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Differential Expression of CD45 Isoforms Is Controlled by the Combined Activity of Basal and Inducible Splicing-regulatory Elements in Each of the Variable Exons*[S]

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The human *CD45* gene encodes five isoforms of a transmembrane tyrosine phosphatase that differ in their extracellular domains as a result of alternative splicing of exons 4–6. Expression of the CD45 isoforms is tightly regulated in peripheral T cells such that resting cells predominantly express the larger CD45 isoforms, encoded by mRNAs containing two or three variable exons. In contrast, activated T cells express CD45 isoforms encoded by mRNAs lacking most or all of the variable exons. We have previously identified the sequences within *CD45* variable exon 4 that control its level of inclusion into spliced mRNAs. Here we map the splicing-regulatory sequences within *CD45* variable exons 5 and 6. We show that, like exon 4, exons 5 and 6 each contain an exonic splicing silencer (ESS) and an exonic splicing enhancer (ESE), which together determine the level of exon inclusion in naïve cells. We further demonstrate that the primary activation-responsive silencing motif in exons 5 and 6 is homologous to that in exon 4 and, as in exon 4, binds specifically to the protein heterogeneous nuclear ribonucleoprotein L. Together these studies reveal common themes in the regulation of the *CD45* variable exons and provide a mechanistic explanation for the observed physiological expression of CD45 isoforms.

In order to attain proteomic and functional complexity, higher eukaryotes have developed numerous mechanisms to amplify the informational content of their relatively limited genomes. One such mechanism, alternative splicing, allows for the production of multiple unique mRNAs from a single gene through the differential inclusion of exons. Since the variant mRNAs produced by alternative splicing can each potentially encode for a functionally distinct protein, alternative splicing is recognized to be a ubiquitous and critical mechanism for regulating cellular function (1, 2).

The importance of regulated alternative splicing in humans is exemplified by the *CD45* gene, which encodes a transmembrane protein-tyrosine phosphatase (3). CD45 phosphatase activity is critical for intracellular signaling in T cells in response to antigen stimulation, as indicated by the severe immunodeficiency observed in CD45-deficient mice and humans (4–7). Five isoforms of the CD45 protein are expressed in humans as a result of alternative inclusion of exons 4–6 (see Fig. 1*A*). In human T cells, the expression of the CD45 isoforms is tightly regulated throughout development and upon activation (3). In particular, naive peripheral T cells express significant amounts of the larger isoforms of CD45, which have high phosphatase activity and maintain the T cell receptor in a state primed for antigen recognition (8). Subsequent to antigen stimulation, however, there is a marked shift in the processing of *CD45* pre-mRNA, such that the variable exons are predominantly excluded from the final message, resulting in expression of the smaller CD45 isoforms (9, 10). The smaller isoforms of CD45 are more prone to homodimerization than the larger isoforms (8, 11). Importantly, in the dimeric state the intracellular phosphatase activity of CD45 is inhibited by steric constraints (8). Therefore, the shift to smaller CD45 isoforms upon activation of T cells reduces CD45 phosphatase activity and is thought to contribute to the attenuation of T cell signaling (8, 12). Such a model for the importance of *CD45* alternative splicing in maintaining T cell homeostasis is supported by studies that have correlated defects in *CD45* splicing regulation with susceptibility to various autoimmune diseases, including multiple sclerosis, systemic sclerosis, and autoimmune hepatitis (13–15).

To fully understand and predict the differential expression of CD45 isoforms in T cells, we need a complete characterization of the determinants of *CD45*-regulated splicing. As for most examples of alternative splicing, the splice site sequences at the intron-exon boundaries surrounding the *CD45* variable exons deviate significantly from the consensus recognition sites for the splicing machinery or "spliceosome" (16). Thus, auxiliary sequences (*i.e.* enhancers or silencers) within or flanking the variable exons are likely to play a critical role in determining the efficiency of spliceosome binding and the resulting exon inclusion. Despite a general appreciation of the role of enhancers and silencers in alternative splicing, our ability to predict the presence and activity of such regulatory elements remains limited (17). Therefore, experimental dissection of *CD45* variable exons is required to identify the regulatory motifs that influence CD45 isoform expression. Previously, we have identified the regulatory sequences that control inclusion of *CD45* variable exon 4, including a motif referred to as the activation-responsive sequence (ARS) consensus that is required for exon silencing upon T cell activation (18, 19). In this study, we have extended our understanding of the splicing-regulatory sequences controlling CD45 isoform expression by systematically mapping all of the exonic regulatory sequences that control inclusion of *CD45* exons 5 and 6 in resting and activated T cells. Consistent with the physiologic distribution of CD45 isoforms, we find that exon 5 contains a strong enhancer (ESE)[3] and a weak silencer (ESS), whereas exon 6 contains two strong ESSs and a weaker ESE. In addition, we show that, as with exon 4, exons 5 and 6 each contain a sequence sufficient to confer activation-induced exon

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table I.

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[3] The abbreviations used are: ESE, exonic splicing enhancer; ESS, exonic splicing silencer; RT, reverse transcription; ARS, activation-responsive sequence; PMA, phorbol 12-myristate 13-acetate; FR, -fold repression; hnRNP, heterogeneous nuclear ribonucleoprotein; PTB, polypyrimidine tract-binding protein.

repression, which binds hnRNP L and obligatorily contains the ARS consensus motif. Taken together, we conclude that CD45 isoform expression is determined by the combinatorial effect of the basal and inducible regulatory activities encoded within each of the three variable exons.

EXPERIMENTAL PROCEDURES

Minigenes—Constructs CD9, SC5, SC6, and SC14 were described previously (19). Constructs CD5 and CD6 contain a BglII-flanked region of ~500 nucleotides of genomic sequence encompassing *CD45* exons 5 and 6 that was removed from SC5 and SC6 and substituted for the exon 9 sequences in CD9. Constructs SC-E5 and SC-E6 were generated by inserting PCR-derived 120-nucleotide fragments internal to exons 5 and 6 into the Glo1 construct described previously (19). Block scanning mutations within SC5 and SC6 were done using PCR to swap blocks of 20 nucleotides within exon 5 to TCAGTATGACTCTCAGTATG or 15 nucleotides within exon 6 with TCAGTATGACTCTCA. Constructs shown in Figs. 4 and 5 to test single or double copies of individual regions of exons 5 and 6, were made by ligation of double-stranded DNA oligonucleotides corresponding to the given sequence (shown in figures) into the MluI site of SC-glo. Constructs E5:R1–5, E5:R1–5(mARS), E6:R5–8, and E6:R5–8(mARS) were also made by insertion of the corresponding synthetic oligonucleotides into the MluI site of SC-glo. SC-glo is identical to the construct "glo" described previously (20).

