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In Vitro Splicing of Cardiac Troponin T Precursors

EXON MUTATIONS DISRUPT SPLICING OF THE UPSTREAM INTRON*

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A single cardiac troponin T (cTNT) gene generates two mRNAs by including or excluding the 30-nucleotide exon 5 during pre-mRNA processing. Transfection analysis of cTNT minigenes has previously demonstrated that both mRNAs are expressed from unmodified minigenes, and mutations within exon 5 can lead to complete skipping of the exon. These results suggested a role for exon sequence in splice site recognition. To investigate this potential role, an *in vitro* splicing system using cTNT precursors has been established. Two-exon precursors containing the alternative exon and either the upstream exon or downstream exon were spliced accurately and efficiently *in vitro*. The mutations within the alternative exon that resulted in exon skipping *in vivo* specifically blocked splicing of the upstream intron *in vitro* and had no effect on removal of the downstream intron. In addition, the splicing intermediates of these two precursors have been characterized, and the branch sites utilized on the introns flanking the alternative exon have been determined. Potential roles of exon sequence in splice site selection are discussed. These results establish a system that will be useful for the biochemical characterization of the role of exon sequence in splice site selection.

Most genes express a single pre-mRNA that is spliced using invariant (constitutive) splice sites to generate a single mRNA. However, a large number of genes express multiple mRNAs via the use of alternative splice sites during pre-mRNA splicing. Alternative splice site selection is often regulated according to cell type or developmental stage and provides a mechanism by which single genes express diverse protein isoforms in a tightly regulated fashion. How the splicing machinery can distinguish alternative and constitutive splice sites and can affect regulated splice site selection remains largely unknown (reviewed in Ref. 1).

Constitutive and alternative splicing appear to employ the same basic mechanism of intron removal which has been elucidated using exogenous RNA precursors in cell free splicing reactions (reviewed in Ref. 2). Intron removal occurs in two steps. First, cleavage at the 5′ splice site occurs concomitant with formation of a 2′,5′ phosphodiester bond between the 5′ end of the intron and an adenosine (named the branch site) usually located 18–38 nucleotides upstream of the 3′ splice site. This step produces a free 5′ exon and a lariat intermediate containing the intron and 3′ exon. In the second step, the two exons are ligated together concomitant with cleavage at the 3′ splice site producing a spliced product and a released lariat intron. Splicing is catalyzed by a multisubunit complex, the spliceosome, which is composed of four ribonucleoprotein particles (snRNPs U1, U2, U4/U6, and U5), the pre-mRNA, and multiple auxiliary proteins (reviewed in Refs. 2–4). The splicing reactions depend upon the presence of relatively short conserved *cis* elements within the introns at the 5′ and 3′ splice sites as well as the branch site (2, 5–9). The spliceosome assembles in a stepwise manner as its components bind to these *cis* elements. Many of these interactions have been characterized by genetic and *in vitro* reconstitution experiments (2–4).

A number of alternatively spliced precursors have been analyzed using *in vitro* splicing systems (10–23). One feature to emerge from these studies is the common use of unconventional branch sites in alternatively spliced introns including multiple branch sites, distal branch sites, and/or branch site residues other than adenosine (10–17). For several RNAs, utilization of unconventional branch sites correlates with some aspects of alternative splicing. For example, within the SV40 early pre-mRNA splicing of two 5′ splice sites to a shared 3′ splice site correlates with utilization of different branch sites suggesting that branch site selection is involved in regulating alternative 5′ splice site selection in this system (15, 16). Within the rat α-tropomyosin gene, two mutually exclusive alternative exons are prevented from splicing together by the close proximity of a distal branch site to the 5′ splice site (10). However, this does not appear to be generally applicable to mutually exclusive alternative exons (11, 12). Branch site selection is an attractive regulatory mechanism for regulated alternative splice site selection, however, whether it is determinative is unknown.

A role for exon sequence in splice site selection is indicated by experiments performed both *in vivo* and *in vitro* on a large number of genes (24–39, 64). The first two and last three nucleotides of the exon are contiguous with the conserved 3′ and 5′ splice site sequences, respectively (5, 6), and are likely to function as components of these *cis* elements. Of particular interest are exon mutations that are internal to the exon/intron junctions which disrupt splicing. Such mutations might be inactivating novel *cis* elements required for splice site selection. These potential *cis* elements have not been well characterized. Interestingly, most examples of internal exon mutations that disrupt splicing *in vivo* are adjacent to alternative splice sites (24–28, 30–36). Only a few such cases have been reported for constitutive exons (38, 64). An *in vitro* system that reproduces the effects of exon mutations would be useful for biochemical investigation of the role of exon sequence in splice site selection.

I have been using the chicken cardiac troponin T (cTNT)[1]

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[1] The abbreviations used are: cTNT, cardiac troponin T; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; snRNP, small nuclear ribonucleoprotein particle; DTT, dithiothreitol; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

gene as a model for the investigation of developmentally regulated alternative splicing. Expression of the cTNT gene is limited to cardiac and skeletal muscle (40, 41). The gene expresses a single pre-mRNA that contains 17 constitutive exons and one 30 nucleotide alternative exon, exon 5, which is either included into or excluded from mature mRNA (41). cTNT mRNAs undergo a developmentally regulated transition such that exon inclusion predominates in early embryonic heart and exon exclusion predominates in the adult heart (41).

Transient transfection of cTNT minigenes has previously demonstrated that minigene pre-mRNAs retain all of the *cis* elements required for alternative splicing as both mRNAs including and excluding exon 5 are expressed (31, 42). Nucleotide substitutions within the alternative exon, distinct from the splice site *cis* elements, result in complete skipping of the exon *in vivo* (31). Exon mutations do not affect mRNA stability indicating that they specifically disrupt exon inclusion during pre-mRNA splicing (31). To characterize removal of the introns flanking the alternative exon, an *in vitro* splicing system for cTNT precursors has been established. In this paper I report that the mutations within exon 5 that lead to exon skipping *in vivo* specifically affect splicing of the upstream intron *in vitro*.

MATERIALS AND METHODS

Recombinant DNA—Recombinant DNA methods were preformed according to Sambrook *et al.* (43). Restriction enzymes, T4 DNA polymerase, and calf intestine alkaline phosphatase were purchased from Boehringer Mannheim, T4 DNA ligase from New England Biolabs, polynucleotide kinase from Pharmacia LKB Biotechnology Inc. and AMV reverse transcriptase from Life Sciences. Sequencing was performed on plasmid DNA using Sequenase (U. S. Biochemical).

