

A CD45 Polymorphism Associated with Multiple Sclerosis Disrupts an Exonic Splicing Silencer*

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Previous studies have identified a single nucleotide polymorphism that significantly increases the splicing of variable exon 4 in transcripts of the human protein-tyrosine phosphatase CD45. Strikingly, the presence of this polymorphism correlates with susceptibility to the autoimmune disease multiple sclerosis. In this study we investigated the mechanism by which the polymorphism enhances splicing of CD45 exon 4. We found that at least four distinct splicing regulatory elements exist within exon 4 and that the strongest of these elements is an exonic splicing silencer (designated ESS1), which is disrupted by the polymorphism. We show that ESS1 normally functions to repress the weak 5' splice site (ss) of CD45 exon 4. The ESS1 sequence also suppresses the splicing of a heterologous 5'ss and associates with a specific complex in nuclear extracts. We further demonstrate that ESS1 is juxtaposed to a purine-rich enhancer sequence that activates the use of the 5'ss of exon 4. Thus, proper functioning of the immune system is dependent on a complex interplay of regulatory activities that mediate the appropriate splicing of CD45 exon 4.

CD45 is a transmembrane protein-tyrosine phosphatase that is expressed on all nucleated hematopoietic cells. In T cells, CD45 functions to maintain the T-cell receptor in a primed state, allowing for activation of the T-cell receptor upon interaction with an antigen-presenting cell (reviewed in Refs. 1 and 2). In mice that contain a knock-out of CD45, the few T cells that survive development are no longer reactive to antigen, and immune function is impaired (3, 4). Similarly, CD45-deficient humans have severe combined immunodeficiency disease (5, 6). Conversely, T cells that express a constitutively active form of CD45 are hyper-reactive to antigen, and mice containing such cells are prone to develop autoimmune disease (7). Thus, both CD45 activity and regulation of this activity are critical for the proper functioning of the immune system.

The gene encoding human CD45 encompasses 33 exons. Three of these exons (exons 4, 5, and 6) are alternatively spliced, giving rise to at least five distinct isoforms that differ in their extracellular domain. The alternative splicing of CD45 is highly regulated in both a tissue-specific manner and upon activation of T cells, although the mechanism(s) by which regulation occurs is largely unknown (1). The various expressed CD45 isoforms do not differ with regard to their intracellular

phosphatase domains. However, it has been proposed that alternative exon usage does indirectly influence phosphatase activity by altering the dimerization of CD45 (7). It has recently been shown that at least some isoforms of CD45 exist as dimers and that in these dimers the phosphatase activity of each molecule is blocked by steric hindrance (7). Because the alternate isoforms of CD45 differ in the primary sequence of the extracellular domain, as well as the extent of *O*-glycosylation and sialation of this domain, it is likely that they also differ in their ability to dimerize. In particular, exclusion of the variable exons, such as that which occurs upon T cell activation, is predicted to decrease the dimerization of CD45, resulting in a decrease in CD45 function. Thus, the regulation of CD45 splicing may be one mechanism by which an immune response is down-regulated following an initial antigen challenge.

Exon 4 is the most tightly regulated of the CD45 variable exons (1). In naive T cells, a significant portion of the translated CD45 is encoded by mRNA that includes exon 4. In contrast, the majority of the CD45 protein expressed in activated and memory T cells is encoded by mRNA that lacks exon 4. Several years ago it was shown that a small percentage of the human population expresses aberrantly high levels of exon 4-encoded CD45 isoforms in all cell types (8, 9). This misregulation of exon 4 inclusion correlated with the presence of a C to G polymorphism at nucleotide 77 (C77G) of exon 4, which is silent with respect to coding sequence (10). Subsequently, we and others demonstrated that engineering the C77G polymorphism into a CD45 minigene is sufficient to confer abnormally high expression of exon 4 in mature mRNA ((11, 12) and see Fig. 1*B*). Importantly, consistent with the hypothesis that regulation of CD45 splicing is critical for the appropriate function of the immune system, the C77G polymorphism was recently shown to correlate with development of multiple sclerosis. This conclusion is based on the observation that the prevalence of the C77G polymorphism is significantly greater among multiple sclerosis patients than in healthy controls (13). Moreover, this polymorphism co-segregates with disease in at least four independent families (13). Therefore, we were interested in understanding how the regulation of CD45 splicing is altered by the C77G change within exon 4.

In general, the splicing of any given exon is primarily determined by the sequences at the 3' and 5' exon-intron boundaries (splice sites). These splice site sequences function as binding sites for small nuclear ribonuclear particles (snRNPs),¹ which form the core of the spliceosome and direct RNA cleavage and ligation (for review see Ref. 14). However, in mammals the

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¹ The abbreviations used are: RNP, ribonuclear particle; snRNP, small nuclear ribonuclear particles; nt, nucleotide(s); ss, splice site; ESS, exonic splicing silencer; RT-PCR, reverse transcription-polymerase chain reaction; WT, wild type; ESE, exonic splicing enhancer; SR, serine-arginine rich; HIV, human immunodeficiency virus.

sequences at the 3' and 5' splice sites are poorly conserved and are often insufficient for optimal recognition by the snRNPs (15, 16). Therefore, the splicing of many mammalian exons is dependent on the presence of additional sequences within the exon itself or within the flanking intron. Numerous splicing enhancers have been identified that promote exon splicing by helping to recruit snRNPs to the splice sites and/or by promoting polyadenylation (reviewed in Refs. 17–19). In addition, other sequences have been shown to inhibit splicing of a given exon via mechanisms that include the steric hindrance of closely neighboring splice sites, sequestration of snRNPs, and sequestration of splice sites (17, 19). The activity of such enhancing and inhibitory sequence elements may be constitutive or may be regulated by specific trans-acting factors, thereby allowing for regulated splice site use (19). In fact, it has recently been proposed that the splicing of most, if not all, mammalian exons are influenced by at least some constitutive enhancer or inhibitor elements in addition to the canonical splice sites (16, 20). In this study, we found that indeed multiple, opposing, splicing regulatory elements exist within exon 4 of CD45. Moreover, we show that the multiple sclerosis-associated C77G mutation disrupts the strongest of these elements, namely an exonic silencer element that functions by inhibiting the use of the 5' splice sites (ss) of exon 4.

