

# The Silent Sway of Splicing by Synonymous Substitutions\*<sup>§</sup>

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**William F. Mueller<sup>‡</sup>, Liza S. Z. Larsen<sup>§</sup>, Angela Garibaldi<sup>‡</sup>, G. Wesley Hatfield<sup>‡§</sup>, and Klemens J. Hertel<sup>‡§1</sup>**

From the <sup>‡</sup>Department of Microbiology and Molecular Genetics and the <sup>§</sup>Institute for Genomics and Bioinformatics, University of California Irvine, Irvine, California 92619

**Background:** The effects of silent mutations on pre-mRNA splicing are poorly understood.

**Results:** Silent mutations can significantly influence exon inclusion and are under purifying selection.

**Conclusion:** Splicing and coding pressures have co-evolved to maintain sufficient exon inclusion levels.

**Significance:** Modified species alignment approaches can be used to identify silent mutations that may alter exon inclusion.

Alternative splicing diversifies mRNA transcripts in human cells. This sequence-driven process can be influenced greatly by mutations, even those that do not change the protein coding potential of the transcript. Synonymous mutations have been shown to alter gene expression through modulation of splicing, mRNA stability, and translation. Using a synonymous position mutation library in *SMN1* exon 7, we show that 23% of synonymous mutations across the exon decrease exon inclusion, suggesting that nucleotide identity across the entire exon has been evolutionarily optimized to support a particular exon inclusion level. Although phylogenetic conservation scores are insufficient to identify synonymous positions important for exon inclusion, an alignment of organisms filtered based on similar exon/intron architecture is highly successful. Although many of the splicing neutral mutations are observed to occur, none of the exon inclusion reducing mutants was found in the filtered alignment. Using the modified phylogenetic comparison as an approach to evaluate the impact on pre-mRNA splicing suggests that up to 45% of synonymous SNPs are likely to alter pre-mRNA splicing. These results demonstrate that coding and pre-mRNA splicing pressures co-evolve and that a modified phylogenetic comparison based on the exon/intron architecture is a useful tool in identifying splice altering SNPs.

Pre-mRNA splicing is an essential process necessary for both proper gene expression and the generation of transcript diversity throughout metazoans (1). Intron removal, directed by sequence signals within the pre-mRNA, is catalyzed by the spliceosome, a large ribonuclear protein complex (2). The interaction of splicing regulatory elements and their trans-acting binding partners (hnRNPs, SR proteins, and small nuclear ribonucleoproteins)<sup>2</sup> determines where and how splicing takes

place (3). Mutations within binding sites of these trans-acting factors will likely alter the identity of the resulting mRNA (4).

The presence of sequence elements that influence splicing throughout the transcript suggests that organisms that rely on high fidelity splicing will be under evolutionary pressure to maintain optimal splice signals within the pre-mRNA molecule (5). Indeed, it has previously been shown that splicing regulatory elements exhibit positive selection, that intron/exon boundaries have a decreased frequency of single nucleotide polymorphisms (SNPs), and that certain codons are generally preferred around intron/exon junctions (6–8). These observations suggest that the spliceosome has to recognize exons by using sequences that are co-evolving with the amino acid sequence code to generate an RNA molecule that both translates and properly splices. However, it is difficult to uncouple sequence requirements imposed by evolutionary coding pressures from sequence requirements necessary to generate the appropriate mRNA through splicing.

Splicing requires sequence elements from both the intron and the exon for proper intron excision. Within the exon, those sequence elements are restricted in sequence by the need for correct protein coding. Given the diversity of the genetic code in the third or wobble position, it has been possible in a few cases to separate splicing constraints from coding constraints (9, 10). Here, we characterize the contribution of splicing evolutionary pressures through the identification of synonymous mutations that specifically alter the efficiency of intron removal. We created a library of synonymous mutations across exon 7 of the *SMN1* gene to identify positions that influence exon inclusion using deep-sequencing approaches. Phylogenetic comparisons with organisms that have a similar *SMN1* exon 7 architecture demonstrated a selection against exon inclusion-reducing mutants, clearly demonstrating that evolutionary pressures exist at wobble positions to uphold efficient splicing of this crucial exon. A survey of human synonymous SNPs showed that 45% contain potentially splice altering nucleotide substitutions. These observations suggest that a large proportion of synonymous SNPs can cause defects in splicing. Thus, filtering multiple species alignments by exon architectural similarity represents a novel strategy to identify exonic splicing regulatory elements and synonymous mutations that disrupt proper pre-mRNA splicing.

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<sup>1</sup> To whom correspondence should be addressed. Tel.: 949-824-2127; Fax: 949-824-8598; E-mail: khertel@uci.edu.