In Vivo Splicing Analysis—Transfections of minigenes, stable clone selection, stimulations, RNA isolation, and analysis of minigene splicing by RT-PCR were done as described previously (19).

UV Cross-linking—^{32}P-Labeled RNA was generated by *in vitro* transcription with T7 polymerase (Promega) in the presence of [^{32}P]CTP. Templates for *in vitro* transcription were generated by cloning double-stranded DNA oligonucleotides encoding E6R5–8 with or without mARS mutations or E5R1–5 with or without mARS mutations downstream of a T7 promoter in the vector pSPT7 (20). Generation of nuclear extract from JSL1 cells and UV cross-linking assays were done as described previously (20).

RNA Interference—Small interfering RNAs, transfection, and analysis by RT-PCR and Western blot were done identically to that described recently by Rothrock *et al.* (20).

RESULTS

The relative expression of the five CD45 isoforms in human peripheral T cells results from the differential inclusion of each of the three variable exons within the *CD45* gene. Variable exons 4 and 6 are only included in a fraction (roughly 40–60%) of mRNAs expressed in resting T cells, whereas exon 5 is expressed in almost all of these mRNAs (10). Upon antigen stimulation, inclusion of exons 4 and 6 is barely detectable, whereas approximately half of the mRNAs still include exon 5 (10). This pattern of *CD45* expression (diagrammed in Fig. 1*A*) suggests differences in the relative strengths of either the splice sites or auxiliary regulatory sequences within or surrounding exons 4 and 6 *versus* exon 5. In addition, whereas the inclusion of exons 4 and 6 is clearly repressed upon T cell activation, it has been debated whether the splicing of exon 5 is inherently regulated or is a stochastic event influenced by the neighboring effects of the other two variable exons (3).

To gain a deeper understanding of the mechanisms controlling CD45 isoform expression, we have sought to identify and characterize the sequences that determine inclusion of *CD45* variable exons 4–6. Previously, we have identified an ESS and ESE within *CD45* exon 4 that together result in the partial inclusion phenotype of this exon in resting

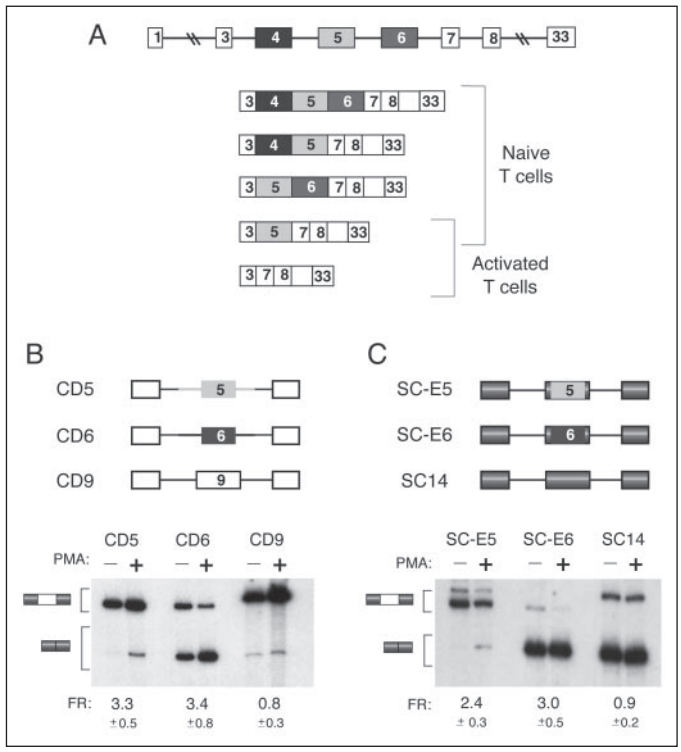

FIGURE 1. **Sequences within CD45 exons 5 and 6 are sufficient to confer the pattern of exon inclusion observed in the endogenous gene.** *A*, schematic of the variably spliced region of the *CD45* gene and resulting isoforms. Variable exons 4–6 are denoted by *black*, *light gray*, and *dark gray boxes*, respectively. Constitutive exons are indicated by *white boxes*. Predominantly expressed isoforms in resting (naive) and activated T cells are as indicated. *B*, RT-PCR of mRNA derived from minigenes stably expressed in resting (−*PMA*) and activated (+*PMA*) JSL1 cells. *Unmarked white boxes* in minigenes correspond to constitutive exons 3 and 7 from *CD45*, which flank variable exons 5 and 6 or control exon 9. Three-exon (*3E*) and two-exon (*2E*) products are as indicated and were confirmed by cloning and sequencing. As described in previous studies (19), the change in isoform ratio upon cellular activation is expressed as an FR value (FR = $((3E/2E)_{-PMA}/(3E/2E)_{+PMA})$) such that FR = 1 indicates no change in splicing between the two conditions. FR values in this and all figures are derived from at least four independent clones in at least two separate stimulations. Statistical analysis of the data demonstrated a high level of significance ($p < 0.001$) between the FR calculated for CD9 and either CD5 or CD6 (see supplemental Table I for full statistical information). *C*, RT-PCR of minigene-derived RNA as in *B*. First and last exons, plus all introns, correspond to sequences from the heterologous unregulated human β-globin gene (indicated by *gradient shading*). The splice sites of the middle exons for each minigene are also derived from β-globin, whereas internal sequences of the middle exon in constructs SC-E5 and SC-E6 are from *CD45* exons 5 and 6, respectively. As in *B*, the difference between SC14 and SC-E5 or SC-E6 was determined to be statistically significant ($p < 0.001$), as shown in supplementary Table I.

T cells (18). The activity of the exon 4 ESS, but not ESE, is further enhanced upon T cell activation and is necessary and sufficient for the decreased inclusion of exon 4 in activated cells (19). Comparison of the sequence of the activation-inducible ESS within exon 4 with sequences in exons 5 and 6 indicated the presence of a conserved element we call the ARS motif. The ARS motif in *CD45* exons 4–6 consists of the sequence MCYYGCAMGCA ("long repeat") in tandem with a shorter copy (MCYYGCA), in which M represents cytosine or adenine and Y is any pyrimidine (19). In all three *CD45* variable exons, mutation of the GCA core of the ARS repeats abolishes activation-induced exon repression; however, only in exon 4 has the ARS motif been determined to be the sole activation-sensitive regulatory element (19).