Cell Culture, Nuclear Extract, and S100 Cytoplasmic Extract Preparation—Spinner cultures of HeLa S3 cells (from American Type Culture Collection) were grown in RPMI 1640 (GIBCO) supplemented with 7% fetal calf serum (Hyclone). Nuclear extracts and S100 extracts were prepared as described by Dignam *et al.* (44).

Plasmid Construction—The E45 plasmid (Fig. 1) contains the cTNT genomic fragment from the 5′-2 minigene (42): an artificial *Bam*HI site in exon 4 to a natural *Mae*III (made blunt-ended using T4 DNA polymerase) site located four nucleotides downstream of exon 5 was subcloned into pKS+ vector (Stratagene) *Bam*HI to a *Sal*I site (made blunt-ended using T4 DNA polymerase). The intron and exon sizes are indicated in Fig. 1. Introduction of the E5–1 and E5–3 mutations into the 3′-2 minigene by oligonucleotide-directed mutagenesis was described previously (31). E45 E5–1 and E45 E5–3 plasmids were derived as above from the 3′-2 minigenes containing these mutations (31). The sequence of exon 5 and the adjacent 3′ splice site was confirmed in all plasmid preparations used for RNA transcription.

The E56 plasmid (Fig. 2) contains a cTNT genomic fragment from a minigene, μSPE, that contains exons 4, 5, and 6 and expresses high levels of both mRNAs including and excluding exon 5 *in vivo*.[2] An *Spe*I site was introduced into the last eight nucleotides of exon 6 by three nucleotide substitutions. This modification has no effect on the alternative splicing in transient transfection assays.[2] The cloned cTNT fragment includes from four nucleotides upstream of exon 5 (*Dde*I site made blunt-ended) to the *Spe*I site in exon 6 subcloned into KS+ *Hin*dIII (blunt-ended)/*Spe*I. The intron and exon sizes are indicated in Fig. 2. The E56 E5–1 and E56 E5–3 plasmids were similarly derived from μSPE minigenes containing the respective mutations. The sequence of exon 5 and the adjacent 3′ splice site was confirmed in all plasmid preparations used for RNA transcription.

The E46ND plasmid (see Fig. 5) was derived from a μSPE minigene containing a collapse of a *Nco*I site in intron 4 to a *Pst*I site in intron 5 following treatment with T4 DNA polymerase. The intron and exon sizes are indicated (see Fig. 5).

RNA Synthesis and in Vitro Splicing Reactions—Capped RNAs were synthesized *in vitro* on linearized plasmids using T3 or T7 RNA polymerase and [α-^{32}P]GTP essentially as described by Krainer (45).

[2] T. A. Cooper, unpublished observations.

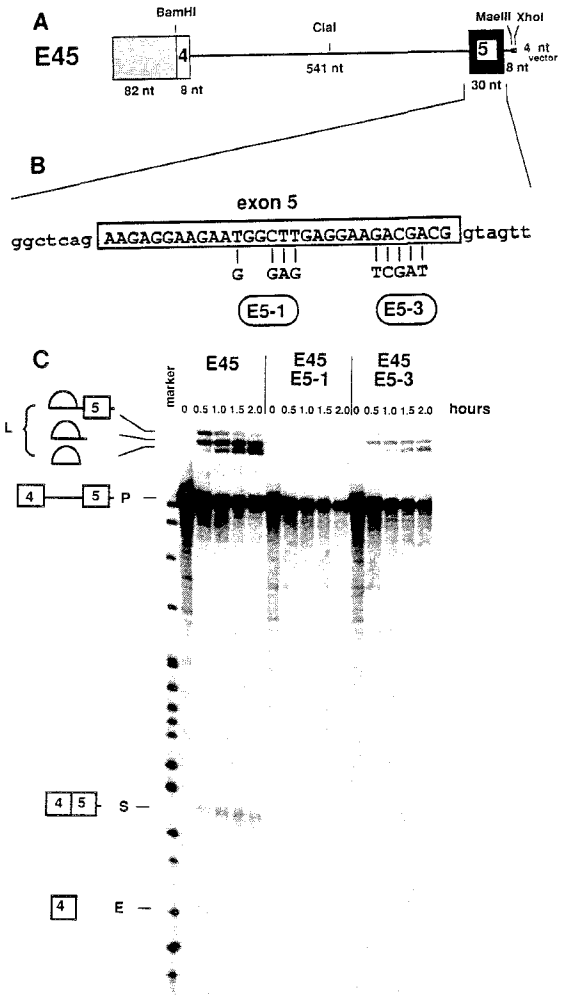

FIG. 1. ***In vitro* splicing of cTNT exons 4 and 5.** *A*, schematic diagram of precursor RNA synthesized *in vitro* (not to scale) (see "Materials and Methods" for details of cloning). Transcripts terminate at the *Xho*I site. The *shaded region* upstream of exon 4 represents vector sequence included in the RNA transcript. *B*, sequence of cTNT exon 5 (*boxed*) and adjacent splice sites. The nucleotide substitutions of the E5-1 and E5-3 mutations are indicated. The three precursors are identical except for the indicated nucleotide substitutions. *C*, time course of *in vitro* splicing reactions for cTNT transcripts E45, E45 E5-1, and E45 E5-3 run on a 4% sequencing gel. The products and intermediates are schematically indicated: *L*, lariat intermediates; *P*, precursor; *S*, spliced exons 4 and 5; *E*, exon 4 intermediate.

Full-length transcripts were isolated from denaturing urea-polyacrylamide gels. *In vitro* splicing assays were performed at 30 °C for the times indicated. A standard assay contained: 1 mM $MgCl_2$, 625 μM ATP, 20 mM creatine phosphate, 2.6% polyvinyl alcohol, 12 mM HEPES (pH 8.0), 12.4% glycerol, 10–15 ng of precursor RNA (3×10^4 cpm), 60 mM KCl, 0.12 mM EDTA, 0.5 mM DTT, 10–25 units of RNasin (Promega), and 10 μl of nuclear extract (9–11 μg of protein/μl) in a 25-μl volume. Following incubation, reactions were treated with proteinase K and recovered as described (45).

