Sequence Divergence Associated with Species-specific Splicing of the Nonmuscle β-Tropomyosin Alternative Exon*

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Alternative splicing of vertebrate β-tropomyosin transcripts ensures mutually exclusive expression of internal exons 6A and 6B in nonmuscle and skeletal muscle cells, respectively. Recently, we reported that this splicing regulation requires species-specific elements, since the splicing profile for the chicken, rat, and Xenopus β-tropomyosin alternative exons is not reproduced in transfection experiments when heterologous myogenic cells are used. By analyzing the splicing pattern of hybrid chicken/rat β-TM constructions transfected into both quail and mouse cell lines, we demonstrate that chicken β-tropomyosin exon 6A is flanked by stronger splicing signals than rat exon 6A, thus leading to the misregulation of splicing in heterologous cells. We have characterized three splicing signals that contribute to this difference: 1) nonconsensus nucleotide differences at positions +4 and +6 in the donor site downstream of exon 6A, 2) differences in the pyrimidine composition between the branch site and acceptor site upstream of exon 6A, and 3) a pyrimidine-rich intronic exon 6A splicing enhancer present upstream of exon 6A only in the chicken β-TM gene. The functional divergence between splicing signals in two homologous vertebrate genes reveals species-specific strategies for proper modulation of splicing of alternative exons.

Splicing of pre-mRNA in eukaryotes requires the accurate pairing of 5’ and 3’ splice sites in order to obtain functional mRNA. The selection of splice donor and acceptor sites involves the interaction between loosely conserved sequence elements present at the exon/intron borders with small nuclear ribonucleoproteins and several auxiliary factors to form the functional spliceosome (reviewed in Refs. 1 and 2). However, it is now clear that such sequence elements alone are not sufficient to define exon/intron borders and that a variety of additional sequence and structural elements present in both exons and introns are important for efficient utilization of splice sites (3–8).

Alternative splicing of vertebrate β-tropomyosin (β-TM)1 pre-mRNA provides an interesting model system for the study of the mechanism of splice site selection. For the chicken β-TM gene, we have shown that all of the sequences necessary to splice exon 6B specifically in skeletal muscle cells (quail differentiated myotubes) and exon 6A in all other cell types (quail myoblasts and fibroblasts) are present in a minigene consisting of the genomic sequences from exon 5 to exon 7 (9, 10). A complex combination of sequence and structural elements has been shown to be responsible for the repression of exon 6B in nonmuscle cells (11–15). In quail myotubes, exon 6B is derepressed and we have shown that competition between the exon 6A and 6B splice sites for pairing with the exon 5 and 7 splice sites favors utilization of exon 6B (16). Therefore, exon 6A, like other alternatively spliced exons, is associated with suboptimal splice sites that are sensitive to competition from flanking splice sites. Furthermore, in nonmuscle cells, efficient utilization of the splice sites flanking exon 6A requires an intronic splicing enhancer (S4), which consists of a 33-nucleotide polypyrimidine-rich tract located 37 nt downstream of exon 6A (12, 17).

The structural organization and expression pattern of the vertebrate β-TM genes is highly conserved, and parallel studies of the chicken and rat β-TM genes indicate that the exon 6B repressor elements are conserved and that exon 6A splice sites in both species are inefficiently spliced relative to the consensus sequences (15, 18–21). However, when equivalent β-TM minigene constructions from three different vertebrate species, namely chicken, rat, and Xenopus, are transfected into heterologous cell backgrounds, splicing of both exons 6A and 6B is deregulated while constitutive exons within the same constructions are accurately spliced (22). In particular, exons 6A and 6B of the rat β-TM gene are not recognized by the splicing machinery in quail fibroblasts and myogenic cells, whereas exons 6A of the Xenopus and chicken β-TM genes become constitutive exons since they are included in mature transcripts in mouse myogenic cells, irrespective of the state of differentiation of the cells. These results are interesting since differences between the splicing machinery among vertebrates have not been reported previously. In contrast, sequence differences inhibiting splicing of vertebrate introns in invertebrate systems and vice versa have been characterized, including differences in the consensus splice site sequences and differences in the size and nucleotide composition of introns (23–28).