EXPERIMENTAL PROCEDURES

Plasmids and RNA—The minigene constructs used in this study were all derived from the previously described minigene MG4, which is subcloned into the *MluI*-*ClaI* sites of the stable expression vector pAWneo3 (12). The two-intron substitution constructs, QC1–QC10 and C77G, were all synthesized using the QuikChange Kit (Stratagene). The resultant mutant minigenes were fully sequenced and reinserted into a fresh pAWneo3 vector to ensure that no extraneous mutations existed that might alter the splicing of the minigene. The single-intron constructs encompassing exons 3 and 4 or 4 and 7 were synthesized using PCR to truncate exon 4. For exon 3–4 constructs a PCR primer was used that terminates exon 4 at nucleotide 181 followed by a *ClaI* restriction site. For exon 4–7 constructs a PCR primer was used that contains an *MluI* restriction site followed immediately by exon 4 beginning at nucleotide 18. The C77G, QC2, QC3, QC4, and QC5 mutations were then introduced into the truncated minigenes as described above for the three-exon minigene. The 5' ss UP mutations were also introduced into the exon 3–4–7 and 4–7 constructs using the QuikChange mutagenesis method. For the CD45 exon 4-globin exon 2 chimera, the CD45 portion contains exon 4 (beginning at nucleotide 18) and ~200 nucleotides (nt) of normal flanking downstream intron (up to a naturally occurring *BglII* site) were ligated to an engineered *BglII* site within the normal β -globin intron ~60 nucleotides upstream of exon 2. For the ESS1 insertion into the β -globin transcript, complementary oligonucleotides were synthesized that contain the entire ESS1 sequence (nt 29–88) with or without the C77G change, flanked by *NcoI* sites. The oligos were annealed and inserted into the natural *NcoI* site located 92 nucleotides upstream of the 5' ss of globin exon 1 (20).

The plasmid templates for RNA transcription were generated as follows. Complementary oligos corresponding to nucleotides 40–90 of CD45 exon 4 were annealed and inserted into the *SmaI* site of a variant of pSP64 (Promega), which has a T7 promoter immediately adjacent to the *SmaI* site, to give the plasmid pT7-ESS1. pT7-ESS1-C77G was created in the same way except that the synthesized oligos contain the C77G polymorphism. The plasmid Rx2 was described previously (21).

RT-PCR Analysis—All RT-PCR assays were done exactly as detailed previously (12). The conditions were determined empirically to result in a signal that was linear with respect to input RNA. RT-PCR reactions were resolved on 5% denaturing polyacrylamide gels and quantitated using a phosphorimaging device (Fuji).

In Vitro Binding Reactions—Nuclear extract was prepared from JSL1 cells using a standard protocol (22). The competitor RNAs, WT, C77G, and Dsx, were synthesized from the plasmids pT7-ESS1, pT7-ESS1-C77G, and Rx2, respectively, by transcription with T7 RNA polymerase (Promega). For the radiolabeled ESS1 RNA probe, [³²P]CTP was added during transcription. Following transcription, all RNAs were gel-purified. To assay binding, ESS1 probe RNA was incubated with the JSL1 nuclear extract under standard splicing conditions (22) for 15 min at 30 °C in the presence or absence of cold competitor RNA. The reac-

tions were then placed on ice, and heparin was added to a final concentration of 0.5 mg/ml. Reactions were loaded on a prerun 4.5% polyacrylamide 0.5× TBE gel and run at 150 V for 1.5 h at room temperature. Gels were dried and analyzed by autoradiography.

RESULTS

The effect of the C77G polymorphism on the splicing of CD45 exon 4 can be explained by one of two general models. Either the polymorphism disrupts a sequence that normally represses the recognition of exon 4, or alternatively the change from C to G at position 77 creates a novel splicing enhancer element. The former possibility is supported by data from a linker scanning mutagenesis of CD45 exon 4, which was designed to determine the sequences involved in regulating the exclusion of this exon from transcripts expressed in thymocytes (23). That study identified three regions corresponding to nt 8–11, 40–92, and 126–137 that, when mutated, resulted in the aberrant inclusion of exon 4. Although the identification of nt 40–92 as repressing sequences is consistent with a model in which the C77G polymorphism disrupts a splicing repressor element, there are two important caveats to this original study. First, in the linker scanning substitutions there was some modification of exon length and no indication of whether the substituting sequences had any inherent effect on splicing (23). Secondly, that study was done prior to the discovery of the C77G polymorphism, so a direct comparison has not been done between the linker scanning results and the effect of the C77G mutation.