To confirm the importance of the ARS consensus motif in exons 5 and 6 and determine whether these exons contain additional sequences that mediate their observed pattern of expression in T cells, we examined the splicing of exons 5 and 6 using chimeric minigenes expressed in T cell-derived JSL1 cells. We have previously shown that the JSL1 cell line recapitulates all aspects of *CD45* alternative splicing (10); thus,

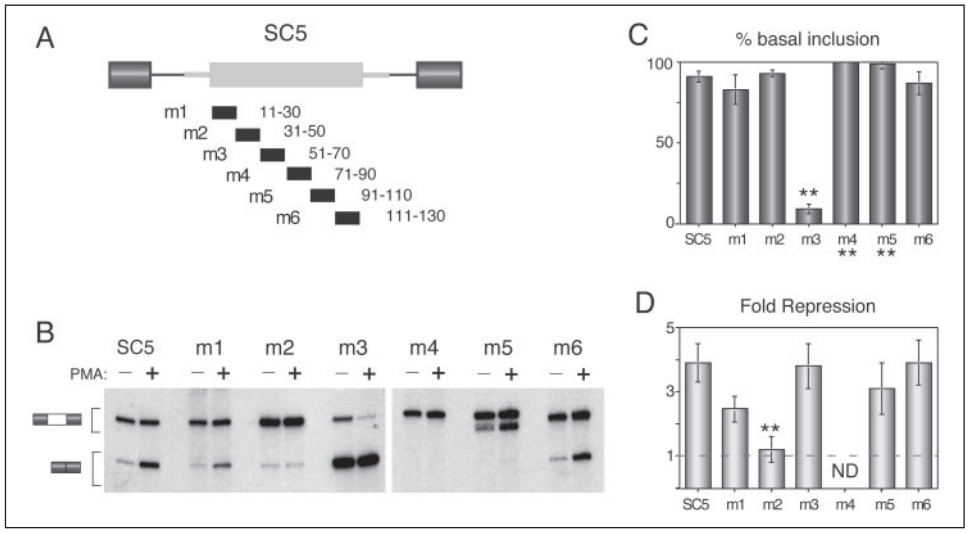

FIGURE 2. ***CD45* exon 5 contains multiple sequences that influence its pattern of inclusion.** *A*, schematic of block scanning mutagenesis of exon 5 in the context of the SC5 minigene, which contains all of *CD45* exon 5 and surrounding intron (*light gray*) flanked by intron and exon sequences from the human β-globin gene (*gray gradient*). The locations of substitutions relative to the start of exon 5 are given for each mutant (m1–m6). *B*, RT-PCR analysis of minigene-derived RNA as described in the legend to Fig. 1. *C*, quantitation of percentage of basal inclusion (*i.e.* percentage of 3E product) in resting cells for each minigene. Values shown are derived from at least four independent clones assayed in at least two separate experiments. *D*, -fold repression observed for each minigene upon PMA stimulation of cells. -Fold repression values shown were calculated as described in the legend to Fig. 1*B*. For *C* and *D*, the *bars* marked by *double asterisks* were significantly different from the wild-type SC5 control ($p < 0.001$) as detailed in supplemental Table I.

these cells provide a useful background for identifying sequences that regulate exon splicing in resting and activated cells. We first sought to confirm whether or not the splicing of *CD45* exons 5 and 6 was in fact regulated upon T cell activation and whether this regulation was inherent to the individual exons. When *CD45* exon 5 and a few hundred nucleotides of flanking intron are fused directly between *CD45* constitutive exons 3 and 7, we indeed observe a pattern of exon 5 expression consistent with that observed in the endogenous gene (Fig. 1*B*, *CD5*). RT-PCR analysis of minigene-derived mRNA reveals almost complete inclusion of exon 5 in resting cells but partial repression of this exon upon activation. For this minigene and all others in this study, we quantify the change in isoform expression upon treatment with PMA by calculating a -fold repression (FR) value. FR is equal to the change in the ratio of three-exon product to two-exon product between resting and activated conditions, such that an FR of >1 indicates the efficiency with which the inclusion of an exon is repressed upon treatment with PMA (see the legend to Fig. 1). Parallel analysis of exon 6 reveals partial skipping of exon 6 in resting cells and increased skipping in activated cells, whereas *CD45* exon 9 (a constitutive exon used as a control) is almost completely included under both cell conditions (Fig. 1*B*, CD6 and CD9). Therefore, the splicing of CD5, CD6, and CD9 is consistent with the observed inclusion of exons 5, 6, and 9 in endogenously expressed *CD45* mRNAs, demonstrating that exons 5 and 6 are regulated independently of each other.

To further delineate the sequences most relevant to exon expression, we next engineered minigenes that contain the bulk of sequences internal to exons 5 or 6, cloned into an unregulated minigene derived from the human β-globin gene (Fig. 1*C*, SC-E5 and SC-E6 *versus* SC14). Importantly, the SC-E5 and SC-E6 constructs contain no intronic sequences from *CD45*; nor to they contain the intron/exon junction sequences from *CD45* exons 5 and 6, yet the splicing patterns of the SC-E5 and SC-E6 minigenes are highly similar to that observed for CD5 and CD6, respectively. The central exon of SC-E5 is mostly included in mRNAs expressed in resting cells, whereas the central exon of SC-E6 is efficiently skipped under resting conditions. The relatively lower amount of three-exon product observed in SC-E6 *versus* CD6 can be attributed to the weak splice sites of the SC14 background. Of greater significance is the fact that mRNAs from both SC-E5 and SC-E6 show increased exon skipping upon PMA stimulation, whereas the control SC14 mRNA does not (Fig. 1*C*, − *versus* + PMA). Therefore, although we cannot completely rule out a potential role of intronic regulatory sequences, we conclude that the physiologic pattern of expression of exon 5 and 6 is primarily determined by sequences internal to these exons.

To identify individual ESS or ESE sequences within *CD45* exons 5 and 6, we next undertook a systematic mutagenesis of each of these exons (Figs. 2 and 3). We replaced regions of 15 (exon 6) or 20 (exon 5) nucleotides of exonic sequence with heterologous sequence previously shown to have no splicing-regulatory activity (21). For each exon, the substitutions, designated m1–m6 for exon 5 or m1–m9 for exon 6, were generated in the context of a minigene that contained all of exon 5 or exon 6 and a short stretch of flanking intron inserted between two constitutive exons of the β-globin gene (SC5 or SC6; Figs. 2*A* and 3*A*). SC5 and SC6 are identical to constructs studied previously (19) and were used as a background for the mutagenesis instead of SC-E5 and SC-E6, since we can more readily detect both two-exon and three-exon products from SC5 and SC6, thus allowing more accurate comparison between wild type and the mutants.