In order to determine whether sequence divergence between the chicken and rat β-TM genes could account for the observed misregulation of splicing in heterologous systems, we generated hybrid constructions between the chicken and the rat β-TM minigenes and transfected them into both quail and mouse cell lines. Here we demonstrate that sequence elements necessary for species-specific regulation of exon 6A splicing are present in the introns flanking exon 6A, while the exonic sequences of these two genes are interchangeable. Our experiments reveal, in particular, a significant disparity in the splicing efficiency of chicken and rat exon 6A, which can be...
attributed to nonconsensus nucleotide differences at the 5' and 3' splice sites flanking exon 6A and to a newly identified pyrimidine-rich splicing enhancer for exon 6A of the chicken β-TM gene.

MATERIALS AND METHODS

Constructions—The chicken wild-type β-TM minigene plasmid pBS/SV-βjalt contains a genomic β-TM fragment spanning exons 5-7 and has been described previously (10). The rat wild-type genomic β-TM fragment spanning exons 5-8 (29) was subcloned into the same SV40 expression vector used for the chicken β-TM sequences (10). To generate the P2 construction, the 5' half of rat β-TM intron 5 (the intron between exons 5 and 6A, except for the first 10 bp), exon 6A, and the first 17 bp of rat intron 6A were PCR-amplified from the rat wild-type genomic DNA. As a control, the P1 env construction was generated by PCR amplification of the entire 5' half of rat β-TM intron 5 (the last 50 bp, followed by rat exon 6A and the first 17 bp of rat intron 6A, overlap extension PCR (17)). The P3, the 3' half of chicken β-TM intron 5 (the last 63 bp) was PCR-amplified from the wild-type chicken minigene with a 5' oligonucleotide primer, which also contained sequences from rat intron 5 including an EcoRII site, and a 3' primer, which also contained sequences from the 5' end of rat exon 6A including a PvuII site. In a separate PCR reaction, P3 sequences from exon 5 to 17 bp downstream of exon 6A were amplified and the amplification product was digested by EcoRII in rat intron 5 and PvuII in rat exon 6A. The 5' and 3' fragments released by this digestion were ligated to the 5' and 3' ends of the first PCR product, also digested with EcoRII and PvuII, such that chicken β-TM exon 5 and the first 10 bp of chicken intron 5 are followed by the first 17 bp of rat intron 5 (the first 72 bp downstream of rat exon 5), the 3' half of chicken intron 5, rat exon 6A, and the first 17 bp downstream of rat exon 6A. This ligation product was PCR-amplified and cloned into the Δ1 KpnI site. For constructions P4–6, intron 5, exon 6A, and the 5' end of intron 6A of constructions P1, P3, and P2, respectively, were PCR amplified with oligonucleotide primers that replaced the first 17 bp of rat β-TM intron 6A by the first 9 bp of chicken β-TM intron 6A. For P7, chicken β-TM exon 5 and intron 5 were fused to rat β-TM exon 6A and the first 17 bp downstream of this exon by overlap extension PCR mutagenesis.

In order to generate the P1 construction, KpnI sites were introduced 6 bp downstream of exon 5 and 17 bp downstream of exon 6A of the rat wild-type β-TM minigene plasmid by oligonucleotide-directed mutagenesis on single-stranded DNA (Rkpn) (17, 32). The modifications in Rkpn do not alter the splicing pattern of this construction with respect to the rat wild-type β-TM minigene (data not shown). Subsequently, the chicken intron 5 and rat exon 6A sequences of P7 were PCR-amplified with primers that introduced the chicken donor site sequence into exon 6A and KpnI sites at either end of this PCR product in order to replace the KpnI fragment of Rkpn.

The P1don and P1env constructions were generated by PCR mutagenesis of the P1 construction with oligonucleotides containing the sequence modifications (see Fig. 4A) and KpnI sites for dicing into Δ1.