<sup>2</sup> The abbreviations used are: hnRNP, heterogeneous nuclear ribonucleoprotein; SMN, survival of motor neuron; SMA, spinal muscular atrophy; nt, nucleotide; PhyloP, part of the PHAST package (Phylogenetic Analysis with Space/Time models) used through UCSC Genome Browser to evaluate phylogenetic conservation.

## Experimental Procedures

**Cell Culture and Transfection of HeLa Cells**—HeLa cells used in this work were maintained at 37 °C in a monolayer in Dulbecco's high glucose modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 4 mM L-glutamate, and 1 mM Na-pyruvate. Their cell confluence was kept to ~80% or less before splitting cells. Cells were transfected according to the manufacturer's specifications for Lipofectamine 2000 (Invitrogen) for plate sizes of 10-cm 6-well plates with 3-cm wells, or 15-cm plates.

**Creation/Transfection of SMN1 Exon 7 Library Mutations**—We used previously described SMN mini-genes containing exons 6, 7, and 8 with shortened introns between each exon (11–13). To generate a library of synonymous mutations across exon 7, we created a series of mutagenesis primers to make a site-directed saturation mutagenesis library of exon 7. This library is comprised of synonymous site-directed mutations spanning the 54-nucleotide exon 7 region of the *SMN1* gene. All possible combinations of synonymous mutations were generated within a sliding 2-codon window such that the gene amino acid sequence remained the same and all possible synonymous mutations were created.

Sets of oligonucleotide primers were used to generate the mutations to each synonymous position in exon 7. To generate the library with fewer per-base errors, oligonucleotide sets used were kept as short as possible and the mutagenesis reactions were split into three separate pooled reactions (sequences of oligonucleotides used are available upon request). The first reaction contained a pool of 8 forward and 8 reverse oligonucleotides (oligos 1–8), the second reaction contained a pool of 10 forward and 10 reverse oligonucleotides (oligos 9–18), and the third reaction contained a pool of 12 forward and 12 reverse oligonucleotides (oligos 19–30), all of which contain a double randomized NNN NNN codon at its center such that the two amino acids are simultaneously mutated (but only to synonymous mutations).

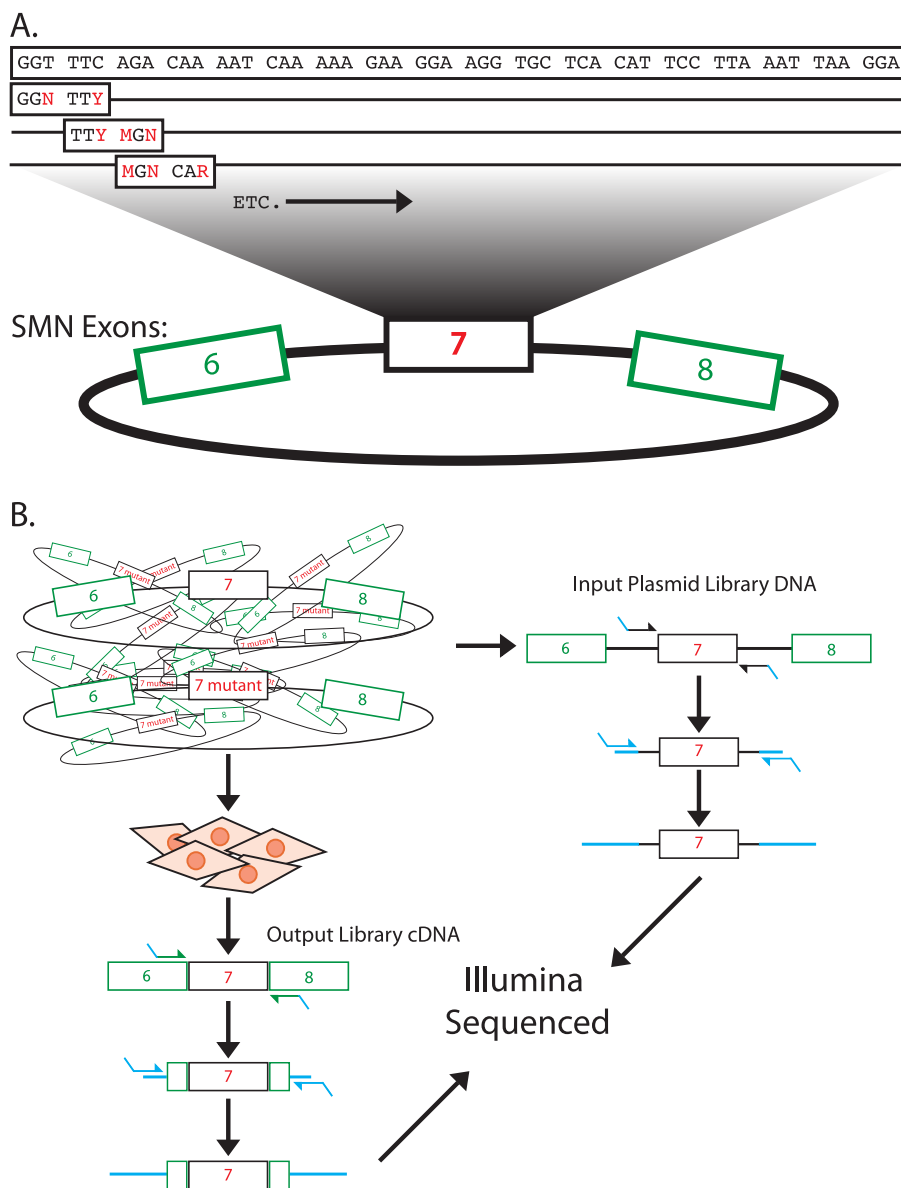
PCR amplifications for each mutagenesis pool of *SMN1* exon 7 were performed in 50- $\mu$ l reactions containing: 50 ng of the plasmid based pCi *SMN1* mini-gene template, 5 units of Pfu Ultra-High Fidelity DNA polymerase (Stratagene); 400  $\mu$ M dNTPs; 1 $\times$  Pfu Ultra-High Fidelity reaction buffer; and 0.03  $\mu$ M of each complementary mutant primer pair. Primer extension and PCR amplification reactions were carried out by: 10 min denaturation at 95 °C, followed by 16 cycles of 15 s at 95 °C, 40 s at 55 °C, 3 min at 72 °C, and a final step of 10 min at 72 °C. The 50- $\mu$ l PCR products were digested with 30 units of DpnI for 3 h at 37 °C to remove the methylated template plasmid. 5  $\mu$ l of the digestion reaction was used for transformation of DH5 $\alpha$  cells and plated onto LB-agar plates (100  $\mu$ g/ml of ampicillin). At this point, a plasmid library containing saturated site-directed mutated target regions is generated. Colonies were selected and suspended in 5 ml of LB medium (100  $\mu$ g/ml of ampicillin) and incubated at 37 °C overnight. 15% glycerol stocks were created, and larger 200-ml flasks of LB medium (100  $\mu$ g/ml of ampicillin) were inoculated and incubated at 37 °C overnight. Plasmid libraries were purified from the large cultures using the Nucleobond Midi-prep Purification system. Plasmid libraries were