Identification of Lariat Intermediates and Branch Site Mapping—Reaction products were analyzed on denaturing urea-polyacrylamide gels. Size determinations were made according to molecular weight using DNA markers. RNA intermediates containing a lariat structure were identified by aberrant migration on different percentage polyacrylamide gels (46, 47). In addition, the presence of lariat structures was confirmed by treatment with debranching activity in S100 extracts (48). To map the sites of branch formation, lariat intermediates were gel-isolated and subject to primer extension analysis. Oligonucleotides complementary to intron 4 or exon 5 (Fig. 3) or intron 5 or exon 6 (Fig. 4) were 5′-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Following heat inactivation of the kinase reaction at 65 °C for 10 min, the labeled primers were used directly for primer

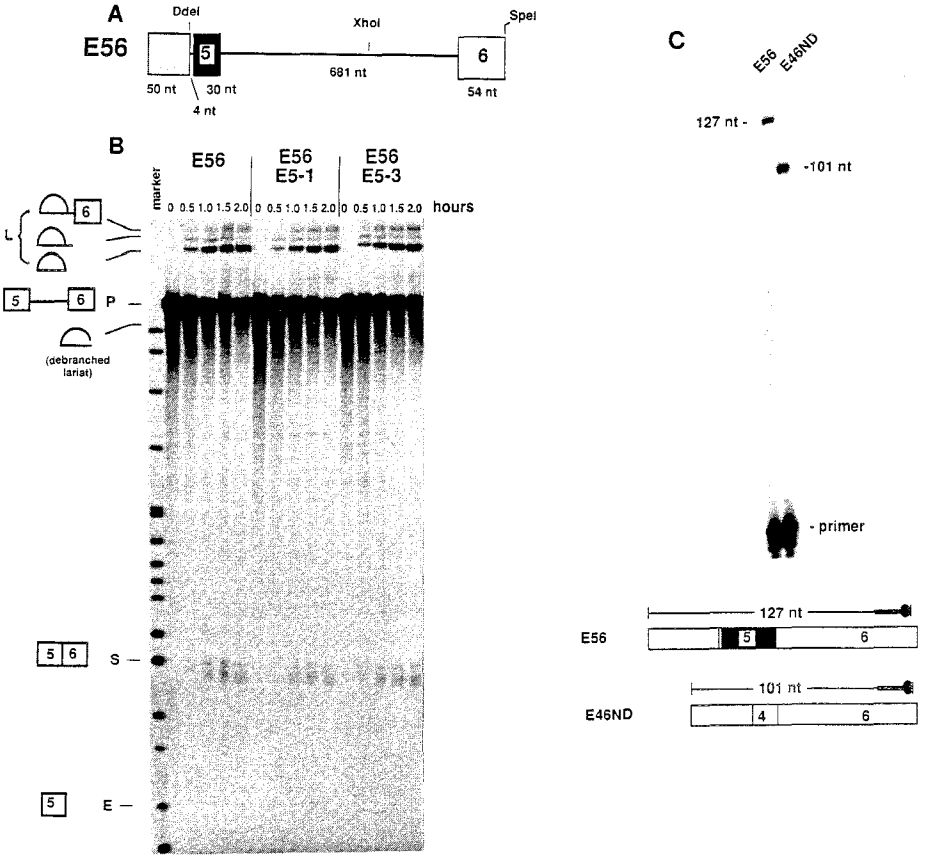

FIG. 2. ***In vitro* splicing of cTNT exons 5 and 6.** *A*, schematic diagram of the E56 precursor (not to scale). Transcripts end at the *SpeI* site at the 3′ end of exon 6. *B*, time course of splicing reactions of the three E56 precursors containing unmodified exon 5 or the E5-1 or E5-3 mutations run on a 4% sequencing gel. The products and intermediates are schematically indicated: *L*, lariat intermediates; *P*, precursor; *S*, spliced exons 5 and 6; *E*, exon 5 intermediate. The spliced E56 product runs as a smear due to degradation by 3′ exonuclease activity (see text). *C*, primer extension was performed on gel-isolated E56 and E46ND (Fig. 5) products. The entire product smears were isolated and used for primer extension analysis. The *bottom* shows a diagram of the expected primer extension products from correctly spliced products. Sizes were determined using a sequencing ladder in adjacent lanes (not shown).

extension. Primers were annealed with branched or debranched lariat intermediates at 65 °C for 10 min in 300 mM NaCl, 40 mM Tricine (pH 8.0), and 0.1 mM EDTA in 10 μl. Tubes were transferred to 42 °C and the extension reaction was initiated by adding an equal volume of mixture to give final concentrations of 100 mM Tris (pH 8.3), 12 mM $MgCl_2$, 10 mM DTT, 0.5 mM each deoxynucleotide triphosphates, and 8 units (per reaction) avian myeloblastosis virus reverse transcriptase. After 30 min, the reaction is stopped by addition of 0.7 volumes of 12 mM EDTA and 5 M ammonium acetate prior to ethanol precipitation. The pellet is redissolved in 0.5 M NaOH/0.1 mM EDTA and treated at 65 °C for 5 min to hydrolyze RNA. Following neutralization by addition of 0.7 volumes of 5 M ammonium acetate with carrier tRNA, ethanol precipitation, and a 70% ethanol wash, the extension products are separated on an 8% denaturing urea-polyacrylamide gel.

RESULTS

Templates for Cardiac Troponin T Precursors Used for in Vitro Splicing—Templates containing exons 4 and 5 (E45, Fig. 1*A*) or exons 5 and 6 (E56, Fig. 2*A*) were constructed for *in vitro* RNA synthesis. Use of these two-exon precursor RNAs allowed separate analysis of the effect of exon 5 mutations on removal of the upstream and downstream introns. Templates were constructed in Bluescript vectors using cTNT genomic fragments from minigenes previously shown to express pre-mRNAs that are correctly and efficiently spliced *in vivo* (31, 42). The details of cloning are described under "Materials Methods." The E45 template differs from genomic DNA by the insertion of a *ClaI* linker into a *PvuII* site in intron 4 and substitution of three nucleotides within exon 4 to create a *BamHI* site (Fig. 1*A*). The E56 template differs from genomic DNA by an *XhoI* linker in a *SmaI* site in intron 5 and a *SpeI* site at the 3′ end of exon 6 created by three nucleotide substitutions (Fig. 2*A*). None of these changes has a significant effect on splicing *in vivo* (31, 42).[2]

Three variants for both the E45 and E56 templates were constructed. These variants contained the unmodified exon 5, exon 5 containing the E5–1 mutation, or exon 5 containing the E5–3 mutation (shown in Fig. 1*B* and Ref. 31). The E5–1 mutation contains a four nucleotide substitution within exon 5 that is 11 nucleotides from the 3′ splice site and 13 nucleotides from the 5′ splice site. The E5–3 mutation contains a substitution of five consecutive nucleotides located two nucleotides from the 5′ splice site. *In vivo*, the E5–1 mutation leads to complete skipping of exon 5 during minigene pre-mRNA splicing and the E5–3 mutation significantly reduces exon 5 inclusion compared to minigenes containing the unmodified exon 5 (31).

