predominate over those excluding exon 5. This result, which is under investigation, is reproducible with a variety of minigenes in cultured skeletal muscle. Despite the preferential inclusion of wild type exon 5 in cultured skeletal muscle cells, mRNAs that include the mutated alternative exon are not detected for mutations E5-1 and E5-2. For mutation E5-3, mRNAs excluding exon 5 clearly predominate over those including the alternative exon. A longer exposure indicates low levels of E5-3 mRNAs that include exon 5 in fibroblast cultures consistent with S1 results (data not shown). Lane to lane variability is due to differences in transfection efficiency. Therefore, nucleotide substitutions within cTNT exon 5 prevent or reduce its recognition in both muscle and non muscle cells and lead to disruption of cTNT pre-mRNA alternative splicing.

DISCUSSION

Alternative splicing of cTNT pre-mRNA is disrupted by nucleotide substitutions within the alternative exon

Our results with the cTNT minigene demonstrate that as few as four nucleotide substitutions within the 30 nucleotide alternative exon leads to its exclusion from processed

mRNA. Indeed, three separate mutations which alter four or five nucleotides within three different regions of the alternative exon either eliminate or dramatically reduce inclusion of the exon into mRNA (E5-1, E5-2, and E5-3; Figures 2, and 5). The result with the E5-1 mutation was confirmed in two different minigene constructs (Figures 2 and 4). The possibility that mRNAs including the mutated exon 5 are produced but are selectively degraded has been ruled out by the results presented in Figure 3: mRNAs containing a mutated exon 5 are of comparable stability to the mRNAs containing the wild type sequence. Therefore, the three mutations within exon 5 have disrupted cis-acting component(s) required for recognition of the cTNT alternative exon 5 as a bona fide exon (see below).

The I5-51 mutation increases the match of the 5' splice site flanking the alternative exon to the consensus sequence (3) and increases its complementarity to the 5' end of U1 snRNA. This mutant expresses a substantial increase in the level of mRNAs that include the alternative exon (Figure 2). This result is consistent with several studies which demonstrate that splicing efficiency is directly related to the match of the 5' splice site to the consensus sequence (30-35), particularly the extent of complementarity between the 5' splice site and U1 snRNA (30). The result with the I5-51 mutant could be due to increased interaction of the 5' splice site of intron 5 with U1 snRNA, disruption of the same element(s) defined by the exon mutations, or disruption of a novel cis element. In addition, we cannot rule out the possibility that the 160 bp deletion within intron 5 affects alternative splicing. However, this would appear unlikely as this region was part of a larger deletion that did not affect alternative splicing (18). Further analysis is required to understand the specific role of the 5' splice site of intron 5 in exon 5 alternative splicing.

In contrast to the effects of nucleotide substitutions within the alternative exon and its flanking 5' splice site, the two nucleotide substitutions introduced into the 3' splice site flanking the alternative exon had no effect on its alternative splicing (Figure 2B). The I4-31 mutation expresses reduced levels of mRNA including exon 5 due to the insertion of 12 additional nucleotides within intron 5, however, a second I4-31 mutant lacking the intron 5 modification (I4-31#25, Figure 2) expresses equivalent levels of both products similar to the parental SM-1. Therefore, cTNT alternative splicing does not depend upon the wild type sequence within the flanking 3' splice site.

The mutated alternative exon is not processed in either muscle or non-muscle cells

We have previously demonstrated that muscle-specific factors in trans are not required for alternative splicing of the cTNT alternative exon as both muscle and non-muscle cells constitutively express both SM-1 mRNAs (18). The lack of tissue specificity in the recognition of alternative splice sites in vivo has been demonstrated for several pre-mRNAs (36-38). One exception is the pre-mRNA from the rat fast skeletal troponin T gene which is evolutionarily related to cTNT. The fast skeletal troponin T pre-mRNA contains multicombinatorial alternative exons which require muscle-specific factors for inclusion into processed mRNA. In non-muscle cells, these alternative exons (and some constitutive exons) are not included in mRNAs from transfected genes (39). Similarly, while we have found that specific identification of cTNT exon 5 as an alternative exon

occurs in all cell types transfected thus far (that is, both splice products are expressed), muscle and non-muscle cells differ in the relative ratio of mRNAs that include and exclude exon 5. In cultured skeletal muscle, mRNAs from both the endogenous and transfected genes favor inclusion of the alternative exon as compared to non-muscle cells in which both mRNAs are expressed at equivalent levels (Figure 5 and unpublished data). This effect indicates cell-specific differences in the trans regulation of cTNT alternative splicing in cultured cells and is currently under investigation. Together, the results from cTNT and fast skeletal TNT indicate that qualitative or quantitative differences between muscle and non-muscle cells could lead to cell-specific differences in the recognition of the mutated cTNT alternative exon. However, the results for E5-1, E5-2, and E5-3 were the same in both muscle and non muscle cells: exon 5 inclusion is not detectable for E5-1 and E5-2 and is dramatically reduced for E5-3 (Figure 5). Therefore, nucleotide substitutions within the alternative exon prevent its recognition during pre-mRNA splicing in both muscle and non-muscle cells.