then combined in equal molar amounts to make one master library.

**Transfection and Sequencing Library Preparation**—The approach for the generation of sequencing libraries is outlined in Fig. 1. To generate a library of spliced mRNAs, HeLa cells at ~80% confluence in 10-cm plates were transfected with 22  $\mu$ g of the master plasmid library using Lipofectamine 2000 for 6 h in serum-free medium at 37 °C. After 6 h, the Lipofectamine medium was removed and medium-containing serum was added. The cells were incubated another 18 h at 37 °C. Total RNA was purified from the cells using TRIzol reagent (Ambion) according to the manufacturer's recommendations and treated with DNase (Invitrogen). Reverse transcription using SuperScript II reverse polymerase (Invitrogen) was carried out using library plasmid-specific primers (pCI backbone forward primer: GCTAACGCAGTCAGTGCTTC; pCI backbone reverse primer: GTATCTTATCATGTCTGCTCG) and 4  $\mu$ g of RNA. The cDNA reaction was then cleaned up using a Qiagen PCR purification kit according to the manufacturer's recommendations. Approximately 0.5  $\mu$ g of purified cDNA was then amplified using primers specific to exon 6 and exon 8 (output cDNA forward primer: CCCTACACGACGCTCTTCCGATCTCATGAGTGGCTATCATACTGGC; output cDNA reverse primer: CCTGCTGAACCGCTCTTCCGATCTGTCA-TTTAGTGCTGCTCTATGC; Seq. tail forward primer: AAT-GATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT; Seq. tail reverse primer: CAA-GCAGAAGACGGCATAACGAGATCGGTCTCGGCATTCC-TGCTGAACCGCTCTTCCGATCT) that also had tails containing the initial segment of Illumina-specific sequencing primers in a 50- $\mu$ l reaction containing 400 nM dNTPs using proofreading DNA polymerase. PCR amplification reactions were carried out by: 5 min denaturation at 95 °C, 3 cycles of: 15 s denaturing at 95 °C, 30 s primer annealing at 55 °C, 20 s of extension at 72 °C, and a final extension of 5 min at 72 °C. This initial PCR library amplification was then run out on a 1.5% agarose gel to remove exon exclusion events and gel purified using a Qiagen Gel extraction kit. The purified PCR product was then amplified again for 12 cycles using the same PCR cycle conditions as described above, except using a full-length Illumina sequencing primer. The products of this reaction were separated by 1.5% agarose gel electrophoresis and the PCR product band was purified using the Qiagen gel extraction kit according to the manufacturer's recommendations. This final library was analyzed for quality on an Agilent Bioanalyzer 2100, quantified using a Nanodrop (ND-1000 Thermo), and then diluted to 10 nM and submitted for sequencing on the Illumina Hi-seq platform. The spliced mRNA pool is referred to as the "output pool."