Nucleotide Substitutions within the Alternative Exon Block Splicing of the Upstream Intron but Not the Downstream Intron—To determine the effect of exon 5 mutations on *in vitro* splicing of exons 4 and 5, capped precursor RNAs were synthesized *in vitro* from templates containing the unmodified exon 5 (E45) and mutated exon 5 (E45 E5–1 and E45 E5–3). The three RNAs synthesized from these templates are identical except for the nucleotide substitutions within exon 5 shown in Fig. 1*B*. RNAs were synthesized to the same specific activity and gel isolated. Conditions for optimal splicing in HeLa nuclear extracts was empirically determined using the E45 precursor (see "Materials and Methods"). The *in vitro* splicing reactions of the E45, E45 E5–1, and E45 E5–3 substrates shown in Fig. 1*C* were run simultaneously using the same reagents. Aliquots of the splicing reactions were removed at the times indicated, RNA was extracted, treated with proteinase K, and the reaction products and intermediates were separated on a 4% sequencing gel.

The unmodified E45 precursor produces the expected bands precisely corresponding in size to correctly spliced product

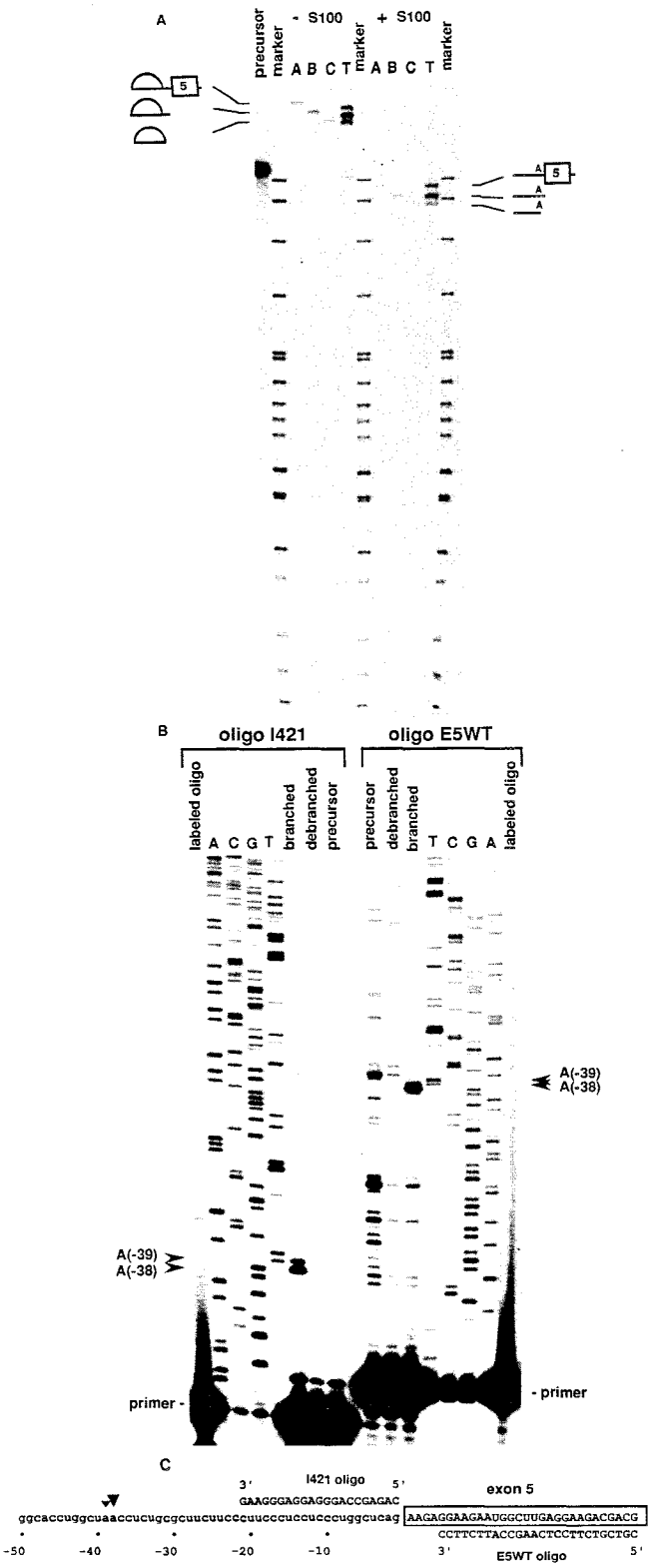

FIG. 3. **Characterization of lariat intermediates for the E45 precursor and branch site determination upstream of cTNT exon 5.** *A*, identification of lariat intermediates of E45 splicing. Lariat intermediates were isolated either separately (*lanes A–C*) or together (*lane T*). Half of the sample was treated with S100 extract containing debranching activity (+S100), the other half was left untreated (−S100) before electrophoresis on a 4% sequencing gel. The lariat intermediates were identified by the size of the linearized RNA using the molecular weights of DNA size markers (a ^{32}P-labeled *Hpa*II digest of pBR322). The position of the branch site relative to the 3′ end of the linearized lariats is indicated by an A. *B*, branch site mapping on isolated E45 lariats. Primer extension was performed on branched and debranched isolated lariats as well as isolated untreated precursor using two oligonucleotides complementary to exon 5 or intron 4 (E5WT and I421, shown in *C*). The cDNA products were run on 8% sequencing gels adjacent to dideoxy sequencing reactions primed by the kinased ^{32}P-labeled oligo on the E45 plasmid. cDNA extension products terminate one nucleotide preceding the branch site. *C*, nucleotide sequence of exon 5 and the adjacent 3′ splice site. Exon 5 is *boxed*. The positions of the complementary oligonucleotides used for branch site mapping are shown. The positions of the major (A(−38)) and minor (A(−39)) branch sites determined in *B* are indicated by *arrowheads*.

and the released first exon, as diagramed in Fig. 1*C*. The three bands that migrate slower than the precursor are lariat intermediates (see below). Therefore, the E45 precursor is correctly and efficiently spliced *in vitro*. The slight smearing of the E45 product band is due to degradation from 3′ exonucleases present in the nuclear extract (see below).

In contrast to the E45 precursor, *in vitro* splicing of the E45 E5–1 precursor is almost completely blocked (Fig. 1*C*).