For exon 5, our mutagenesis scan reveals three primary regions of interest (Fig. 2). Mutation of region 3 results in an almost complete switch from three-exon to two-exon product in resting cells (m3; Fig. 2, *B* and *C*), although this mutation has no effect on the ability of PMA to induce further exon repression (m3; Fig. 2, *B* and *D*). Hence, the effect of the m3 mutation suggests that region 3 may function as a basal ESE (see Fig. 4). In contrast, mutation of region 2 has no consequence on exon inclusion in resting cells but renders the splicing entirely resistant to PMA stimulation (m2; Fig. 2, *B–D*). Importantly, region 2 contains both of the ARS motif repeats in exon 5 (see Fig. 5*D*). Thus, the effect shown here of substitution m2 is consistent with the splicing phenotype observed previously upon the introduction of point mutations within the ARS motif of exon 5 (19). Substitution of region 1 also causes a slight reduction in the PMA-induced -fold repression of exon 5, most likely due to disruption of the sequence context immediately adjacent to the ARS motif. However, beyond m2 and m3, the only other mutation that significantly alters the splicing pattern of exon 5 is substitution of region 4 (m4). Exon 5 is included in all of the transcripts derived from the m4 minigene, implicating region 4 as a sequence that normally functions to partially repress exon inclusion (Fig. 2, *B* and *C*). Since inclusion of exon 5 is absolute in m4-derived mRNAs, we cannot meaningfully quantitate a -fold repression value for this minigene (Fig. 2*D*). Therefore, we cannot differentiate between a direct role of region 4 in activation-induced exon repression *versus* region 4 acting to keep the overall level of splicing sufficiently suboptimal to render it sensitive to activation-induced repression via region 2.

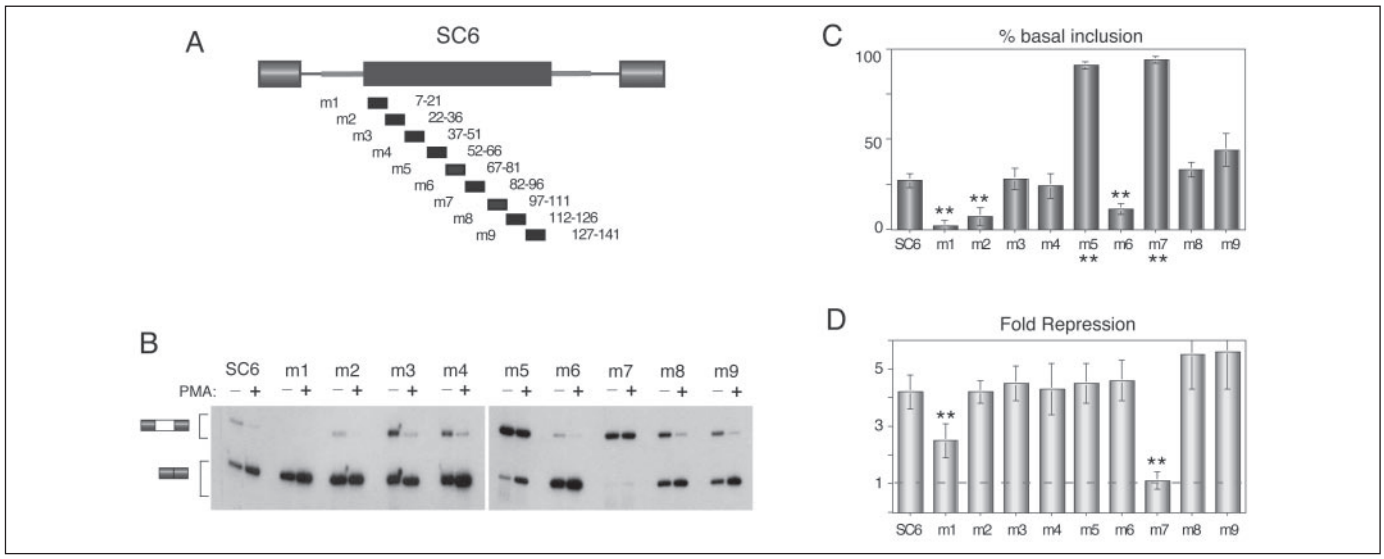

FIGURE 3. ***CD45* exon 6 contains multiple sequences that influence its pattern of inclusion.** *A*, schematic of block scanning mutagenesis of exon 6 in the context of the SC6 minigene, which contains all of *CD45* exon 6 and surrounding intron (*dark gray*) flanked by intron and exon sequences from the human β-globin gene (*gray gradient*). The locations of substitutions are given for each mutant (m1–m9). *B*, RT-PCR analysis of minigene-derived RNA as described in the legend to Fig. 1. *C*, quantitation of percentage of basal inclusion (*i.e.* percentage of 3E product) in resting cells for each minigene. Values shown are derived from at least four independent clones assayed in at least two separate experiments. *D*, -fold repression observed for each minigene upon PMA stimulation of cells. -Fold repression values shown were calculated as described in the legend to Fig. 1*B*. For *C* and *D*, *bars* marked by *double asterisks* were significantly different from the wild-type SC6 control ($p < 0.001$) as detailed in supplemental Table I.

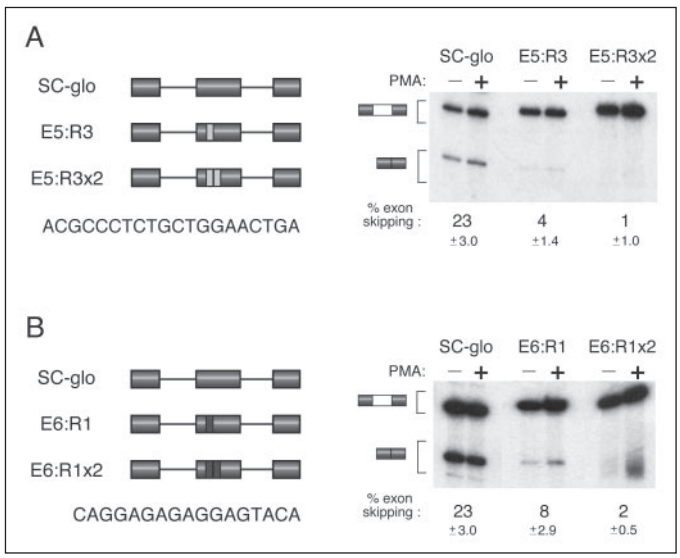

FIGURE 4. ***CD45* exon 5 contains a potent ESE with an atypical sequence, whereas exon 6 contains a canonical purine-rich ESE.** *A*, RT-PCR analysis of minigene-derived RNA as described in the legend to Fig. 1. A single or double copy of region 3 (R3) from exon 5 (sequence shown *under* minigene constructs) was inserted into a β-globin minigene (SC-glo). The percentage of two-exon product (*i.e.* percentage of exon skipping) is shown for each minigene under resting conditions. Percentage of exon skipping values are the average of at least four independent clones assayed in at least two separate experiments, and the S.D. is shown. The difference between the percentage of exon skipping observed for R3 and R3 × 2 gave a *p* value of 0.001. No significant effect of stimulation was observed for any of the minigenes (FR < 1.0 ± 0.3). *B*, RT-PCR analysis of minigene-derived RNA as described in the legend to Fig. 1. A single or double copy of region 1 (R1) from exon 6 (sequence shown *under* minigene constructs) was inserted into a β-globin minigene (SC-glo). Percentage of exon skipping values are calculated as described in *A*, and S.D. is shown. The difference between the percentage of exon skipping observed for R1 and R1 × 2 gave a *p* value<0.04. No significant effect of stimulation was observed for any of the minigenes (FR < 1.4 ± 0.3). Additional statistical details for all constructs are given in supplemental Table I.