Spacing changes affect cTNT alternative splicing

It is interesting that a 12 nucleotide insertion within a 650 nucleotide intron 50 nucleotides downstream from the alternative exon can quantitatively affect alternative splice site selection. Because this region can be deleted without affecting alternative splicing (18), it is unlikely that a required sequence has been disrupted by the insertion. It is more likely that the altered spacing between exons has affected the use of alternative splice sites. Changes in intron size has been shown to effect the splicing efficiency of some alternative and constitutive exons (13-16, our unpublished observations). Such results emphasize the advantages of nucleotide substitutions rather than insertions or deletions to determine the sequence-specific requirements for alternative splicing.

A role for exon sequence in alternative exon recognition

Our results indicate a major role for exon sequence in the recognition of the cTNT alternative exon as a bona fide exon. This is a provocative result as the elements known to be required for pre-mRNA splicing are generally restricted to introns (3-5). Exons do not appear to contain conserved consensus sequences separate from those of the splice sites. In a recent literature search for mutations that disrupt constitutive splicing, the vast majority altered the sequence of the 5' or 3' splice sites. Only three examples of such mutations were located within exons (40-42) and two of these altered the first or last two exon nucleotides which are within splice site consensus sequences (41, 42). While in vitro cis competition assays have demonstrated a role for exon sequence in splice site selection for β-globin exons 1 and 2 (15, 32), in the absence of cis competition, the vast majority of the β-globin exon 2 can be deleted (to the first 4 to 20 nucleotides depending on the system used) or replaced with non-exon sequence without eliminating splicing in vitro (43-45). Within other genes, exons as small as 6 and 7 nucleotides have been found to splice efficiently in vivo (17, 46, 47) suggesting that a minimum of exon sequence is required for splicing of some constitutive exons. Furthermore, RNase protection or chemical footprinting studies consistently demonstrate protection of intron sequence (except for a few nucleotides at the

intron/exon boundary) (48-57). One study, however, found protection of regions within exon sequence (51). In general, specific elements within exons that are required for pre-mRNA splicing have not been defined.

It is not clear whether the mutations within cTNT exon 5 have disrupted signals required specifically for alternative splicing or have disrupted "general" splicing signals present in both alternative and constitutive exons. However, in comparison to constitutive exons, splicing of the cTNT alternative exon is unusually sensitive to disruption of exon nucleotide sequence. This sensitivity may reflect the presence of cis elements required to distinguish exon 5 as an alternative exon. Several studies suggest that intact exon sequence may be a requirement for alternative splice site recognition in some pre-mRNAs. For example, deletions or insertions within exon sequence of the SV40 late transcript have been found to alter the efficiency with which alternative splice sites are used (7). In addition, mutations which are thought to prevent use of a female-specific alternative 5' splice site within the Drosophila doublesex gene have been identified as insertions or deletions within adjacent exon sequence (58). In results similar to those presented here, transfection analysis of the human fibronectin gene has determined that removal or inversion of an 81 nucleotide internal segment of the 270 nucleotide EDIIIA alternative exon (which, like cTNT exon 5, is either included or excluded) prevents expression of mRNAs including the exon as assayed by RNA blots (10). Interestingly, comparison of the EDIIIA alternative exons from avian and mammalian fibronectin genes indicates that the nucleotide sequence of the alternative exon is slightly more conserved (86%) than the encoded protein sequence (79%) possibly reflecting a role for the exon nucleic acid sequence in its alternative splicing (59). Similarly, cis elements required for alternative splicing have been defined within an alternative exon of the leukocyte common antigen pre-mRNA (12).

Presumably, cTNT exon 5 mutations prevent inclusion of the exon by either disrupting pre-mRNA secondary/tertiary structure or by preventing interaction between exon 5 and one or more trans-acting factors. A search for higher order RNA structure reveals no striking potential base-pairing that would be disrupted by the mutations within exon 5. Indeed, we have previously found that deletion of regions containing complementarity to exon 5 does not prevent alternative splicing of cTNT minigene pre-mRNAs (18). However, the difficulty in predicting pre-mRNA intramolecular base-pairing from nucleotide sequence alone prevents us from ruling out a role for pre-mRNA higher-order structure in the results presented here. An alternative explanation is that factor(s) in trans act in a positive fashion to include exon 5 into mRNA during pre-mRNA processing. In the most straightforward model, factor(s) would bind to the alternative exon itself in a sequence specific manner. If this model is correct, wild type and mutant exon 5 RNAs should differ in their ability to bind to nuclear factors in vitro.

Exon recognition represents only one component of a process leading to exon 5 alternative splicing. Previous results suggest that multiple pre-mRNA cis elements are required to define exon 5 as an alternative exon (18). In addition, cTNT alternative splicing in vivo is under developmental regulation (17) which requires regulatory factors acting in trans. Clearly, the selection of a specific

exon for alternative splicing and the modulation of the ratio of the two splicing pathways according to developmental stage involves multiple regulatory features. The identification of specific nucleotides required for alternative exon recognition represents an important first step in understanding this process.

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