RESULTS

Exonic Chicken and Rat β-TM sequences Are Interchangeable, while Intronic Sequences Are Responsible for Deregulated Splicing in a Heterologous Cell Context—In stably transfected quail fibroblasts (QT6 cell line), the chicken wild-type β-TM minigene generates ~95% spliced transcripts containing exons 5, 6A, and 7 (Fig. 1, A and B, lanes 1 and 2), while the wild-type β-TM minigene produces mostly mature transcripts that skip directly from exon 5 to 7 (Fig. 1B, lanes 5 and 6) and only 8% of the mature transcripts contain exon 6A (Fig. 1, A and B, lanes 5 and 6, only observed with longer exposures). In order to identify which sequences in the rat β-TM gene prevent utilization of rat exon 6A in this cellular context, we generated hybrid constructions between the chicken and rat β-TM minigenes (RP1 and P1, Fig. 1A). In the P1 construction, all β-TM sequences derive from the rat minigene except for intronic sequences flanking exon 6A, which derive from the chicken minigene. In the P1 construction, all β-TM sequences derive from the chicken minigene except for exon 6A and its flanking intronic sequences, which derive from the rat minigene. The RP1 construction is spliced like the chicken wild-type β-TM minigene in mouse C2 myotubes. In contrast, the RP1 hybrid transcript shown in Fig. 1B, lane 7, only 8% of the mature transcripts contained exon 6A (>95%, Fig. 1, A and B, lanes 7 and 8). These results exclude all the exonic rat β-TM sequences present in RP1, as well as all the splice sites associated with all the exons except exon 6A, from being responsible for rat exon 6A skipping in quail fibroblasts. Conversely, the high rate exon 6A inclusion for RP1 indicates that the minimum chicken β-TM sequences required for efficient exon 6A splicing in quail cell lines are the intronic sequences immediately flanking this exon in RP1 (Fig. 1A). The P1 construction, on the other hand, behaves like the rat wild-type β-TM minigene when transfected into quail fibroblasts: a minority (12%, Fig. 1A) of the mature transcripts contain exon 6A, while the majority of the transcripts skip from exon 5 directly to exon 7 (Fig. 1B, lanes 3 and 4). These results indicate that intronic rat β-TM sequences flanking exon 6A in P1 (Fig. 1A) are not recognized by the splicing machinery in quail fibroblasts.

In mouse C2 myotubes, the rat β-TM minigene generates mostly mRNA spliced directly from exon 5 to exon 6B, while exon 6A is included in approximately 10% of the mRNA (Fig. 1, A and C, lanes 5 and 6). In contrast, inclusion of chicken β-TM exon 6A is high (80%) in these cells (Fig. 1, A and C, lanes 1 and 2). Analysis of C2 stable transfectants containing the P1 hybrid construction showed that P1 is regulated like the rat β-TM minigene (~5% exon 6A inclusion; Fig. 1, A and C, lanes 3 and 4). Therefore, the rat β-TM intronic sequences flanking exon 6A in P1 are sufficient to obtain proper down-regulation of exon 6A in mouse C2 myotubes. In contrast, the RP1 hybrid construction exhibits high (95%) exon 6A inclusion (Fig. 1, A and C, exon 6A is included in approximately 10% of the mRNA (Fig. 1, A and C, lanes 5 and 6). In contrast, inclusion of chicken β-TM exon 6A is high (80%) in these cells (Fig. 1, A and C, lanes 1 and 2). Analysis of C2 stable transfectants containing the P1 hybrid construction showed that P1 is regulated like the rat β-TM minigene (~5% exon 6A inclusion; Fig. 1, A and C, lanes 3 and 4). Therefore, the rat β-TM intronic sequences flanking exon 6A in P1 are sufficient to obtain proper down-regulation of exon 6A in mouse C2 myotubes. In contrast, the RP1 hybrid construction exhibits high (95%) exon 6A inclusion (Fig. 1, A and C,
lanes 7 and 8) like the chicken β-TM minigene. This result indicates that chicken β-TM intronic sequences immediately flanking exon 6A in RP1 are responsible for deregulated splicing, since chicken exon 6A in mouse myogenic cells. Taken together (see Fig. 1A), our results show that in both cellular contexts, quail fibroblasts and mouse myotubes, the presence of chicken β-TM intronic sequences flanking exon 6A (chicken wild-type minigene and RP1) confers exon 6A inclusion, while that of rat β-TM intronic sequences flanking exon 6A (rat wild-type minigene and P1) leads to exon 6A skipping.