We have previously developed and characterized a cell line JSL1, which faithfully reproduces the pattern of CD45 splicing that is observed in mature, naive T cells (12). In addition, we have identified a CD45 minigene consisting of variable exon 4 flanked by constitutive exons 3 and 7 that, when stably expressed in JSL1 cells, shows a pattern of exon 4 inclusion consistent with that seen in the endogenous CD45 gene (12). Importantly, when the C77G polymorphism is engineered into our CD45 minigene, it results in a dramatic increase in the inclusion of exon 4 ((12) and Fig. 1B). Therefore, our minigene and cell line provide a good model system to study in more detail the effect of the C77G polymorphism on the splicing of CD45 exon 4.

To understand why the C77G polymorphism increases the inclusion of exon 4, we first wanted to map potential exonic regulatory elements within exon 4 in the context of our minigene. To do this we systematically changed blocks of 20 nt within exon 4 to a heterologous 20-mer sequence (Fig. 1A). The heterologous sequence we used for these substitutions is a sequence from the IgM gene that was previously shown to lack intrinsic splicing enhancer activity (24). The resultant mutant minigenes, as well as the wild type counterpart, were transfected into the JSL1 cells, and individual clones that stably expressed the desired minigene were isolated and expanded. To determine the relative splicing of exon 4 in these various minigenes, we analyzed total cellular RNA by an RT-PCR assay, which we have described previously (12). To ensure that any differences seen were not the result of random clonal variation, we analyzed four to six independent clones for each minigene. In each case we detected no significant variation among the independent clones; however, for simplicity, in this study we only show two to three representative clones for each minigene.

Consistent with our previous results, 40–50% of transcripts from the wild type minigene contain exon 4 ((12) and Fig. 1, B and C). Strikingly, the splicing of exon 4 is dramatically stimulated when nucleotides 29–48, 49–68, or 69–88 are substituted with the heterologous sequence, similar to the effect of the C77G polymorphism (compare Fig. 1C, QC2, QC3, and QC4, and Fig. 1B). The stimulation of exon 4 splicing observed in constructs QC2–QC4 is unlikely to be a consequence of the

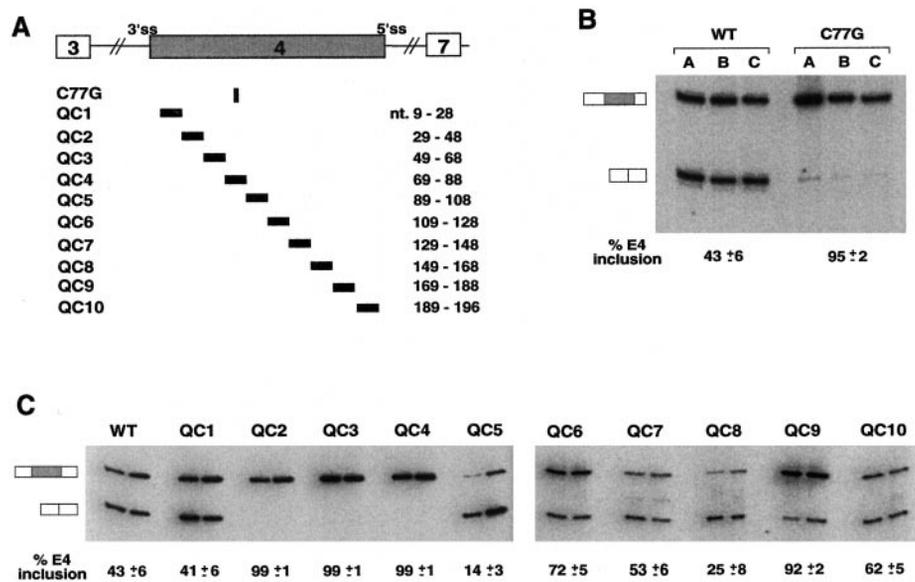


FIG. 1. Consequence of mutations within CD45 exon 4 on splicing efficiency. *A*, schematic of the minigenes used for experiments in this figure. The wild type minigene includes variable exon 4 flanked by the constitutive exons 3 and 7. Exons are represented by *open* or *shaded* boxes, introns are represented by *lines*. The 3'ss and 5'ss of exon 4 are located as indicated. The introns are not drawn to scale, as indicated by the *cross-hatches*. For the mutant versions of the minigene, the *black boxes* underneath exon 4 represent the relative size and location of the substitutions made in the exon. The first and last nucleotide of each substitution are listed to the *right* of the *black boxes*. The substitutions involved replacing a block of 20 nucleotides with the sequence TCAGCATGACTCTCAGCATG except for the C to G transversion at nucleotide 77 and the substitution QC10 that replaced nt 189–196 with the sequence CATGACTC. *B*, RT-PCR analysis of three representative clones of JSL1 cells stably expressing either the WT minigene shown in *panel A* or that containing the C77G mutation. RT-PCR analysis for these samples and all others presented in this study was performed such that the amount of product detected was linear with respect to input RNA as described previously (12). Identification of bands, as indicated to the *left* of the *gel*, was confirmed by subcloning and sequencing the PCR product. *C*, RT-PCR analysis of two representative clones stably expressing the minigene variant indicated. Quantitation of the relative percent of the product that included exon 4 is given *beneath* the *gel* and represents the results from at least three representative clones for each minigene, each harvested and analyzed at least twice.

insertion of the heterologous sequence because the substitution of this sequence at other locations within exon 4 had either the opposite effect (QC5 and QC8) or no effect (QC1, QC7, and QC10) on the inclusion of exon 4 (Fig. 1C). In addition, the results shown here with QC2–QC4 are very similar to those obtained by Streuli and Saito (23) upon the substitution of nucleotides 40–92 with a sequence unrelated to that used in this study. Thus, we conclude that the region of exon 4 encompassing nucleotides 29–88 most likely represents a strong exonic splicing silencer (ESS), which represses the inclusion of this exon.