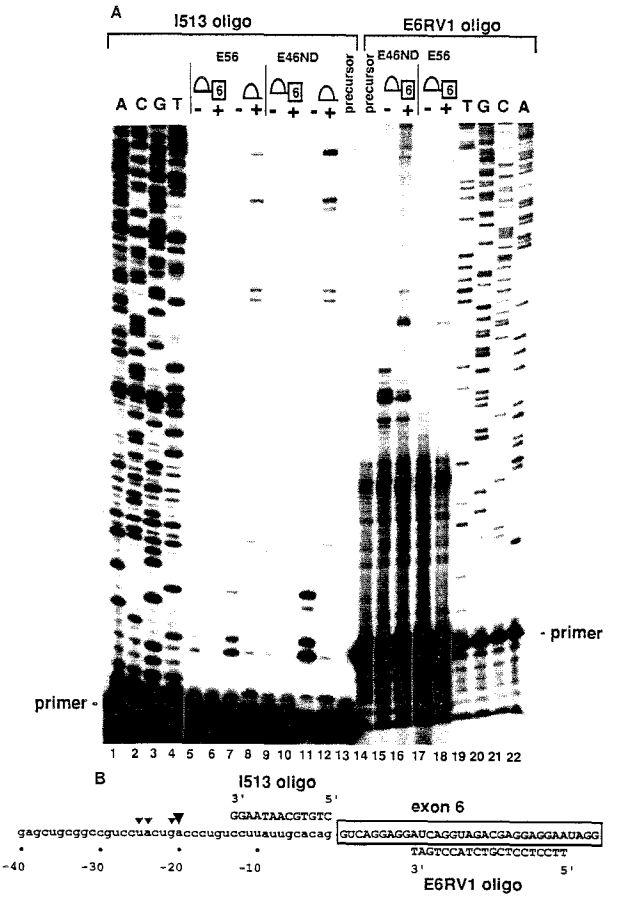

FIG. 4. **Branch site determination on isolated E56 and E46ND lariat intermediates.** *A*, lariat forms of intron-exon 6 and the intron alone (schematically diagramed at the *top* of the *lanes*) were isolated separately from sequencing gels. Primer extension was performed on branched (−) or debranched (+) lariats and isolated untreated precursor using the two labeled oligonucleotide primers shown in *B*. Labeled oligonucleotides were also used to prime dideoxy sequencing reactions on the E56 plasmid. Primer extension products and sequencing reactions were run on an 8% sequencing gel. cDNA extension products terminate one nucleotide preceding the branch site. *B*, nucleotide sequence of exon 6 and adjacent 3′ splice site. Exon 6 is *boxed*. The positions of the complementary oligonucleotides used for branch site mapping are shown. *Arrowheads* indicate the positions of the branch sites.

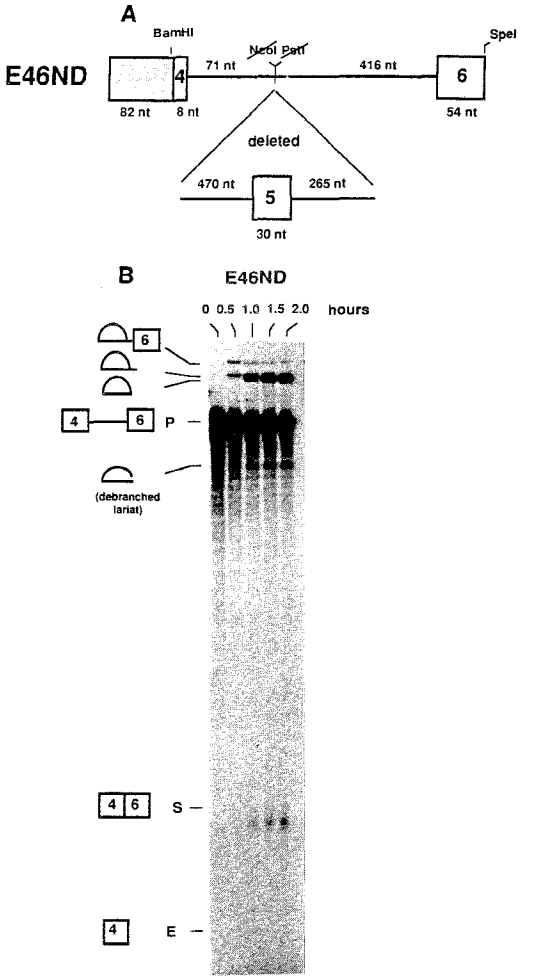

FIG. 5. ***In vitro* splicing of the E46ND precursor.** *A*, schematic representation of the E46ND precursor (not to scale). *B*, time course of accumulation of splice products and intermediates. The products were analyzed on a 4% sequencing gel. The identities of the products are indicated schematically.

Long exposures reveal very low levels of E45 E5–1 lariat intermediates. Bands corresponding to either correctly spliced product or the use of cryptic splice sites are not detected even following incubation times of 4 h and long exposure times (Fig. 1*C* and data not shown). Therefore, a four-nucleotide substitution within exon 5 inhibits splicing of the E45 E5–1 precursor. Furthermore, this mutation blocks the first step in the splicing reaction.

Similarly, splicing of the E45 E5–3 precursor is significantly reduced. Compared to the E45 precursor, E45 E5–3 produces low levels of intermediates and product (Fig. 1*C*). The results presented in Fig. 1*C* have been reproduced using a variety of reaction conditions including four different HeLa nuclear extract preparations from three different laboratories (data not shown). In all cases, the E5–1 mutation prevents splicing of the E45 precursor and the E5–3 mutation substantially reduces splicing compared to the unmodified E45 precursor.

In vitro splicing reactions using the three E56 precursors (E56, E56 E5–1, and E56 E5–3) are shown in Fig. 2*B*. The RNA precursors were synthesized to the same specific activities and gel-isolated and *in vitro* splicing reactions were performed simultaneously using the same reagents. The E56 precursor produces the expected band for the released first exon and a smear just below the expected size for spliced product. To confirm that the E56 precursor produces the correctly spliced product, primer extension was performed on isolated bands forming the smear using an oligonucleotide complementary to exon 6. This analysis produced a single band precisely of the expected size (Fig. 2*C*). Primer extension performed directly on RNA extracted from *in vitro* splicing reactions yielded the same results (data not shown). Therefore, the E56 precursor is correctly spliced *in vitro*. Smearing of the product band most likely results from degradation due to 3′ exonuclease activity in the nuclear extract.

The species having a slower mobility than the precursor on a 4% sequencing gel are lariat intermediates (see below). The band which appears just above the top band of the DNA marker after 60 min is the released intron 5 lariat linearized by debranching activity in the nuclear extract (data not shown).