CD45 exon 6 also contains multiple splicing-regulatory sequences as determined by scanning mutagenesis (Fig. 3). The largest and most significant effects on the basal level of exon 6 splicing are observed upon mutation of regions 1, 5, and 7 (m1, m5, and m7; Fig. 3, *B* and *C*). Virtually all of the mRNA derived from the m1 minigene exclude exon 6, whereas inclusion of exon 6 is the predominant pattern for minigenes m5 and m7. Interestingly, whereas both regions 5 and 7 contain homology to the ARS consensus, region 7 contains the longer ARS motif sequence and is the only sequence within exon 6 absolutely required for PMA-induced alternative splicing (FR = 1; Fig. 3*D*). Whereas we do observe a partial decrease in PMA sensitivity with the exon 6 minigene m1, this is most likely due to a limitation in how much more exon 6 can be repressed in this construct beyond that already occurring in resting cells.

The scanning mutagenesis is indispensable for identifying sequences necessary for the splicing pattern of exons 5 and 6, but it does not confirm that particular sequences are *bona fide* ESE or ESS sequences sufficient to induce exon inclusion or silencing. The mutagenesis shown in Figs. 2 and 3 suggests that region 3 of exon 5 (E5:R3) and region 1 of exon 6 (E6:R1) may function as ESEs, since mutation of both of these regions decreases exon inclusion. Moreover, the sequence encompassed by E6:R1 is highly reminiscent of the well characterized purine-rich ESE motif (see Fig. 4*B*). To test whether E6:R1 or E5:R3 could function as a *bona fide* ESEs, we inserted single or dimeric copies of these sequences within a version of the β-globin minigene that contains a restriction site to facilitate cloning (SC-glo). As shown in Fig. 4, we find that both E5:R3 (Fig. 4*A*) and E6:R1 (Fig. 4*B*) function in a dose-dependent manner to increase exon inclusion, confirming their identity as separable ESE-regulatory elements. Interestingly, E5:R3 is a particularly potent splicing enhancer despite having little, if any, sequence homology to previously characterized ESE motifs.

The scanning mutagenesis in Figs. 2 and 3 also highlights sequences within exons 5 and 6 that are potential basal ESS candidates, since their integrity within exons 5 and 6 serves to repress inclusion of these exons in resting cells. Within exon 6, regions 5 and 7 (E6:R5 and E6:R7) are both predicted to function as ESSs, as is region 4 of exon 5 (E4:R5). Consistent with the scanning mutagenesis results, both E6:R5 and E6:R7 are sufficient to induce exon skipping in a dose-dependent manner when inserted into the SC-glo background (Fig. 5, *A* and *B*; $p < 0.001$ for difference between single and double copies; see supplemental Table I).

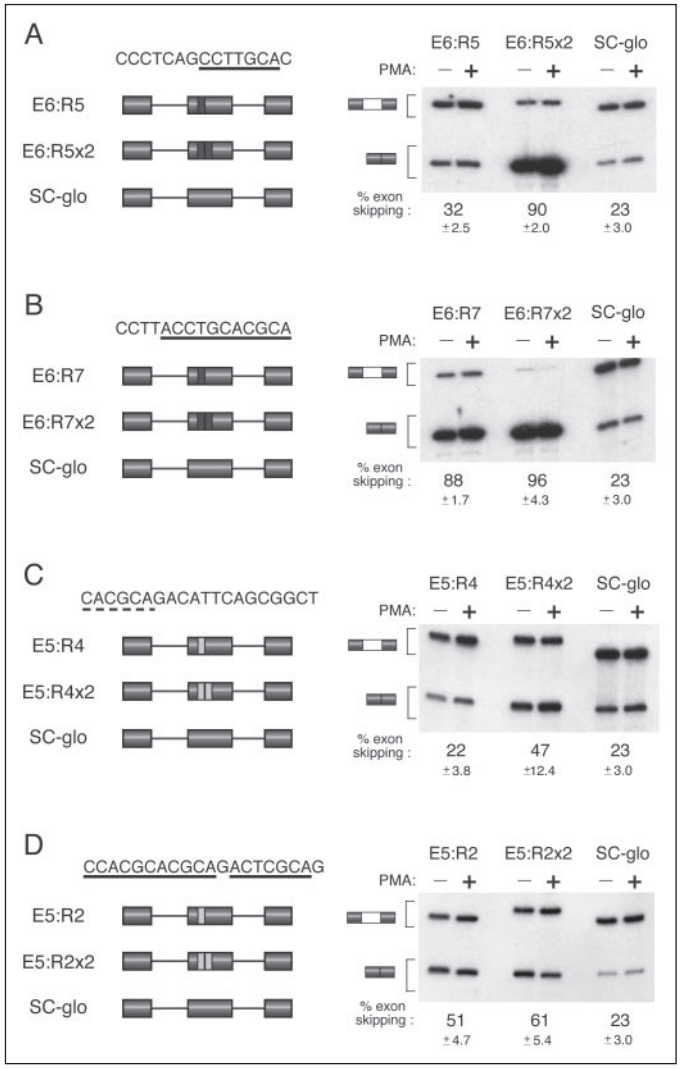

FIGURE 5. ***CD45* exon 6 contains potent ESS sequences, whereas ESS activity from exon 5 is less robust.** RT-PCR analysis of minigene-derived RNAs as described in the legend to Fig. 1. A single or double copy of regions from exons 5 and 6 was inserted into a β-globin minigene (SC-glo). The percentage of two-exon product (*i.e.* percentage of exon skipping) is shown for each minigene under resting conditions. Percentage of exon skipping values are the average of at least four independent clones assayed in at least two separate experiments. Inserted sequences are shown in each *panel* with the short and long versions of the ARS motif indicated by a *solid underline* and the partial match to the short ARS motif denoted by a *dashed underline*. Constructs and statistics for each *panel* are as follows. *A*, insertion of region 5 of exon 6 (E6:R5). No significant effect of stimulation was observed for any of the minigenes (FR $< 1.1 \pm 0.25$). *B*, insertion of region 7 of exon 6 (E6:R7). No significant effect of stimulation was observed for any of the minigenes (FR $< 1.6 \pm 0.2$). *C*, insertion of region 4 of exon 5 (E5:R4). -Fold repression for all constructs was $<1.3 \pm 0.5$. *D*, insertion of region 2 of exon 5 (E5:R2). -Fold repression for all constructs was $<1.0 \pm 0.3$. For all constructs in *A–D*, S.D. (shown) and *p* values (supplemental Table I) were also calculated.