In addition to the strong ESS at nucleotides 29–88 (ESS1), our mutational analysis reveals the presence of multiple additional elements within exon 4 that influence its splicing, albeit more weakly than ESS1 (Fig. 1C). Substitution of nucleotides 169–188 results in the increased inclusion of exon 4. In contrast, the inclusion of exon 4 is decreased significantly upon substitution of nucleotides 89–108 and 149–168, indicating that these sequences may normally function to enhance exon 4 splicing. Therefore, multiple, competing splicing regulatory elements likely exist within CD45 exon 4. Because of our interest in the C77G polymorphism, this study focuses on understanding the role of the ESS1 element and its effect on the flanking splice sites and juxtaposed regulatory element (nt 89–108).

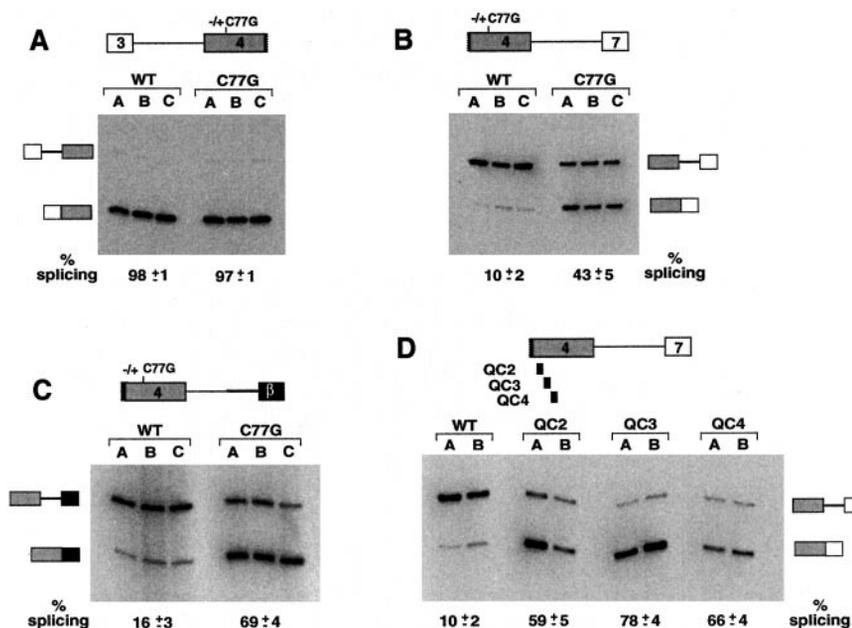
ESS1 Represses Utilization of the Weak 5'ss of Exon 4—The first step in the splicing of an exon involves the recognition of the 3'- and 5'ss (14). Although the recognition of one splice site often facilitates the use of the other through a process known as exon definition, the two splice sites can also be recognized independently and are both potential sites of regulation (16, 25). To determine which of the splice sites of exon 4 is regulated by the C77G polymorphism and by ESS1, we made single-intron minigene constructs in which exon 4 was truncated either just upstream of the 5'ss or just downstream of the 3'ss

(see Fig. 2, *A* and *B*, respectively). Strikingly, RT-PCR analysis of multiple clones that stably express these minigenes indicates that the 3'ss of exon 4 is recognized constitutively (Fig. 2A, WT), whereas splicing of the 5'ss of exon 4 in the absence of the 3'ss is very inefficient (Fig. 2B, WT). Moreover, in the presence of the C77G polymorphism, the use of the 5'ss is dramatically increased (Fig. 2B), whereas this polymorphism has no apparent effect on 3'ss utilization (Fig. 2A). In all cases the RT-PCR assays were done under conditions in which no signal was detectable when reverse transcriptase was omitted, confirming that the slower migrating band is indeed unspliced RNA and not contaminating genomic DNA (data not shown).

To confirm that the 5'ss of exon 4 and not the 3'ss of exon 7 is the sequence that is regulated by the C77G polymorphism, we constructed a minigene chimera consisting of CD45 exon 4 fused to a heterologous exon. The heterologous exon we chose to use is the 3'ss and complete exon 2 of the human β -globin gene, which is spliced constitutively (20). The splicing of the resultant CD45 exon 4-globin exon 2 chimera is inefficient because of the weak 5'ss of CD45 exon 4 (Fig. 2C, WT). However, as predicted, the substitution of the C77G polymorphism into the chimera strongly enhances the splicing of the 5'ss of exon 4 to the heterologous β -globin exon (Fig. 2C, C77G). Finally, to determine whether the C77G polymorphism functions similarly to the other ESS1 mutations, we constructed the QC2, QC3, and QC4 substitutions in the context of the CD45 exon 4-exon 7 minigene. As shown in Fig. 2D, the QC2–QC4 substitutions indeed strongly promote use of the 5'ss of exon 4, indicating that these substitutions are mechanistically similar to the C77G mutation. Taken together, these data indicate that ESS1 inhibits use of the 5'ss of exon 4 and further indicate that the C77G polymorphism allows for inclusion of exon 4 by disrupting the activity of this silencer.