Regulation of Exon 6A Utilization Requires Three Independent Intronic Sequence Elements: the Donor and Acceptor Site Regions Flanking Exon 6A and Sequences Upstream of the Intron 5 Branchpoint—The rat β-TM intronic sequences, present in the P1 construction and responsible for exclusion of exon 6A in quail fibroblasts and mouse myotubes, can be divided into three separate regions (Figs. 1A and 2): 1) the donor site and donor site context immediately downstream of exon 6A, 2) the acceptor site region immediately upstream of exon 6A, and 3) the intron 5 sequences between the donor site and the branchpoint(s). We tested each of these three rat β-TM regions independently for their contribution to rat exon 6A skipping in quail fibroblasts and mouse myotubes. To do so, we exchanged one of these regions within the P1 construction with the corresponding sequences from the chicken β-TM minigene (P2, P3, and P4, Fig. 3A). Analysis of the mature transcripts produced in QT6 stable transfectants containing the P2, P3, and P4 constructions showed significant activation of exon 6A splicing for all three constructions (55%, 85%, and 60%, respectively) relative to the P1 construction (8%) (Fig. 3, A and B, lanes 1–8). These results demonstrate that each of the three rat intronic elements, namely the donor region, the acceptor region, and the intron 5 distal region, contributes to the poor recognition of rat exon 6A in this cellular background. In the P5, P6, and P7 hybrid constructions, different combinations of two of the three rat intronic elements in P1 were exchanged with the corresponding chicken β-TM sequences (Fig. 3A).

As in quail fibroblasts, transfection of the P1–P7 hybrid constructions into mouse C2 myotubes shows that the same three intronic regions flanking exon 6A of the rat β-TM gene are associated with exclusion of exon 6A, while the analogous sequences of the chicken β-TM gene activate splicing of this exon (data not shown). Taken together, our experiments demonstrate the presence of independent exon 6A splicing regulatory signals, not only in the donor and acceptor site regions flanking exon 6A, but also in the region upstream of the branchpoint of intron 5 of the chicken and rat β-TM gene.
changing only the 5' half of rat β-TM intron 5 in P1 with the corresponding region of the chicken β-TM gene (P2, Fig. 3A) leads to an increased splicing efficiency of the downstream rat exon 6A in both quail fibroblasts and mouse myotubes (Fig. 3B and data not shown). These regions of chicken and rat intron 5 differ in size (approximately 143 and 95 nt, respectively) and in the striking presence, only in the chicken β-TM gene, of a stretch of 33 nt comprising 85% pyrimidines and located 25 nt downstream of exon 5. Since we have previously identified a pyrimidine-rich splicing enhancer downstream of chicken β-TM exon 6A, we tested here the possibility that this upstream pyrimidine-rich sequence may also act as a splicing enhancer. The chicken intron 5 33-nt pyrimidine-rich sequence was inserted into the rat β-TM minigene, 26 nt downstream of exon 5 (Rat+Pyr, Fig. 4B), and, for comparison, the complementary purine-rich sequence was inserted at the same position (Rat+Pur. Fig. 4B). In transiently transfected QT6 cells, the rat wild-type β-TM minigene exhibits very low levels of exon 6A splicing (5%, Fig. 4B). In the presence of a purine tract in intron 5, there is no change in the utilization of rat β-TM exon 6A compared to the rat wild-type minigene (Fig. 4B). In contrast, the addition of the pyrimidine stretch leads to 18% inclusion of rat exon 6A in quail cells (Fig. 4B). Therefore, the pyrimidine tract upstream of the branchpoint of chicken intron 5 functions as an exon 6A splicing enhancer when placed within the rat β-TM minigene.

### DISCUSSION

Species Specificity of β-TM Exon 6A Splicing Is the Result of a Disparate Splicing Efficiency—Splicing of the nonmuscle exon 6A of the chicken and rat β-TM gene is strictly regulated when minigene constructions are transfected into quail and mouse myogenic cells, respectively, but not when transfected into a heterologous cell background (22). Our results demonstrate that the exon sequences of the chicken and rat β-TM genes are entirely interchangeable (exon 6A in these two species differs at 13 out of 75 nt). In contrast, the intronic sequences flanking chicken and rat exon 6A have diverged significantly, so that these sequences are not functionally equivalent. In both quail QT6 fibroblasts and mouse C2 myotubes, we show that the rat β-TM exon 6A donor and acceptor sites flanking exon 6A, as well as the intron 5 sequences upstream of the branchpoints, are not efficiently recognized by the splicing machinery, while the cognate chicken β-TM sequences ensure efficient utilization of exon 6A in these cells. The misregulation of exon 6A splicing presented here can therefore be interpreted as being due to a significantly greater splicing efficiency for chicken versus rat exon 6A splice signals. In order to obtain proper splicing regulation of this exon then, a specific adaptation is needed between the intrinsic strength of the regulated splice sites and the “strength” that the nuclear environment can provide in the way of specific splicing factors and/or the balance of general splicing factors. In particular, from our results we can infer that the relatively weaker rat β-TM exon 6A splice sites require at least one potentiating factor for efficient splicing in mammalian nonmuscle cells. A screen for such a factor(s) is under way.