To generate an equivalent sequencing library of the expression plasmid pool, the same procedure (minus transfection and RNA purification) was carried on the plasmid pool using primers specific to introns 6 and 7 (input DNA forward primer: CCC-TACACGACGCTCTTCCGATCTGCTATTTTTTTTAACTTCTTTATTTTC; input DNA reverse primer: CCTGCTGAACCGCTCTTCCGATCTGTAAGATTCACTTTTCATAA-TGCTG). The expression plasmid pool is referred to as the "input pool." PCR analysis of SMN exon 7 mutants for valida-

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**FIGURE 1. Mutation scheme for the *SMN1* exon 7 library preparation.** *A*, all possible silent mutations were generated per in-frame hexamer across the exon. For example, the first two codons depicted are GGT TTC. All three mutations were made in GGT resulting in GGN and combined with all silent mutations in TTC (TTT), resulting in eight combinations including the wild-type sequence. *B*, the library of mutations were sequenced and transfected into HeLa cells. The RNA resulting from the transfection was purified and sequenced. The relative abundance of each mutation was compared between the two pools to generate fitness index values.

tion and comparison of our sequencing experiment was carried out using primers as previously described (11, 17).

**Bioinformatic Analysis of *SMN1* Exon 7 Library Mutations**—We received 54,780,073 single-end reads of 100 nt from our sequencing run. Using Bowtie these reads were aligned to a custom index made from the genomic segment of *SMN1* spanning exons 6 to 8 (14), from the spliced mRNA sequence spanning exons 6, 7, and 8, and from all library mutants made. Thus, the alignment index contained an entry for each library mutation to control for possible mutations or sequencing errors. Only reads that contained the wild-type sequence or library mutations were aligned. Approximately three fourths of the reads were aligned to the custom index. Another 8% of the reads corresponded to exon exclusion reads, and the remaining reads did not align.

The total read count for each library mutation was used to determine the relative representation of each mutation within in the sequenced input and output pools. To evaluate changes in exon 7 inclusion, we calculated WT-normalized differences in mutant output and input ratios according to “fitness index” = (output/WT output)/(input/WT input). These ratios are defined as the fitness index. The average fitness index for a position is the average of the fitness indices for all mutations at that position.

For statistical analysis we compared the output and input reads for each mutation to the non-mutated reads using a Fisher’s Exact test and determined the significance using a Bonferroni correction for comparison between the 138 mutations. In addition to the Fisher’s Exact test, we applied a biological cutoff for significance of the fitness index value. Twenty percent exon



7 inclusion is consistent with mRNA expression reports for SMA type I. An observable difference in the exon inclusion phenotypes between SMA types I and III has been reported (12, 15–17). In SMA type III there is ~70% inclusion of exon 7 compared with WT levels, *versus* the 20% observed in type I. Therefore, we set our biological significance cutoff at this 70% value.

Our analysis relied on the circumstantially validated assumptions that all plasmids transfect and transcribe with the same efficiency and that the resulting RNAs are similarly stable in HeLa cells. A decrease in transcription or transcriptional pausing causing a loss of reads is unlikely, as significant numbers of reads were generated and analyzed corresponding to each mutation (average cDNA reads 209,891/mutation, median: 39,288). This suggests that each mutation was represented within the input plasmid pool, successfully transfected, and successfully expressed. The lowest read count observed for a single mutant was 30 output reads for the mutations in positions 24 and 27. This position is known for being a splicing regulator (Tra2- $\beta$ 1/SRSF10) binding site, suggesting that the loss in reads was due to the loss of an enhancer binding sequence (18). Consistently, low output reads correlated with a loss of a known positive splicing regulator, the gain of a negative splicing regulator, or increasing known local RNA secondary structures. It is not known whether mRNAs generated from the reporter plasmids are translated, and therefore are potential NMD targets. However, because all exon 7 mutations are located within 50 nt of the last exon-exon junction, it is unlikely that the resulting mRNA would be targeted by NMD (19). It is also unknown whether the stability of exon 7 included mRNAs generated from the mini-gene constructs is different from mRNAs lacking exon 7.