The canonical sequence of a mammalian 5'ss is AG/

FIG. 2. ESS1 regulates use of the 5'ss of CD45 exon 4. RT-PCR analysis of RNA from JSL1 clones stably expressing the following minigenes is indicated in each panel. *A*, a minigene that is truncated immediately upstream of the 5'ss of exon 4 and either contains (*C77G*) or lacks (*WT*) the *C77G* polymorphism; *B*, a minigene that begins just following the 3'ss of exon 4 with or without the *C77G* polymorphism; *C*, a minigene in which the 3'ss truncated form of exon 4 and downstream intron is fused to the intron and exon 2 of human β -globin, as indicated by the *dark box and thick line*; *D*, a minigene identical to that in *panel B* but containing the substitutions *QC2*, *QC3*, or *QC4* described in Fig. 1. In all cases RT-PCR was performed as described in Fig. 1. *UP*, an idealized 5'ss consensus sequence.



GUAAGU in which / indicates the point of cleavage (14). Although the 5'ss of CD45 exon 4 contains the most critical of these nucleotides (the GU at positions +1 and +2 relative to the cleavage site and a G at position +5), it differs from the consensus at the other positions in the intron (Fig. 3, *A* and *B*). The differences between the consensus 5'ss sequence and that present at the 5'ss of exon 4 suggest that the 5'ss of exon 4 might be inherently weak. To test this possibility and investigate the influence of 5'ss strength on exon 4 splicing, we constructed minigenes in which the 5'ss of exon 4 was mutated to exactly match the canonical mammalian 5'ss. As shown in Fig. 3, *A* and *B*, respectively, this 5'ss UP mutation results in a significant increase in the splicing of a two-exon single-intron construct and results in the complete inclusion of exon 4 in a three-exon construct. Thus, the 5'ss of exon 4 is inherently weak, and this characteristic is important for its incomplete inclusion and susceptibility to regulation. However, we do note that in the single-intron construct in which the UP mutation does not result in complete recognition of the 5'ss, the presence of the *C77G* polymorphism does allow for further use of the 5'ss (Fig. 3*A*, 90% versus 96% splicing). Therefore, the ESS1 can suppress, albeit weakly, the utilization of an optimized 5'ss.

ESS1 Functions as a Separable Regulatory Element to Repress 5'ss Utilization—As mentioned above, the ESS1 is only one of several exonic elements that appear to influence the splicing of exon 4. We were particularly interested in the presence of a putative exonic splicing enhancer (ESE) positioned immediately downstream of ESS1 (nt 89–108). Previous studies have identified similar cases of a juxtaposed splicing enhancer and inhibitor that function to counterbalance each other (26). The sequence of the ESS1 proximal enhancer element, which we term ESE1, closely resembles that of a canonical purine-rich splicing enhancer. Most well characterized purine-rich enhancers have been shown to enhance the use of a weak 3'ss (24, 27–29). However, there is at least one example of enhancement of a 5'ss by a purine-rich enhancer (30) as well as an example of 5'ss enhancement by a non-purine-rich element (31). Therefore, we wanted to determine whether ESE1 stimulates the use of exon 4 splicing by enhancing 3'ss recognition or alternatively, whether ESE1 functions to counter repression of the 5'ss by the ESS1. As shown in Fig. 4*A*, splicing to the 3'ss of exon 4 is equally efficient in the presence or absence of ESE1. In contrast, disruption of ESE1 by the *QC5* substitution further

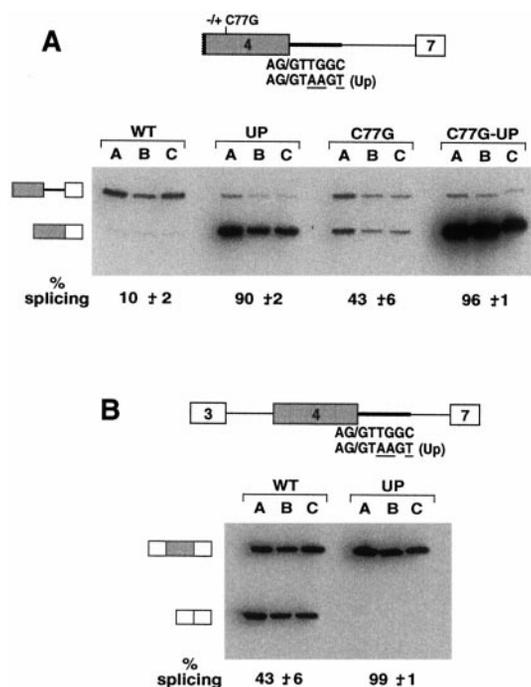
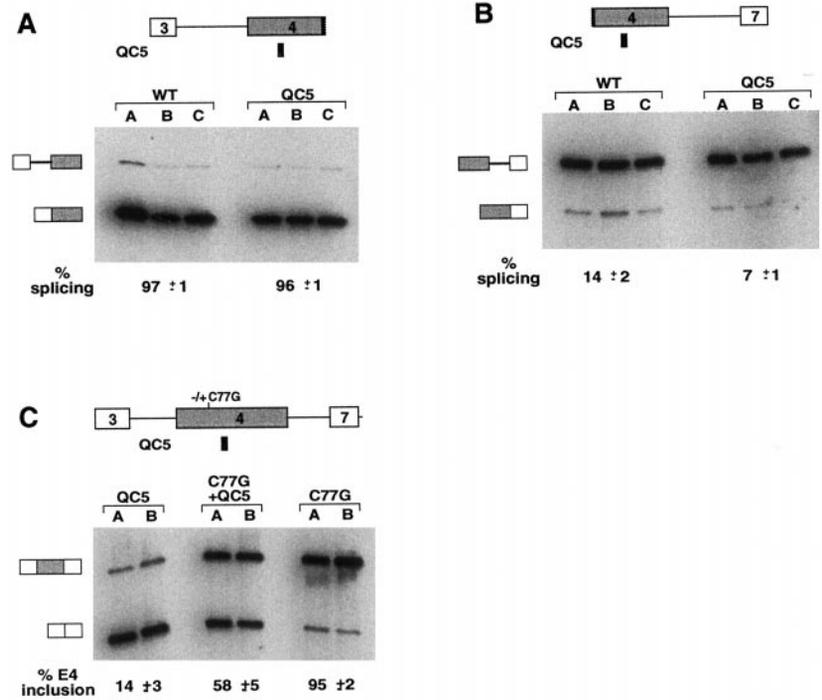