The Disparate Splicing Efficiency between Exon 6A of the Chicken and Rat β-TM Genes Is Associated with Nonconsensus Nucleotides in the Flanking Splice Sites—The donor splice site adjacent to β-TM exon 6A in both chicken and rat is degenerate with respect to the consensus sequence at the same intronic positions (Fig. 2). Both splice sites contain a U instead of a G at the highly conserved position +5 relative to the exon/intron border (present in 80–90% of vertebrate donor sites; Ref. 36). In addition, neither donor site contains the consensus nucleotides at the two positions immediately flanking the U+5 (A and Y are

| 1) donor site and donor site context | consensus donar site | match to consensus | 
|---|---|---|---|
| donor site | chicken | AG/GGUGGUGG | AG/GGUGGUGG | AG/GGUGGUGG | AG/GGUGGUGG | 
| 5/8 | 5/8 | 5/8 | 5/8 |

| 2) acceptor site region | branch sites | match to consensus | distance to 5' splice site |
|---|---|---|---|
| chicken | TUNURAY | CCUCUGAC | 68 | -18 to 40 |
| rat | TUNURAY | CUCUGACU | 68 | -48 |
| pyrimidine tracts | chicken | 79% | 19 |
| length | rat | 76% | 33 |

| 3) distal portion of intron 5 | consensus PyrNC AGGG | CUCUGGCCUCUCUGCCCGCCACACCCGCGGUCUCUCACCCAGG | CUCUGGCCUCUCUGCCCGCCACACCCGCGGUCUCUCACCCAGG |
|---|---|---|---|
| chicken | rat | rat |
| length | 145 | 95 |
| Py stretch | 33nt (85%) | none |

**Fig. 2. Sequence comparison of the splicing elements flanking exon 6A of the chicken and rat β-TM genes.** The donor, acceptor, and branch site sequences are all compared to the vertebrate consensus sequences. The exon/intron borders are indicated by a slash. The donor and acceptor site positions that diverge from the conserved vertebrate sequences are boxed and underlined, respectively. The sequences immediately downstream of the donor site that comprise the donor site context are shown in lowercase letters. Intronic sequences immediately upstream of exon 6A are shown in the section “pyrimidine tracts,” and two of the mapped branchpoints upstream of rat exon 6A are underlined (2D), as well as the first potential branchpoint upstream of chicken exon 6A. The distal portion of intron 5 refers to the sequences between the donor site and branchpoint(s).
found at these two positions in approximately 70% and 60% of vertebrate donor sites, respectively. The sequence context immediately downstream of these donor sites also differs between the two species (Fig. 2). We showed that the two nucleotidic sequence differences at positions +4 and +6 of the donor site alone, and not the donor site context, is responsible for the disparate splicing efficiency of the chicken and rat donor sites in quail cells. We have shown previously that exon 6A splicing in β-TM minigene constructions derived from the owl, Otus scops, and from Xenopus, like that of chicken β-TM exon 6A, is inappropriately activated in mouse C2 myotubes (22). The contribution of the exon 6A donor site to this misregulation of splicing is supported by the fact that both the owl and Xenopus β-TM genes contain the same exon 6A donor site sequence as

FIG. 3. Transcript analysis of quail QT6 fibroblasts transfected with hybrid rat/chicken β-TM constructions. A, schematic representation of β-TM sequences present in hybrid rat/chicken minigene constructions and the results of the quantification of transcripts after stable transfection of these constructions in QT6 fibroblasts. For hybrid constructions P2–P7, only the region that has been modified with respect to P1 is shown. The relative position of the branchpoints upstream of exon 6A is marked with a dot. Otherwise, the figure is organized just as in Fig. 1A. B, RT-PCR analysis of transcripts produced upon stable transfection of QT6 cells with β-TM constructions. The size and structure of the P1-P7 PCR products amplified with the same primer pair are indicated to the left of the gel. The radioactively end-labeled HaeIII-digested φX174 DNA is included as a size marker.