However, the inherent silencing activity of E5:R4 is marginal, since no silencing is observed with a single copy of E5:R4, and only a modest increase in exon repression is observed in the presence of two copies of E5:R4 (Fig. 5*C*; $p = 0.026$ for E5:R4 × 2 *versus* E5:R4). Regions E6:R7 and E6:R5 contain the long (MCYYGCAMGCA) and short (MCYYGCA) components of the ARS motif respectively, whereas E5:R4 contains only an imperfect match to the short ARS motif (see *underlines* in Fig. 5). Thus, it appears that the strength of basal exon repression may correlate with homology to the ARS consensus sequence. A role of the ARS consensus sequence in basal exon silencing is consistent with our previous observation that mutation of the GCA core of the ARS repeats in exons 4 and 6 not only leads to a loss of activation responsiveness but also results in a dramatic increase in basal exon inclusion (19). The one observation inconsistent with a role of the ARS motif in basal exon repression is the observation that substitution or mutation of the ARS-containing region 2 within exon 5 (E5:R2) does not significantly increase exon inclusion in resting cells (m2; see Fig. 2 and Ref. 19). However, we do find that insertion of a single or dimeric copy of the E5:R2 sequence into the SC-glo background is sufficient to promote basal exon skipping (Fig. 5*D*; $p <$ 0.001 and 0.01, respectively). Therefore, we conclude that the ARS sequence does contribute to basal exon silencing, although this activity is somewhat context-dependent.

We also note that the individual ARS-containing regions (E5:R2, E5:R4, E6:R5 and E6:R7) are not sufficient to mediate significant activation-induced exon repression, since the -fold repression values for all constructs shown in Fig. 5 are less than 1.6 (see Fig. 5 legend). In our studies with *CD45* exon 4, we have found that robust activation-induced exon repression requires a 60-nucleotide sequence encompassing the ARS repeats, although ARS repeats themselves are the only specific sequences that are absolutely essential for this activity (19).[4] Therefore, we wanted to identify the minimal sequence from exons 5 and 6 that is sufficient for activation-induced exon repression and confirm the requirement for the ARS repeats in this minimal context.

Similar to our observations with *CD45* exon 4, a 60-nucleotide sequence from exon 6 spanning regions 5–8 is sufficient to mediate activation-induced exon repression of a heterologous unregulated exon (Fig. 6*A*, E6:R5–8). The PMA-induced -fold repression of E6:R5–8 is comparable with that observed for the wild-type exon 6 (see CD6; Fig. 1*B*). The addition of further exon 6 sequences to the E6:R5–8 construct did not increase PMA-induced exon skipping, whereas reduction in the amount of exon 6 sequence to regions 6–8 or regions 5–7 diminished PMA-induced exon repression (data not shown). Importantly, the 60-nucleotide element encompassed by regions 5–8 contains all of the ARS motif elements from *CD45* exon 6. Moreover, mutation of the ARS core GCA sequences within E6:R5–8 eliminates both basal and activation-induced repression (Fig. 6*A*, E6:R5–8mARS). In contrast to what we observe with *CD45* exons 4 and 6, virtually the entire sequence of *CD45* exon 5 is required to confer activation-induced repression on a heterologous exon (Fig. 6*B*, E5:R1–5). However, point mutations within the ARS core completely abolish PMA-induced skipping of the exon 5 chimeric minigene (Fig. 6*B*, E5:R1–5mARS). This requirement for the ARS motif within the minimal regulatory elements of exons 5 and 6 is consistent with what has been observed for the wild-type exons 4–6 (19) and demonstrates the central role of the ARS motif in activation-induced exon skipping.

Recently, we have identified the heterogeneous ribonucleoprotein hnRNP L as the primary protein that associates with the ARS repeats within *CD45* exon 4 and mediates repression of this exon under both resting and activated conditions (20). To determine whether hnRNP L also binds the minimal activation-responsive sequence from *CD45* exons 5 and 6 in an ARS-dependent manner, we synthesized ^{32}P-labeled RNA corresponding to the 60-nucleotide E6:R5–8 sequence or the 100-nucleotide E5:R1–5 sequence by *in vitro* transcription. These RNAs were incubated under splicing conditions with nuclear extract derived from JSL1 cells, and proteins that associate with E6:R5–8 or E5:R1–5 were covalently cross-linked to the RNA by treatment with UV light. Following digestion of the RNA, proteins bound to the RNA remain covalently attached to ^{32}P-labeled nucleotides, such that they can be

[4] J. Nguyen, A. Tong, and K. W. Lynch, unpublished data.