FIG. 3. The 5'ss of CD45 exon 4 is inherently weak. RT-PCR analysis of RNA from JSL1 clones that stably express the minigenes is indicated for each panel. *A*, a minigene that begins just downstream of the 3'ss of exon 4 and contains either completely wild type sequence (*WT*), a change of the normal 5'ss sequence to an idealized 5'ss consensus sequence (*UP*), the *C77G* polymorphism (*C77G*), or both the polymorphism and the idealized 5'ss (*C77G-UP*); *B*, a minigene consisting of the complete exon 4, flanked by exons 3 and 7, that contains either the normal (*WT*) or idealized (*UP*) 5'ss sequence. RT-PCR and quantitation were done as described in Fig. 1.

decreases the inefficient recognition of the 5'ss by 2-fold (Fig. 4*B*). Thus, we conclude that the ESE1 element activates exon 4 inclusion by enhancing 5'ss use.

Given that the ESS1 and ESE1 appear to influence the same step in exon 4 recognition, we next wanted to determine whether the activities of ESS1 and ESE1 were inter-related or separable. For example, if the ESS1 represses 5'ss use by directly suppressing the enhancer activity of ESE1, then when the ESE1 element is absent, disruption of the ESS1 should

FIG. 4. ESS1 regulates use of CD45 exon 4 independent from the activity of ESE1. RT-PCR analysis of RNA from JSL1 clones that stably express the minigenes is indicated for each panel. *A*, a minigene that is truncated just upstream of the 5' splice site of exon 4 either with (QC5) or without (WT) the QC5 substitution described in Fig. 1; *B*, a minigene that begins just downstream of the 3' splice site of exon 4 either with (QC5) or without (WT) the QC5 substitution; *C*, a minigene consisting of the complete exon 4, flanked by exons 3 and 7, that contains either the QC5 substitution (QC5), the C77G polymorphism (C77G), or both mutations (C77G + QC5). RT-PCR and quantitation were done as described in Fig. 1.



have no effect. As shown in Fig. 4C, this prediction is clearly not met. Instead, we observe that disruption of ESS1 by the C77G polymorphism in the background of the ESE1-eliminating substitution QC5 results in a significant increase in exon inclusion (from 14 to 58%). Similarly, elimination of ESE1 in the background of the C77G polymorphism decreases the splicing of exon 4 from 95 to 58%. Therefore, we conclude that the ESS1 and ESE1 function independently of each other to influence utilization of the 5' splice site of exon 4.

To further determine whether the ESS1 truly functions as a separable splicing silencer element, we tested whether the presence of the ESS1 could confer repression on a heterologous splicing construct. When stably expressed in JSL1 cells, exons 1 and 2 of a human β -globin minigene are spliced together with relatively high efficiency (Fig. 5, *Glo*). However, this efficiency is significantly decreased when the ESS1 is inserted ~80 nt upstream of the 5' splice site of exon 1 (Fig. 5, *GloESS*) such that the relative percentage of unspliced transcript increases by more than 3-fold. Importantly, at least half of the ESS1 repression of β -globin splicing is alleviated by the presence of the C77G polymorphism in the ESS sequence (Fig. 5, *GloESM*). The fact that the C77G polymorphism does not entirely eliminate the ESS1-mediated repression of β -globin splicing is consistent with the fact that this single mutation does not increase the use of CD45 exon 4 as much as do the QC2, QC3, and QC4 substitutions (see Figs. 1 and 2), indicating that the C77G polymorphism weakens but does not completely disrupt ESS1 function.

ESS1 RNA Associates with a Specific Complex in Vitro—The observation that ESS1 can repress the splicing of a heterologous substrate strongly suggests that this sequence functions as a binding site for a splicing inhibitory complex. To test this possibility, we incubated an RNA fragment corresponding to the sequence of the ESS1 with nuclear extract from JSL1 cells and resolved the resulting RNA-protein complexes on a native gel. As shown in Fig. 6, we indeed detected a complex that associates with the ESS1 RNA. To determine the specificity of the ESS1-associated complex, we repeated the binding reactions in the presence of cold competitor RNA. Importantly, a dimer of the splicing regulator element from the *Drosophila doublesex* gene, which is of the same approximate length and