**Comparative Alignment Analysis of SMN1 Exon 7 Mutations and Synonymous SNPs**—The position of SMN1 exon 7 was identified through the UCSC Genome Browser. The UCSC Table Browser was then used to download the PhyloP Score by position across the exon (position chr5: 70,247,768 to 70,247,821) using the Comparative Genomics Group and the Conservation Track in the Vertebrate 46 way alignment table SMN1. PhyloP conservation scores allow a measure of conservation by position. Multiple alignment data were generated by taking this same genomic position into the Ensembl! database (GRCh37) and selecting the comparative genomics (text option) tool for all 36 eutherian mammals in the Ensembl! Genome Sequence Viewer. This was also used to validate intron/exon architecture. Intron/exon architecture similarity was determined through sequence analysis using the comparative genomics tool. Organisms with similar exon length ( $\pm 6$  nt) and splice site sequences were compared. Species that exhibit similar exon/intron architecture were kept in the alignment. Species exhibiting differing exon/intron architecture were excluded from the alignment. In the case of the SMN alignment, all primates were included in the intron/exon architecture filter, however, this excluded multiple species from rodents to the alpaca. The same approach was used for filtering SNP-based species alignments.

Synonymous SNPs associated with human disease were taken from the literature and from the Entrez dbSNP web

browser and added to synonymous human SNPs downloaded using the Ensembl! PerlAPI (20). The 40 disease associated SNPs and 40 Ensembl! SNPs were randomly selected and analyzed using the Ensembl! genome browser comparative genomics alignment tools as was done for the SMN exon analysis. Eighty more human synonymous SNPs from non-coding exons, plus an additional 82 synonymous SNPs with a single exon were also acquired using the Ensembl! PerlAPI and analyzed in the same manner (supplemental Table S1). Filtered organisms were compared by sequence conservation in an 11-nucleotide window around the SNP position. The numbers of the classified putative splice altering SNPs and neutral SNPs were compared using a  $\chi^2$  test of independence.

## Results

**Analysis of Library Mutations**—To determine the influence of synonymous mutations on exon inclusion, we used the well studied SMN1 mini-gene, spanning exons 6–8 (13, 17, 21) in which exon 7 can be included or excluded depending on the splicing signals within the pre-mRNA. Each set of two neighboring codons in exon 7 was mutated to every possible combination of silent mutations within the context of a hexamer, a minimal binding platform for splicing regulatory proteins (Fig. 1A) (22). The resulting library of plasmids was transfected into HeLa cells and plasmid-specific mRNAs were analyzed by deep sequencing (Fig. 1B). Relative exon inclusion indexes were determined by calculating the abundance of mutations present in the exon 7-included mRNA population, normalized to the abundance in the input plasmid control. Presumably, differences in inclusion indexes reflect differences in exon 7 inclusion levels (Table 1). To test this assumption a small number of mutants (Table 2 and Fig. 2) were tested individually for exon inclusion levels using standard RT-PCR approaches. The near linear relationship between the different experimental approaches demonstrate that changes in the fitness indexes are largely driven by altered pre-mRNA splicing efficiencies. If a mutation was observed more frequently in the spliced mRNA pool than the input plasmid pool, that mutation was considered to be beneficial to exon inclusion. If a mutation was observed less frequently in the spliced mRNA pool compared with the input plasmid pool, that mutation was considered to be inhibitory to exon inclusion.

The statistical significance of an observed change in relative mutant abundance was compared with the wild-type (WT) representation using a Fisher's Exact test with Bonferroni correction. In addition a biological filter was imposed based on previous observations that mRNA levels of SMA type III patients display exon 7 inclusion levels at ~70% of that observed for healthy individuals (12, 15). All mutants that caused a decrease in the fitness index to 70% or less of the unchanged WT value were considered to have a negative effect on exon inclusion. Of 138 mutations tested, 32 met the criteria for significant changes in the fitness index (Table 1).

The fact that only 32 of the 138 mutations evaluated caused significant fitness index reductions demonstrates that the majority of the mutations tested did not appear to cause striking changes in exon inclusion. Most mutations that caused significant changes negatively affected the fitness index. Interest-

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**TABLE 1**

**All mutations analyzed**

Summary of results obtained for all library mutations analyzed. The mutation positions are from the first position of *SMN1* exon 7. The wild-type and mutant nucleotides are separated by “>”. Read counts and exon inclusion ratios were found and calculated as described under “Experimental Procedures”. Mutants highlighted in grey were found to cause a significant reduction in the fitness index value.