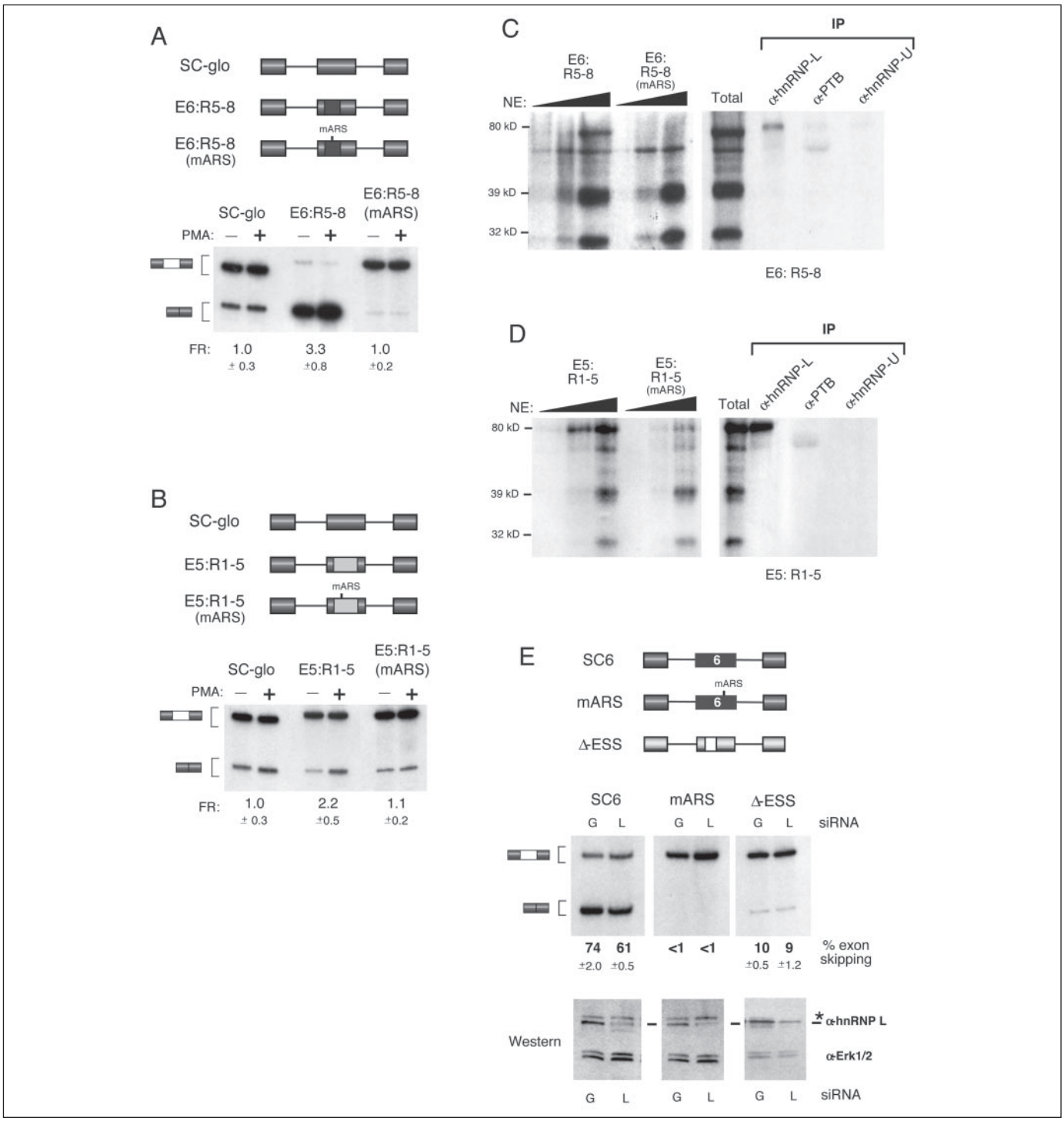

FIGURE 6. **The ARS motif is necessary, but not sufficient, for activation-induced switching and correlates with binding of hnRNP L.** Shown is RT-PCR of mRNA derived from exon 6-derived minigenes (*A*) or exon 5-derived minigenes (B), stably expressed in resting (−*PMA*) and activated (+*PMA*) JSL1 cells. -Fold repression values and S.D. (FR) shown for each construct are calculated from at least four independent experiments. *C* and *D*, UV cross-linking experiments done with uniformly labeled RNAs corresponding to E6:R5–8 with or without mARS mutation (*C*) or E5:R1–5 with or without mARS mutation (*D*). As indicated, some cross-linked samples were subject to immunoprecipitation with antibodies to hnRNP L, PTB, or hnRNP U prior to running of the SDS-polyacrylamide gel. *E*, Western blot of total protein (*bottom panel*) or RT-PCR of mRNA derived from minigenes (*top panel*) from 293 cells transiently transfected with the indicated minigene and pools of small interfering RNAs directed against either hnRNP L (*L*) or green fluorescent protein (*G*). Minigenes have been described previously (19). The percentage of exon skipping is calculated from at least three independent experiments with an S.D. of <2%. The difference observed in the SC6 minigene upon treatment with the small interfering RNAs against hnRNP L *versus* green fluorescent protein is statistically significant ($p = 0.016$). Western blot was done with antibodies against hnRNP L or Erk1/2 as a loading control. The *band* marked by an *asterisk* is a nonspecific interaction.

visualized following resolution on an SDS-polyacrylamide gel. As shown in Fig. 6, *C* and *D*, autoradiography of SDS-polyacrylamide gels following the UV cross-linking assay reveal that both E6:R5–8 and E5:R1–5 bind to four prominent proteins that migrate at ~75, 55, 38, and 30 kDa. Strikingly, only the binding of the 75-kDa protein is reduced by mutations in the ARS repeats (Fig. 6, *C* (E6:R5–8 *versus* E6:R5–8mARS) and *D* (E5:R1–5 *versus* E5:R1–5mARS)). The specificity of the 75-kDa protein for the wild-type ARS sequences was also confirmed by binding

studies done in the presence of mutant or unrelated competitor RNAs (data not shown).

Importantly, the ARS-specific 75-kDa protein bound to both E5:R1–5 and E6:R5–8 was identified as hnRNP L based on the ability of the cross-linked protein to be selectively precipitated by antibodies specific for hnRNP L but not another hnRNP family member, hnRNP U (Fig. 6, *C* and *D*, *right panels*). Antibodies specific for another hnRNP protein, PTB, precipitate the 55-kDa protein bound to E6:R5–8 or E5:R1–5. The binding of PTB to exons 5 and 6 in an ARS-independent manner is consistent with our previous observation that the ESS1 of *CD45* exon 4 binds to PTB in addition to hnRNP L but that the association of PTB with ESS1 is not dependent on the integrity of the ARS motif (20). Therefore, we find that the minimal activation-responsive sequences from *CD45* exons 5 and 6 both associate with a similar profile of proteins, as does the activation-responsive sequence from *CD45* exons 4. Most importantly, both the binding of these activation-dependent regulatory sequences to hnRNP L and their activity in cells is dependent on the integrity of the ARS core repeats.

Since the JSL1 cells are not amenable to RNA interference, we cannot confirm the functional requirement for hnRNP L in activation-induced splicing repression (data not shown). However, we can at least partially confirm a functional role of hnRNP L in basal exon 6 silencing by performing RNA interference knockdown of hnRNP L in the heterologous 293T cell line. hnRNP L is highly abundant in 293T cells; however, by transfecting the cells with a pool of small interfering RNAs targeted to hnRNP L message, we can achieve ~50% reduction in hnRNP L protein (Fig. 6*E*, *bottom panel*). Significantly, even this modest reduction in hnRNP L is sufficient to result in a decrease in exon 6 skipping from a co-transfected minigene (Fig. 6*E*, *top panel*, $p = 0.0162$), as has been observed previously with exon 4 (20). In contrast, no effect of hnRNP L knockdown on exon 6 splicing is observed upon mutation of the ARS consensus (Fig. 6*E*); nor does knock-down of hnRNP L alter splicing of a related *CD45*-derived construct that lacks any ARS core sequences (ΔESS; Fig. 6*E*). Unfortunately, we cannot determine whether knockdown of hnRNP L has a similar stimulatory effect on exon 5 inclusion, since this exon is essentially completely included in the control 293T cells, similar to the JSL1 cells (see Fig. 2). Nevertheless, the similarities in splicing regulation of exons 4–6, including binding of hnRNP L, imply that parallel or overlapping mechanisms are involved in determining the level of inclusion of each of these exons within the final mRNA.