Interestingly, E56 precursors that contain either the E5–1 or the E5–3 mutations within exon 5 are spliced with the same efficiency and kinetics as the unmodified E56 precursor (Fig. 2*B*). The nucleotide substitutions within exon 5 have no effect on splicing of exons 5 and 6. Therefore, exon 5 mutations that prevent or reduce exon inclusion *in vivo* specifically interfere with splicing of exons 4 and 5 *in vitro*. Furthermore, the degree to which the two different exon mutations interfere with splicing of exons 4 and 5 *in vitro* correlate with their effects *in vivo*.

Characterization of Lariat Intermediates and Branch Site for Intron 4—In general, the position of branch site formation upstream of alternative splice sites occur either at multiple sites and/or at sites that are relatively distant from the 3′ splice site compared to constitutive splice sites (10–17). Branch site formation upstream of the cTNT alternative exon 5 was therefore of interest. *In vitro* splicing of the E45 precursor generated three bands with mobilities that were less than the precursor (see Fig. 1*C*) indicating that they represented splicing intermediates containing lariat structures. These bands were identified as the only potential lariat intermediates generated during E45 splicing by analysis of whole splicing reactions on different percentage acrylamide gels before and after incubation with HeLa S100 extracts which contains debranching activity (48) (data not shown). To characterize the E45 lariat intermediates, bands were isolated and either left untreated or treated with HeLa S100 extract and run on a 4% sequencing gel (Fig. 3*A*). In Fig. 3*A*, bands that were isolated separately are labeled *A*-*C* from slowest to fastest migration prior to debranching. All three bands were also isolated together (labeled as *T*). The change in migration of these bands following debranching results from linearization and confirms the presence of a lariat structure. The sizes of the linearized bands correspond precisely to those expected for the intermediates diagramed in Fig. 3*A*. The slowest migrating band (*A*) represents the intermediate containing intron 4 and exon 5. Band *B* represents the released intron 4 lariat following joining of exons 4 and 5. The size of the linear band *C* is 504 nucleotides, the distance from the first nucleotide of the intron to the branch site (see below). Therefore, band *C* represents released intron 4 lariat the 3′ end of which has been degraded to the branch site nucleotide by 3′ exonuclease activity present in the nuclear extract.

To determine the branch sites upstream of the cTNT alternative exon, primer extension analysis was performed on isolated lariat intermediates of the E45 precursor. The presence of branched nucleotides block reverse transcriptase such that the cDNA extends to the residue preceding the branched nucleotide (47). The three lariat intermediates were isolated together and half of the sample was treated with HeLa S100 extract (debranched, Fig. 3*B*), the other half was used directly

for primer extension (branched, Fig. 3*B*). Separate primer extension experiments were performed using two labeled oligonucleotides. Oligo E5WT anneals within exon 5 and oligo I421 anneals to the last 21 nucleotides of intron 4 (Fig. 3*C*). In addition to isolated branched and debranched lariats, primer extension was also performed on isolated precursor to identify the "strong stops" of reverse transcriptase on the linear RNA. cDNA products were analyzed on an 8% denaturing acrylamide gel adjacent to dideoxy sequencing reactions primed by the indicated labeled oligonucleotide on the E45 plasmid template (Fig. 3*B*).

As shown in Fig. 3*B*, primer extension from each oligonucleotide produced two bands on branched templates that are not produced on debranched templates and do not correspond with reverse transcriptase strong stops on untreated precursor. Comparison with the adjacent dideoxy sequencing reactions indicates that the branched nucleotides correspond to two adjacent A residues at positions 38 and 39 nucleotides upstream from the end of intron 4 (A(−38) and A(−39)). This corresponds to the closest consensus branch site sequence upstream of exon 5 having the sequence GGCUAAC (the *underlined A* corresponds to A(−38)) (7–9). It is unclear whether the signal corresponding to position −39 is due to lariat formation at this A residue or to template-independent addition of one nucleotide during primer extension (49, 50). However, the relative intensity of the two bands clearly indicates that position −38 is the preferred branch site. Branch sites at positions other than A(−38) and A(−39) could not be detected.

Characterization of Lariat Intermediates and Branch Site for Intron 5—Splicing intermediates for the E56 precursor, indicated in Fig. 2*B*, were identified and characterized as described above for E45 (data not shown). The branch site upstream of exon 6 was determined on E56 lariat intermediates by primer extension using a labeled oligonucleotide complementary to exon 6 (E6RV1) and a short oligonucleotide complementary to the last 13 nucleotides of intron 4 (I513) (Fig. 4*B*). Splicing intermediates corresponding to intron 5 lariat and intron 5-exon 6 lariat were isolated separately on a 4% sequencing gel and either treated with HeLa S100 or used directly for primer extension as above. Primer extension products were run on an 8% sequencing gel adjacent to dideoxy sequencing reactions primed by the respective labeled oligonucleotides on the E56 plasmid (Fig. 4*A*).

Primer extension using the E6RV1 oligo on the isolated intron 5-exon 6 lariat produced very little signal (Fig. 4*A*, *lanes 17* and *18*). Several attempts using three different oligos complementary to exon 6 and isolated lariats or whole splicing reactions failed to detect definitive signals from branched nucleotides (data not shown). The reason for this is unknown but is likely to be due to several factors including the low abundance of the intron 5-exon 6 intermediate (see Fig. 2*C*) and the sensitivity of this RNA to exonuclease activity present in the nuclear extract. Branch sites were detected, however, in primer extensions using an oligo (I513, Fig. 4*B*) that anneals to the 3′ end of intron 5 (Fig. 4*A*, *lanes 5–8*). A major branch site was mapped to position −20 with minor branch sites at −21, −24, and −25 relative to the 3′ splice site. The same branch sites were detected at the same relative levels on the isolated intron 5-exon 6 lariat (*lanes 5* and *6*) and the intron 5 lariat (*lanes 7* and *8*). The finding that all four branch sites were detected on the isolated intron 5 lariat as well as the intron 5-exon 6 intermediate indicates that branch formation at any of these four sites is able to proceed to the second step of splicing.

The predominant branch site at −20 is an adenosine residue which is within a good match to the consensus branch site sequence (7–9). Interestingly, two nonconsensus nucleotides are utilized in branch site formation: a guanosine adjacent to the major branch site at position −21 and a uridine at −25. As above, it is unclear how much of the signal from G(−21) is due to template-independent nucleotide addition. However, branch site formation at U(−25) has been confirmed in three separate assays and is also detected as an intermediate during splicing of exons 4 and 6 (see below).