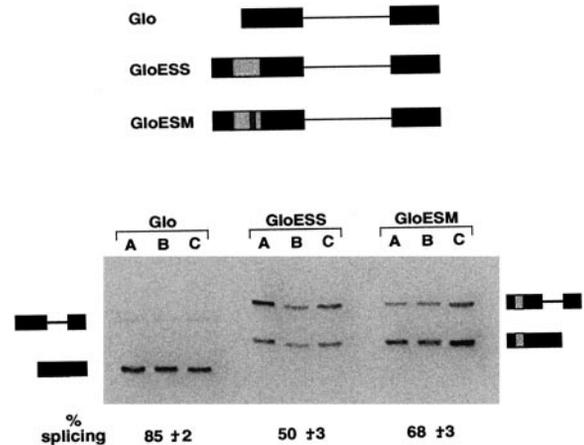


FIG. 5. ESS1 confers repression on a heterologous substrate. RT-PCR analysis of RNA from three representative JSL1 clones that stably express a human β -globin minigene (*Glo*), a minigene in which the nucleotides 29–88 from CD45 exon 4 were inserted within the first β -globin exon (*GloESS*), or a minigene in which exon 4 nucleotides 29–88 containing the C77G polymorphism were inserted into the first β -globin exon (*GloESM*), is displayed. RT-PCR and quantitation were done as described in Fig. 1.

nucleotide content (CA-rich) as the ESS1, does not compete for binding of the complex even when present at 100-fold excess (Fig. 6, *rightmost lane*). Our splicing data further suggest that if the ESS1-associated complex is functionally significant, the C77G polymorphism would inhibit or weaken binding of the complex to the RNA. Consistent with this prediction, the ESS1-bound complex is competed ~10-fold more efficiently by wild type ESS1 RNA than by that which contains the C77G polymorphism. We conclude that the ESS1 sequence likely functions by binding to a protein or complex of proteins that inhibits the use of the downstream 5' splice site and that the C77G polymorphism stimulates 5' splice site recognition by disrupting the association of the inhibitory complex with the RNA. Work is currently in progress to identify the components of this ESS1-specific complex.

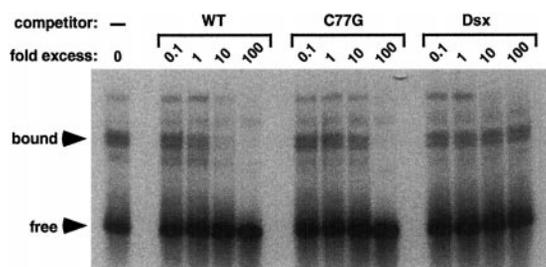


FIG. 6. A specific complex assembles on ESS1 RNA *in vitro*. A radiolabeled RNA fragment corresponding to nucleotides 40–90 of exon 4 (GUGACCCUUACCUCACACACCACUGCAUUCACCCGCAAGCACC UUUG) was incubated with nuclear extract from JSL1 cells, and reactions were resolved on a native gel. Incubation of probe with nuclear extract was done either in the absence (0) or presence of unlabeled competitor as indicated above each lane. WT competitor was identical to the RNA used as the probe, C77G competitor differed from the probe only in that it contained the C77G polymorphism (located at the position *underlined* in the above sequence), and Dsx competitor was a 52-nt sequence that contained a dimer of the *dsx* repeat element (UCUCAAUCAACAUCGCGAGATCCUCUCAAUCAACA) plus flanking sequence.

DISCUSSION

The presence of the C77G polymorphism in human CD45 exon 4 has been shown to dramatically alter the splicing of CD45 and has been correlated with susceptibility to the autoimmune disorder multiple sclerosis. In this study we demonstrate that the C77G polymorphism functions by disrupting an exonic splicing silencer element (ESS1), which normally represses the use of the 5' splice site of exon 4. It has long been observed that nonsense mutations frequently lead to exon skipping, a phenomenon that has recently been attributed to the disruption of ESEs (32, 33). In addition, it has been shown that missense or silent point mutations may similarly result in exon skipping because of the disruption of an ESE (18, 32) or may cause increased exon usage by creating or strengthening an ESE (34). However, to our knowledge, this is the first report of a naturally occurring point mutation that disrupts the function of a *bona fide* ESS. Moreover, this study provides a striking example of how a translationally silent point mutation can have profound biological consequences by altering the splicing of critical genes.

In addition to characterizing the role of ESS1 in the regulation of CD45 exon 4, we also demonstrated the presence of additional splicing regulatory elements within this exon. These additional elements include two enhancer sequences at nucleotides 89–108 and 149–168 as well as a second, weaker silencing element at nucleotides 169–188, as modeled in Fig. 7. The results of our linker scanning analysis of CD45 exon 4 correspond well with a previously published study focused on identifying negative regulatory elements within exon 4 (23). The boundaries of ESS1 as described here (nt 29–88) closely overlap one of the sequence elements that Streuli and Saito (23) determined to be critical for exon 4 exclusion in thymocytes (nt 40–92). The additional elements they identified as repressor sequences (nt 8–11 and 126–137) do not appear to have a significant effect on splicing in our hands. However, none of our substitutions mutated either of these regions completely. Similarly, the additional sequence that we find to repress exon 4 (nt 169–188) was not mutated in the previous study (23). Finally, we also identified two splicing enhancer elements within CD45 exon 4. At the time of the earlier study, there was no precedent for the existence of splicing enhancers; thus, the potential presence of such elements in CD45 exon 4 was not investigated.