Mutation and Position	Input Read Count	Output Read Count	Inclusion Index Value	Log Inclusion Index	Mutation and Position	Input Read Count	Output Read Count	Inclusion Index Value	Log Inclusion Index
Wildtype, No Mutations	2969387	6464990	1.00	0.00	34:T>A, 35:C>G, 36:A>T*	92751	133695	0.66	-0.18
3:T>A*	65023	174024	1.23	0.09	34:T>A, 35:C>G, 36:A>C	127089	274765	0.99	0.00
3:T>C*	72883	213355	1.34	0.13	34:T>A, 35:C>G, 36:A>T, 39:T>C*	17411	233	0.01	-2.21
3:T>G	104141	213785	0.94	-0.03	34:T>A, 35:C>G, 36:A>C, 39:T>C*	53816	1956	0.02	-1.78
3:T>A, 6:C>T*	28855	13109	0.21	-0.68	36:A>C*	63524	178900	1.29	0.11
3:T>C, 6:C>T*	47776	27248	0.26	-0.58	36:A>G*	105766	299431	1.30	0.11
3:T>G, 6:C>T*	68754	19519	0.13	-0.88	36:A>T*	219252	506661	1.06	0.03
6:C>T*	231830	195369	0.39	-0.41	36:A>T, 39:T>C*	10722	2188	0.09	-1.03
6:C>T, 7:A>C*	63130	163781	1.19	0.08	36:A>G, 39:T>C*	22456	8618	0.18	-0.75
6:C>T, 7:A>C, 9:A>T*	71615	185308	1.19	0.07	36:A>C, 39:T>C*	41583	25936	0.29	-0.54
6:C>T, 7:A>C, 9:A>C*	87558	237283	1.24	0.10	39:T>C*	56096	18783	0.15	-0.81
6:C>T, 7:A>C, 9:A>G*	89105	263489	1.36	0.13	39:T>C, 40:T>A, 41:C>G*	7176	34778	2.23	0.35
6:C>T, 9:A>G*	202960	95344	0.22	-0.67	39:T>C, 40:T>A, 41:C>G, 42:C>T*	7581	22007	1.33	0.12
7:A>C	215137	697820	1.49	0.17	39:T>C, 42:C>T*	3922	11742	1.38	0.14
7:A>C, 9:A>T*	239937	633391	1.21	0.08	39:T>C, 42:C>A*	4682	16120	1.58	0.20
7:A>C, 9:A>G*	252521	882474	1.61	0.21	39:T>C, 42:C>G*	4901	19685	1.84	0.27
7:A>C, 9:A>C*	308880	910247	1.35	0.13	40:T>A, 41:C>G*	14984	19322	0.59	-0.23
7:A>C, 9:A>T, 12:A>G*	110159	320179	1.33	0.13	40:T>A, 41:C>G, 42:C>T*	8090	4891	0.28	-0.56
7:A>C, 9:A>C, 12:A>G*	114606	337835	1.35	0.13	40:T>A, 41:C>G, 42:C>T, 43:T>C	1286	2975	1.06	0.03
7:A>C, 9:A>G, 12:A>G*	142308	458433	1.48	0.17	40:T>A, 41:C>G, 42:C>T, 43:T>C, 45:A>T	1206	1980	0.75	-0.12
7:A>C, 12:A>G*	106081	305749	1.32	0.12	40:T>A, 41:C>G, 42:C>T, 43:T>C, 45:A>C	3094	10505	1.56	0.19
9:A>G*	518071	1048763	0.93	-0.03	40:T>A, 41:C>G, 42:C>T, 43:T>C, 45:A>G	3510	11954	1.56	0.19
9:A>G, 12:A>G*	258109	526741	0.94	-0.03	40:T>A, 41:C>G, 42:C>T, 45:A>G	8597	17255	0.92	-0.04
12:A>G*	479922	999508	0.96	-0.02	40:T>A, 41:C>G, 43:T>C	7745	18558	1.10	0.04
12:A>G, 15:T>C*	225435	542544	1.11	0.04	40:T>A, 41:C>G, 43:T>C, 45:A>T	1627	4423	1.25	0.10
15:T>C*	462016	1074101	1.07	0.03	40:T>A, 41:C>G, 43:T>C, 45:A>G*	3808	17866	2.15	0.33
15:T>C, 18:A>G*	110917	288864	1.20	0.08	40:T>A, 41:C>G, 43:T>C, 45:A>C*	3963	17870	2.07	0.32
18:A>G	377050	802943	0.98	-0.01	40:T>A, 41:C>G, 45:A>G	3724	11039	1.36	0.13
18:A>G, 21:A>G*	272585	706508	1.19	0.08	42:C>A*	5994	18705	1.43	0.16
21:A>G*	314749	774770	1.13	0.05	42:C>T*	7417	22495	1.39	0.14
21:A>G, 24:A>G*	58143	146114	1.15	0.06	42:C>G*	13930	52007	1.71	0.23
24:A>G*	179832	290267	0.74	-0.13	42:C>A, 43:T>C	536	2379	2.04	0.31
24:A>G, 27:A>T*	18298	30	0.00	-3.12	42:C>T, 43:T>C	574	2342	1.87	0.27
24:A>G, 27:A>C*	31048	3203	0.05	-1.32	42:C>G, 43:T>C*	3213	16193	2.31	0.36