Branch Site Utilization during Splicing of Exons 4 and 6—*In vivo*, exon 6 is spliced to either exon 5 or exon 4 as a result of the alternative splicing of exon 5. To determine whether the "skip" splice that joins exons 4 and 6 utilizes the same or different branch sites as splicing of exons 5 and 6, a two-exon precursor containing exons 4 and 6 was synthesized from a template in which introns 4 and 5 were fused at the *Nco*I and *Pst*I sites, respectively (E46ND, Fig. 5*A*). As indicated in Fig. 5*A*, this construct retains the first 71 nucleotides of intron 4 and the last 416 nucleotides of intron 5 and removes exon 5, 470 nucleotides of the upstream intron, and 265 nucleotides of the downstream intron. A time course of the products and intermediates formed during processing of the E46ND precursor *in vitro* is shown in Fig. 5*B*. As with the E56-spliced product, the E46ND product is subject to degradation due to exonuclease activity in the nuclear extract. However, primer extension analysis on isolated product confirms that exons 4 and 6 are spliced correctly *in vitro* (Fig. 2*C*). Lariat intermediates for E46ND were initially identified by anomalous migration on different percentage acrylamide gels and sensitivity to debranching activity in HeLa S100 extract (data not shown). The identity of the bands indicated in Fig. 5B was determined from the size of linearized lariat intermediates following debranching reactions (data not shown).

The location of the branch site nucleotides employed during splicing of the E46ND precursor was determined by primer extension on intron 5-exon 6 and intron 5 lariats isolated separately as described above for the E56 precursor. This determination is shown in Fig. 4*A*. Primer extension using both the E6RV1 and I513 oligos produces extension products corresponding to branched nucleotides at positions −20, −21, −24, and −25 (Fig. 4*A*, *lanes 9–12*, *15*, and *16*). These are the same branch sites detected using the E56 lariats as templates (see above). In addition, A(−20) is the major site of branch formation. Furthermore, the same pattern of branch formation has been detected on isolated lariats derived from the skip splice during processing of a precursor containing exons 4, 5, and 6 (data not shown). Therefore, the same branch sites are utilized to the same relative levels regardless of whether exon 4 or exon 5 splices to exon 6 *in vitro*.

In vitro splicing reactions of the E56 and E46ND precursors shown in Figs. 2 and 5 generate relatively high levels of linearized lariat during the splicing reaction (Figs. 2*B* and 5*B*). If different branch sites have different sensitivities to the debranching activity in nuclear extract, the relative levels of the primer extension products detected from isolated lariats may not be representative of branch site utilization. Those branch sites that are debranched during the splicing reaction will be underrepresented in primer extension of isolated lariats. However, lariats used for branch site mapping were derived from splicing reactions that contained low levels of debranched lariats relative to branched lariats. Therefore, the results presented in Fig. 4*A* should accurately reflect branch site utilization for the E56 and E46ND precursors.

DISCUSSION

cTNT Exon 5 Mutations Specifically Disrupt Splicing of Exons 4 and 5 in Vitro—Previous results from transfection

analysis of cTNT minigenes indicated that sequence within the alternative exon, distinct from the known splice site *cis* elements, has a role in splice site recognition (31). To investigate this role, an *in vitro* splicing system using cTNT precursors has been established. The effect of exon 5 mutations on the removal of the upstream and downstream flanking introns was investigated separately using two RNA precursors containing either exons 4 and 5 or exons 5 and 6. The results reported in this paper demonstrate that the exon 5 mutations that block exon inclusion *in vivo* specifically block splicing of the upstream intron *in vitro* (Fig. 1) and have no effect on splicing of the downstream intron (Fig. 2). In addition, the degree to which two different exon mutations affect splicing *in vitro* correlate with their effects *in vivo* supporting the relevance of these results to splice site selection *in vivo*. For example, the E5-1 mutation, which leads to complete skipping of the exon *in vivo* (31), essentially blocks splicing of exons 4 and 5 (Fig. 1) while the E5-3 mutation, which significantly reduces but does not eliminate exon 5 inclusion *in vivo* (31) reduces *in vitro* splicing of exons 4 and 5 compared to unmodified precursor (Fig. 1). This is the first system in which the effects of mutations within a cassette alternative exon have been characterized *in vitro*.

Several different exon 5 mutations containing different nucleotide substitutions throughout the exon have been found to eliminate or reduce inclusion of exon 5 *in vivo* (31 and data not shown). It is unlikely that several mutations of different sequence composition would fortuitously introduce secondary structure or "poison" sequences that could artificially interfere with splicing *in vitro* and *in vivo* (51, 52). In addition, exon 5 mutations do not introduce sequences that resemble splice sites that could interfere with splicing (55) or termination codons which could interfere with a potential link between translation and splicing and/or RNA transport (53, 54).

The E5-1 mutation is of particular interest since it disrupts sequence within the exon that is clearly distinct from the conserved splice site consensus sequences (5, 6). Results both *in vivo* and *in vitro* indicate that internal sequence within exon 5 serves as a positive-acting element required for splice site recognition. Any model to define the mechanism by which internal exon sequence promotes splice site utilization must involve either binding by *trans*-acting factors and/or formation of secondary and tertiary structures. Since exon 5 mutations inhibit splicing of the upstream intron but not the downstream intron, the most straightforward model is that exon sequence promotes recognition of the immediately adjacent 3′ splice site. This model is similar to one of the potential models proposed for the female-specific exon within the *Drosophila doublesex* (*dsx*) gene in which a positive-acting element within the exon may serve to promote utilization of a "weak" 3′ splice site (as well as a polyadenlyation site) (36, 56).

A role for exon sequence in promoting 5′ splice site recognition is also possible. Recent studies have demonstrated that the efficiency of intron removal *in vitro* can be influenced by the relative strength of the 5′ splice site of the downstream intron (57, 58) and correlates with the efficiency with which U1 snRNP binds to the downstream 5′ splice site (22). These results support a model in which splicing factors bound to one splice site can promote binding of factors to the other splice site by interaction across the exon (59). Therefore, a second possibility for the results described here is that sequence within exon 5 directly promotes recognition of the adjacent 5′ splice site which indirectly affects splicing of the upstream intron. Indeed, the 5′ splice site flanking cTNT exon 5 is relatively weak (ACG:GTAGTT) and mutating this site to match the consensus converts exon 5 from an alternative exon to a constitutive exon *in vivo* (31). Exon 5 sequence could function to complement this weak 5′ splice site. Similarly, elements within the exon could promote recognition of both splice sites by enhancing communication between them. It has recently been proposed that small exons (such as cTNT exon 5) may require one dominant splice site to prevent interference from simultaneous complex formation at both flanking splice sites (62). Sequence within exon 5 could serve this function by enhancing recognition of one splice site.