FIG. 4. A, RT-PCR analysis of transcripts produced upon transient transfection of QT6 cells with P1-derivative constructions containing modifications within the donor site region immediately downstream of exon 6A. The sequences at the 3' exon/intron border of exon 6A in these constructions, which represent the only differences between these constructions, are shown below the gel. The size and structure of the amplified products are indicated to the left of the gel. The quantification represents the percentage of total transcripts that contain exon 6A, as averaged from the results of several independent transient transfections showing a variation of less than 20%. B, RT-PCR analysis of transcripts produced upon transient transfection of QT6 cells with rat β-TM-derivative constructions, which contain the insertion, 26 nt downstream of exon 5, of either the pyrimidine stretch (Rat + Pyr) present in intron 5 of the chicken β-TM gene or the complementary sequence (Rat + Pur). The sequences inserted are indicated below the gel. The percentage inclusion of exon 6A was calculated as indicated in A.
the homologous chicken gene.2

Recognition of the intronic portion of the donor site sequence is thought to occur sequentially by base pairing first with U1 snRNA, and, subsequently, with U6 snRNA over the course of the splicing reaction (37–44). The G at intronic position +5 of the donor site is thought to be important for both of these events. Given that little is known about donor site recognition, there are several possibilities for the significantly different efficiency of splicing at the chicken and rat exon 6A donor sites. The repetition GUAGUA in the rat exon 6A donor site may present two possible binding sites for U1 snRNA which could interfere with splicing at both sites. Alternatively, there may be more favorable alternative sites for U6 snRNA base pairing for the chicken than for the rat exon 6A donor site. Since vertebrate donor sites can be highly divergent relative to the consensus sequence, it is likely that additional RNA-RNA and RNA-protein interactions are needed to specify the donor site in these organisms. This is probably the case for the donor sites that do not contain a G at position +5, as is the case for the exon 6A donor sites. It is possible that RNA variants and/or splicing factors not yet characterized are involved in recognition of these divergent splice sites.

The rat and chicken exon 6A acceptor site regions (Fig. 2) both contain branch site sequences with 6/6 matches to the consensus sequence, CAG/C cleavage sites that diverge from the consensus (CAG/G) at exonic position +1, which is a purine in 80% of vertebrate acceptor sites (36), and a suboptimal pyrimidine tract interspersed with purines. Roscigno et al. (45) demonstrated that the presence of consecutive uracils substituting pyrimidinetract interspersed with purines. Roscigno in 80% of vertebrate acceptor sites (36), and a suboptimal pyrimidine tract, can induce large variations in the splicing efficiency of the genes that have been characterized to date. Surveys of different subsets of splice sites, for example those associated with alternative exons expressed in neuronal cells, define consensus splice site sequences that differ from those of the general consensus splice sites (47).

Recognition of Chicken β-TM Exon 6A Involves Several Intronic Splicing Enhancers—The introduction of a 33-nt stretch made up of 85% pyrimidines, present 25 nt downstream of chicken but not rat exon 5, into the same position in intron 5 of the rat β-TM minigene, leads to significant activation of rat exon 6A splicing in quail cells. These results indicate the presence of a splicing activating sequence in chicken intron 5, rather than the presence of negative elements in rat intron 5.

Furthermore, the addition of the complementary purine-rich sequence at the same position of rat β-TM intron 5, which has no effect on exon 6A splicing, indicates that the relatively smaller size of rat intron 5 probably does not contribute to the poor splicing efficiency of this intron in quail cells. In addition, deletion of the 33-nt pyrimidine-rich sequence from the chicken wild-type β-TM minigene leads to a decrease in exon 6A utilization.2 Significantly, the owl β-TM intron 5 contains a sequence with 16/20 nt identity to part of the chicken intron 5 pyrimidine-rich splicing enhancer at approximately the same position in intron 5, and Xenopus β-TM intron 5 also has pyrimidine-rich elements upstream of the potential branchpoint.2

There are not many other examples of intronic sequences upstream of a branchpoint contributing to splicing regulation, and the mechanism by which these sequences function is unknown (48–50). We have shown previously that splicing of the chicken exon 6A depends on the presence of a similar pyrimidine-rich intronic enhancer sequence (S4) located 30 nt downstream of exon 6A (12, 17). In the present work, we provide additional evidence that splicing of exon 6A of the chicken β-TM gene relies strongly on the presence of intronic splicing enhancers. In the case of the rat β-TM gene, where neither of these enhancers exist (our results and Ref. 18), a different mechanism for the recognition of exon 6A has to be involved.

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