24:A>G, 27:A>G*	71168	75288	0.49	-0.31	42:C>A, 43:T>C, 45:A>T	902	3068	1.56	0.19
27:A>C*	147704	39043	0.12	-0.92	42:C>T, 43:T>C, 45:A>T	1001	3805	1.75	0.24
27:A>T*	150992	1333	0.00	-2.39	42:C>T, 43:T>C, 45:A>G*	1701	7735	2.09	0.32
27:A>G*	218079	398116	0.84	-0.08	42:C>G, 43:T>C, 45:A>G*	1784	10870	2.80	0.45
27:A>C, 28:A>C*	59948	107050	0.82	-0.09	42:C>T, 43:T>C, 45:A>C*	2102	9964	2.18	0.34
27:A>T, 28:A>C*	87645	153342	0.80	-0.09	42:C>G, 43:T>C, 45:A>C*	2176	11188	2.36	0.37
27:A>G, 28:A>C*	125619	228546	0.84	-0.08	42:C>A, 43:T>C, 45:A>G*	2522	11896	2.17	0.34
27:A>T, 28:A>C, 30:G>C	637	1407	1.01	0.01	42:C>G, 43:T>C, 45:A>T*	4712	22728	2.22	0.35
27:A>T, 28:A>C, 30:G>A	1146	2638	1.06	0.02	42:C>A, 43:T>C, 45:A>C*	9964	45096	2.08	0.32
27:A>G, 28:A>C, 30:G>C	2369	4298	0.83	-0.08	42:C>T, 45:A>G*	4759	19918	1.92	0.28
27:A>G, 28:A>C, 30:G>A	2932	6053	0.95	-0.02	42:C>A, 45:A>G*	9597	43639	2.09	0.32
27:A>T, 28:A>C, 30:G>T*	5086	6751	0.61	-0.21	42:C>G, 45:A>G*	9696	49135	2.33	0.37
27:A>C, 28:A>C, 30:G>T	5450	12751	1.07	0.03	43:T>C*	9503	39639	1.92	0.28
27:A>G, 28:A>C, 30:G>T*	5463	6631	0.56	-0.25	43:T>C, 45:A>G*	7342	39288	2.46	0.39
27:A>C, 28:A>C, 30:G>A	6967	15411	1.02	0.01	43:T>C, 45:A>C*	8064	41261	2.35	0.37
27:A>C, 28:A>C, 30:G>C*	16372	28212	0.79	-0.10	43:T>C, 45:A>T*	11361	44770	1.81	0.26
27:A>G, 30:G>A	18656	46330	1.14	0.06	43:T>C, 45:A>T, 48:T>C*	2525	11256	2.05	0.31
27:A>T, 30:G>A*	46870	27557	0.27	-0.57	43:T>C, 45:A>G, 48:T>C*	7494	39959	2.45	0.39
27:A>C, 30:G>A*	47164	12476	0.12	-0.92	43:T>C, 45:A>C, 48:T>C*	12183	62275	2.35	0.37
28:A>C*	156401	520880	1.53	0.18	43:T>C, 48:T>C*	2715	13250	2.24	0.35
28:A>C, 30:G>A*	59716	195669	1.50	0.18	45:A>G*	11015	49451	2.06	0.31
28:A>C, 30:G>T*	86092	225255	1.20	0.08	45:A>G*, 48:T>C*	31178	138530	2.04	0.31
28:A>C, 30:G>C*	87453	260549	1.37	0.14	48:T>C*	34094	128744	1.73	0.24
28:A>C, 30:G>T, 33:C>T	35527	85210	1.10	0.04	48:T>C, 50:A>G*	25972	100932	1.78	0.25
28:A>C, 30:G>A, 33:C>T*	43630	134625	1.42	0.15	48:T>C, 51:A>G*	7375	656	0.04	-1.39
28:A>C, 30:G>C, 33:C>T*	55156	152696	1.27	0.10	50:A>G*	42309	144136	1.56	0.19
28:A>C, 33:C>T*	105855	314480	1.36	0.13	50:A>G, 54:A>T	3657	6954	0.87	-0.06
30:G>A*	448725	1381667	1.41	0.15	50:A>G, 54:A>C*	11668	41602	1.64	0.21
30:G>A, 33:C>T*	176490	498528	1.30	0.11	50:A>G, 54:A>G*	11677	75474	2.97	0.47
33:C>T*	347154	651408	0.86	-0.06	51:A>G*	14915	856	0.03	-1.58
33:C>T, 34:T>A, 35:C>G, 36:A>C*	17143	12575	0.34	-0.47	51:A>G, 54:A>G*	590	3204	2.49	0.40
33:C>T, 34:T>A, 35:C>G, 36:A>T*	44240	11285	0.12	-0.93	51:A>G, 54:A>C*	4612	942	0.09	-1.03
33:C>T, 36:A>T	10175	19445	0.88	-0.06	51:A>G, 54:A>T*	7808	577	0.03	-1.47
33:C>T, 36:A>C	60282	128984	0.98	-0.01	54:A>G*	1751	12496	3.28	0.52
33:C>T, 36:A>G*	108350	293254	1.24	0.09	54:A>C*	9417	35763	1.74	0.24
*-significant by Fisher's Exact Test; Greyed rows values are 70% or less than WT					54:A>T	9963	18727	0.86	-0.06