It is also possible that mutations within the exon disrupt a natural secondary structure between exon 5 and upstream sequence that is required for splicing of exons 4 and 5. This in contrast to the standard model for secondary structure as an inhibitor of splicing by sequestration of splice sites and/or exons within hairpin structures (51, 52) as recently reported for the chicken β-tropomyosin pre-mRNA (27, 60, 61). A precedent for secondary structure playing a positive role in nuclear pre-mRNA splicing has been described for the adenovirus E1A pre-mRNA in which formation of a hairpin that shortens the distance between a distal branch site and the 3′ splice site is required for splicing (14). As previously described, removal of "obvious" potential secondary structures has no effect on cTNT alternative splicing *in vivo* (31). Therefore, functionally relevant secondary structures that involve cTNT exon 5 are likely to be complex and have not yet been identified.

It is not yet clear whether the requirement for exon sequence is related to exon 5 alternative splicing or is a general feature of at least some alternative and constitutive exons. It is also unclear what role exon sequence plays in the developmentally regulated alternative splicing of exon 5 in striated muscle. While a requirement for internal exon sequence has been described in a few cases of constitutive splice site selection (37, 38, 64), most examples described are within alternative exons (24–36, 39). In addition, we have recently found that a 30-nucleotide heterologous constitutive exon in place of the 30-nucleotide exon 5 is completely skipped *in vivo*,[3] indicating that a constitutive exon does not contain the information required for splice site recognition that is contained within cTNT exon 5. It is tempting to speculate that the mechanism for at least some cases of regulated alternative splice site selection in vertebrates involve the recognition of elements within exons as proposed for the *Drosophila dsx* gene (36, 56). This is a particularly attractive hypothesis for cTNT exon 5 since, except for the weak 5′ splice site (see above), this exon is indistinguishable from a constitutive exon in splice site sequences and branch site utilization (see below). It should be noted that the hypothesis that exon sequence plays a role in regulated alternative splice site selection does not rule out a role for exon sequence in constitutive splice site selection.

Branch Site Determination for cTNT Introns 4 and 5—The predominant branch site upstream of the cTNT alternative exon was found to be an adenosine residue located 38 nucleotides from the 3′ splice site, A(−38) (Fig. 3). A minor branch site was also detected at the adjacent upstream adenosine. Branch site formation at A(−38) was not unexpected because this is the first adenosine upstream of the 3′ splice site and is contained within a sequence that matches the branch site consensus (7–9). In addition, an AG dinucleotide is located 11 nucleotides upstream from A(−38). 3′ splice sites generally correspond to the first AG downstream from the branch site

[3] J. Teng and T. Cooper, manuscript in preparation.

(10, 63) making a branch site upstream of this AG dinucleotide unlikely. These sequence elements within the 3′ end of intron 4 also suggest that A(−38) is likely to be the branch site utilized during splicing *in vivo*.

The utilization of a conventional branch site upstream of the cTNT alternative exon is in contrast to many alternatively used 3′ splice sites in which branch sites are relatively distal to the 3′ splice site, at multiple nonadjacent nucleotides, and/or and at nucleotides other than adenosine (10–17). For example, the EIIIB exon of fibronectin, which is either included or excluded as a cassette like cTNT exon 5, utilizes multiple and distal branch sites at three A residues 76, 70, and 62 nucleotides upstream from the 3′ splice site (13). The results from cTNT exon 5 demonstrate that alternative splicing does not necessarily correlate with the use of unconventional branch sites. This has also been demonstrated for a few other alternatively spliced pre-mRNAs (20–23). Such results emphasize the difficulties in understanding how the splicing machinery distinguishes between constitutive and alternative exons and suggest that either subtle differences exist in the known splicing *cis* elements or novel *cis* elements are involved (see above).

Branch formation at the 3′ splice site adjacent to exon 6 was of interest because, *in vivo*, it serves as a "common acceptor" for two 5′ splice sites. To determine whether the two splicing pathways employ the same or different branch sites, primer extension analysis was performed on lariat intermediates from precursors containing either exons 5 and 6 (E56, Fig. 2) or exons 4 and 6 (E46ND, Fig. 5). The results demonstrate that the same four residues at −20, −21, −24, or −25 nucleotides from the 3′ splice site were utilized as branch sites during processing of both precursors (Fig. 4). In addition, the relative levels at which these four sites were utilized do not differ in the E56 and E46ND precursors: A(−20) is the major branch site and G(−21), A(−24), and U(−25) are minor branch sites (Fig. 4). Therefore, branch site utilization upstream of exon 6 is independent of the 5′ splice site that is involved in the lariat structure. Furthermore, the fact that all four branch sites were detected on the lariat introns, end-products of the splicing reaction, indicates that lariat intermediates that utilize each of the four branch sites proceed through the second step of splicing. These results from cTNT exon 6 are in contrast to those from the SV40 early pre-mRNA in which utilization of different but overlapping branch sites correlates with the use one of two 5′ splice sites (15, 16). However, like cTNT exon 6, a "common acceptor" exon of the rat skeletal muscle β-tropomyosin gene employs a single A at −24 during splicing of either a fibroblast- or skeletal muscle-specific upstream alternative exon (11). Similarly, the same branch site (a single A at −28) is used for three 5′ splice sites in the adenovirus E1A transcript (21). While these differences might reflect different mechanisms associated with different alternative splicing pathways, their significance is presently unclear.

The branch site utilization of intron 5 included three minor branch sites, two which were at nonconsensus nucleotides: a G at −21 from the 3′ splice site which is adjacent to the major branch site and a U at −25. It is unclear why minor branch sites are utilized in the presence of a good match to the consensus at A(−20). It should be noted, however, that substitution of the last 250 nucleotides of intron 5 with comparable regions from heterologous constitutively spliced genes do not affect the accuracy or efficiency of exon 6 splicing *in vivo*. In addition, these mutants retain the ability to alternatively splice exon 5.[4] Therefore, the 3′ end of intron 5 is not essential for the mechanisms involved in alternative splicing of the cTNT pre-mRNA.

In conclusion, the results presented in this paper indicate that cTNT precursors will be useful for the biochemical investigation of the role of exon sequence in splice site selection.

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[4] R. Xu and T. Cooper, unpublished observations.

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