Most previously described splicing regulatory elements involve the regulation of a weak 3' splice site (24, 26, 28, 29). In contrast, we show in this study that both the ESS1 and ESE1 elements

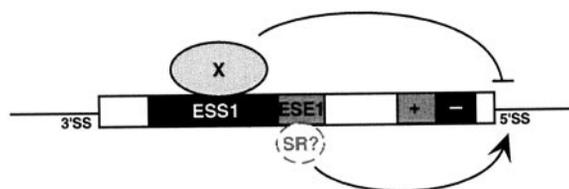


FIG. 7. Model of regulation of CD45 exon 4 splicing by multiple exonic element. Exon 4 and flanking introns are depicted by the *open box* and *straight line*, respectively. ESS1 and the other negative regulatory elements are depicted by *black boxes*, whereas ESE1 and the additional enhancer element are depicted by *gray boxes*. The unidentified complex that associates with ESS1 and represses use of the 5' splice site of exon 4 is indicated by an X. In contrast, ESE1 is likely to interact with SR proteins, as discussed in the text, and functions independently of the ESS1 to activate the 5' splice site. The sites of action of the additional silencer (-) and enhancer (+) are still unknown.

within CD45 exon 4 alter the use of a weak 5' splice site. The ESE1 element appears to fall within the category of the well characterized purine-rich enhancers by virtue of sequence similarity. However, there is only one other example of a purine-rich enhancer functioning similarly to ESE1, namely by enhancing the use of a downstream 5' splice site (30). In addition, there is only one example of an ESS sequence that has been shown to repress the use of a flanking 5' splice site (35). In this example the ESS sequence has been shown to function as a pseudo-5' splice site, which sequesters the splicing machinery into a nonproductive complex. Although we cannot rule out that ESS1 functions in a similar manner, the sequence of ESS1 does not contain any region that would appear likely to function as a pseudo-splice site.

We propose that 5' splice site silencing by ESS1 is mediated by a protein or complex of proteins (indicated as X in Fig. 7), which represses recognition of the downstream 5' splice site. The evidence for such a complex X is two-fold. First, we detected the specific association of a complex with ESS1 RNA *in vitro*, and this complex associates less strongly with the C77G polymorphism version of ESS1 that has decreased silencer activity (Fig. 6). Secondly, the fact that ESS1 can repress the splicing of a heterologous construct (Fig. 5) argues against alternative models for ESS1 function, such as the interaction of this RNA sequence with an RNA element elsewhere in the CD45 pre-mRNA. However, because β -globin exon 1 does appear to contain some enhancer activity (20), we cannot rule out the possibility that ESS1 functions to somehow block the general enhancement of 5' splice sites. Recently, several studies have demonstrated that the A1 member of the family of heterogeneous RNP binds to and influences the function of ESS sequences located in variable exons of the CD44, HIV Tat, and fibroblast growth factor receptor 2 genes (36–38). Preliminary studies indicate that heterogeneous RNP A1 is not a component of the ESS1-associated complex X (data not shown). However, it is possible that other heterogeneous RNP family members might play a role in the function of the ESS1 element.

In addition to the presence of complex X, we further predict that the activity of ESE1 is likely mediated by SR proteins (Fig. 7). Although we currently have no direct evidence for SR association with the ESE1 element, SR proteins have been shown to associate with similar purine-rich enhancer sequences and to mediate the activity of these enhancers (28, 29). Moreover, two groups have shown the effects of overexpression of SR proteins on the splicing of CD45 exon 4 in co-transfection assays, suggesting a possible role of these proteins in regulating exon 4 use (39–41). Finally, splicing changes in CD45 have been observed *in vivo* upon the conditional knock-out of the SR protein SC35 (42).

Lastly, our data indicate that the ESS1 and ESE1 sequences do not regulate the use of the 5' splice site of exon 4 by competing

directly with one another, as shown by the fact that each element can regulate splicing in the absence of the other element (Fig. 5). Thus, in our model, factor X and SR proteins likely can associate simultaneously with neighboring sites on the RNA, as depicted in Fig. 7. The activity of the remaining enhancer (+) and inhibitor (−) elements have yet to be determined, but for the reasons mentioned above, these elements are unlikely to directly influence the activity of ESS1. In summary, our data suggest a model in which the inclusion of exon 4 into mature CD45 RNA is primarily determined by a balance of competing elements/activities that regulate the use of at least the 5′ss of exon 4. In the presence of the C77G polymorphism, the activity of ESS1 is decreased, thus shifting the balance of activity toward the enhancement of exon 4 inclusion.

The use of multiple, antagonistic, regulatory elements in mediating exon inclusion or exclusion is a theme that is becoming increasingly common in the regulation of splicing as more systems are being studied in appropriate detail (19). In particular, the close juxtaposition of ESS1 and ESE1 within CD45 exon 4 is reminiscent of other neighboring, opposing splicing regulatory elements that have been identified in exons within the IgM (26) and CD44 (43) genes. Similarly, several intronic and exonic splicing regulatory elements have been shown to be bipartite in that they are comprised of multiple independent binding sites for factors that cooperate in effecting regulation (30, 44–46). It remains to be determined whether this pattern of closely juxtaposed regulatory elements is simply a consequence of their abundance in many transcripts, or conversely, whether it plays an additional regulatory role in determining splice site choice.

Clearly, identification of the protein(s) bound to ESS1 and to the other regulatory elements within CD45 exon 4 will be required for a complete understanding of the mechanisms by which these elements function. In addition to the alterations of exon 4 inclusion by the C77G polymorphism, the use of this exon is tightly regulated in both a tissue-specific and activation-dependent manner. Given the results we have presented here, the tissue- and activation-induced differences in exon 4 splicing are likely attributable, at least in part, to alterations in the balance of the four regulatory activities we have identified within exon 4. This may occur by directly altering either the expression or activity of the factors that associate directly with the exon 4 regulatory elements, or it may involve independent factors bound to other sites that counteract the activities of the elements we have described in this study. Therefore, the determination of the sequences and factors involved in regulating exon 4 expression in resting, naive T cells (as modeled by our cell line) is a first step toward understanding the more complicated mechanisms of the regulated alternative splicing of CD45 throughout the immune system.

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