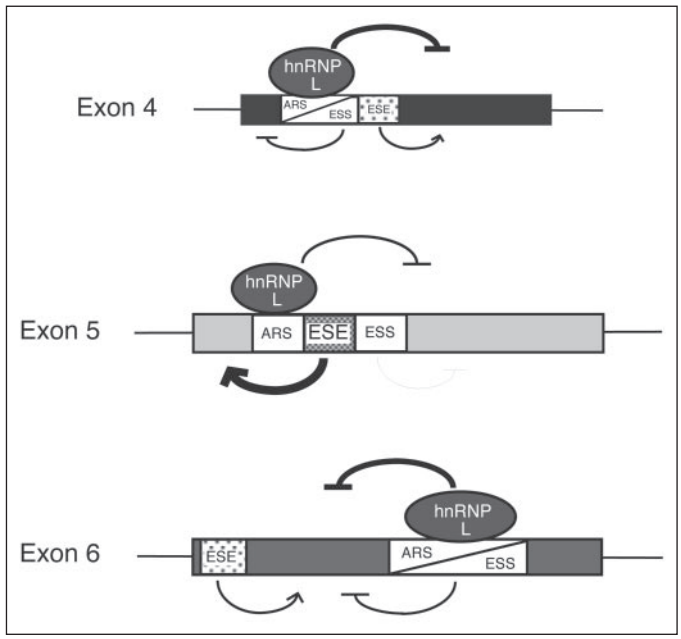

FIGURE 7. **The inclusion of *CD45* exons 4–6 is independently controlled by a similar internal combination of basal and inducible regulatory motifs.** Shown is a schematic of ESEs, ESSs, and ARSs in *CD45* exons 4 (18, 19) and *CD45* exons 5 and 6 (this study). Purine-rich ESEs are designated by *dots*, whereas the noncanonical ESE of exon 5 is shown by a *diamond pattern*. The *arrows* indicate enhancer activity (*i.e.* promoting exon inclusion), whereas *bars* indicate silencer function (*i.e.* repressing exon inclusion). The *widths* of *bars* and *arrows* are suggestive of relative strength of activity. hnRNP L is shown binding to the ARS consensus motifs in each exon.

DISCUSSION

It has long been appreciated that the CD45 tyrosine phosphatase exists in at least five distinct isoforms due to alternative splicing of exons 4–6 of its gene (3). Importantly, the expression of the CD45 isoforms is well documented to be regulated in a cell- and activation-dependent manner, thereby influencing the function of the human immune system (3, 12). In this study, we demonstrate that the critical features of regulation of the three *CD45* variable exons are highly similar, suggesting that common mechanisms are involved in determining the level of inclusion of each of these exons and hence in regulating the isoform expression of CD45 at the cell surface.

Each of the three *CD45* variable exons contains multiple splicing-regulatory sequences that control the ultimate decision of exon inclusion *versus* exon skipping (see Fig. 7). Exons 4 and 6 both contain a canonical purine-rich ESE sequence that promotes inclusion, whereas exon 5 contains a unique sequence that acts as an unusually robust enhancer of exon inclusion. Similarly, all three exons contain ESS elements that counter exon inclusion and promote exon skipping. For exons 4 and 6, the sequence that confers exon skipping in resting cells contains the ARS consensus sequence and also functions to cause increased exon repression upon cellular stimulation. The ARS consensus sequence is additionally present in exon 5 and is required for stimulation-induced repression of this exon, since mutation of E5:R2 causes exon 5 splicing to be refractory to PMA (see Fig. 2). Importantly, the RNA-regulatory factor hnRNP L binds to each of the three *CD45* variable exons in an ARS-dependent manner and is involved in basal repression of exon 6, as we have previously shown for exon 4 (20). Therefore, not only do the *CD45* variable exons share common regulatory sequences and expression patterns, but they are also each targets for at least one common regulatory protein.

Despite the numerous similarities in the regulation of the *CD45* variable exons, there are a few notable differences. First, unlike in exon 4 and 6, the isolated ARS-containing sequence from exon 5 causes only slight exon repression in resting cells (Fig. 5*D*). Moreover, we are not able to identify a minimal region surrounding the ARS core within exon 5 that is sufficient to induce a level of PMA-induced splicing repression similar to that observed in the full exon. The reason for the distinction between ARS consensus activity in the three exons is unclear, but it probably points to additional contextual effects that are yet to be defined. In this regard, it is worth noting that the ARS motif in E6:R5 and E6:R7, as well as in *CD45* exon 4, is flanked by an upstream stretch of 4–5 pyrimidines, whereas the E5:R2 sequence does not contain such a sequence. Moreover, any ARS-mediated basal silencing activity that might exist within E5:R1–5 is probably masked by the strong enhancer activity conferred by E5:R3. Thus, whereas the core ARS motif is clearly necessary for signal-induced changes in splicing, our data suggest that it is not entirely sufficient for either basal or activation-induced exon repression. In addition, we cannot rule out the possibility that intronic sequences normally surrounding exon 5 (and even perhaps exons 4 and 6) may contribute to the regulatory network that influences splicing of these exons.

Nevertheless, our data clearly demonstrate the importance of exonic

sequences in the regulation of *CD45* isoform expression. In particular, the relative strengths of the regulatory sequences that we have characterized in each of the *CD45* variable exons are consistent with the observed physiological expression of the CD45 protein. The ESS activities within exons 4 and 6 are highly robust, whereas exon 5 has a relatively weak ESS but a particularly strong ESE. This difference in the balance of regulatory pressures provides a mechanistic explanation for the observation that exon 5 is included in the vast majority of transcripts produced in resting cells and even many of the transcripts expressed following activation, whereas inclusion of exons 4 and 6 is much less frequent (see Fig. 1).

Finally, in addition to explaining the normal physiologic expression of CD45, a detailed understanding of the splicing-regulatory elements is critical for interpreting the biological effects of polymorphisms and mutations within the *CD45* gene. At least two polymorphisms in *CD45* exon 4 and one in *CD45* exon 6 have already been described in the human population to alter *CD45* isoform expression and have been potentially linked to autoimmune disease susceptibility (13–15, 22–24). These three polymorphisms all fall within or near the regulatory sequences described here or in our previous study (18). Given the importance of the splicing-regulatory sequences within *CD45* exons 4–6, we predict that many more mutations within the *CD45* gene could exist that alter protein expression and immune function. Moreover, our data suggest that mutations within the hnRNP L protein might also influence CD45 isoform expression. Therefore, the data presented here provide a framework for predicting and understanding the effect of human mutations and polymorphisms that may be found in *CD45* and/or alter isoform expression of the CD45 protein tyrosine phosphatase.

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