ingly, the fitness index-altering mutations were observed across the entire SMN exon 7. This observation suggests that optimization of exon inclusion levels for SMN was achieved at multiple exonic locations, providing a sufficient degree of flexibility to respond to localized coding pressures.

*Single Library Mutations*—When single nucleotide mutations were analyzed we observed both increased and decreased

splicing efficiencies (Fig. 2B). Of the 32 mutations that caused significant decreases in the fitness index value, 6 of them were single nucleotide mutations (Table 1). Several of the single point mutations that caused the largest decreases in the fitness index have previously been reported as prominent splice altering mutations. Of 8 mutations previously evaluated in the literature, all are faithfully reproduced using our pooled deep

**TABLE 2**  
Validated mutations

The mutations in this table have been previously reported in the literature or were validated from individual clones isolated from our library pools. Comparison to our data set shows that previous results are replicated faithfully (compare the similarity in changes between fitness index value or log transform with the previously published inclusion values).

Mutation and position	Inclusion index value	Log inclusion index value	Previously analyzed % inclusion for exon 7	Citations
Wild-type, no mutations	1	0	75–100%	11, 12, 20, 23, 31, 32, see Fig. 2
3:T>G	0.94	-0.03	96%	31
3:T>G, 6:C>T	0.13	-0.88	6%	31
6:C>T	0.39	-0.41	26%	23
6:C>T, 7:A>C	1.19	0.08	100%	26, 32
7:A>C	1.49	0.17	100%	26, 32
27:A>C	0.12	-0.92	46%	See Fig. 2
27:A>T	0.00	-2.40	11%	See Fig. 2
27:A>G	0.84	-0.08	89%	See Fig. 2
27:A>T, 30:G>A	0.27	-0.57	76%	See Fig. 2
27:A>G, 30:G>A	1.14	0.06	93%	See Fig. 2
39:T>C	0.15	-0.81	33%	See Fig. 2
39:T>C	0.15	-0.81	24%	20
39>C, 42:C>A	1.58	0.20	91%	See Fig. 2
39:T>C, 42:C>G	1.85	0.27	85%	See Fig. 2
42:C>A	1.43	0.16	84%	See Fig. 2
42:C>G	1.71	0.23	87%	See Fig. 2
45:A>G	2.06	0.31	93%	31
54:A>G	3.28	0.52	100%	20

sequencing approach (Table 2). Similarly, some of the mutations in our data that increased the fitness index value were previously reported in the literature to either destabilize an inhibitory RNA secondary structures or to increase the strength of the splice sites, *i.e.* alter the sequence to be closer to the splicing consensus sequence as measured by maximum entropy score (Fig. 3) (23). This agreement with previously published work further demonstrates that our deep sequencing approach reliably evaluates changes in splicing efficiencies. For example, when the highly conserved residue at position 6 was mutated to a synonymous codon (cytidine to thymidine), thus emulating the known difference in sequence between *SMN1* and *SMN2*, a 2.6-fold decrease in the fitness index was observed, in agreement with exon inclusion differences observed for *SMN1* and *SMN2* (4, 12, 24–27) (Table 1). Increases in fitness index values due to mutations may also be due to pre-mRNA or mature mRNA stability issues.

Furthermore, single nucleotide mutant influences on the fitness index values are nucleotide specific. For example, changing nucleotide position 27 from an adenosine to thymidine or cytidine caused a ~200- or ~8-fold decrease in exon inclusion, respectively (Fig. 2B, *inset*). However, when mutated to guanosine no significant change in the fitness index was observed.

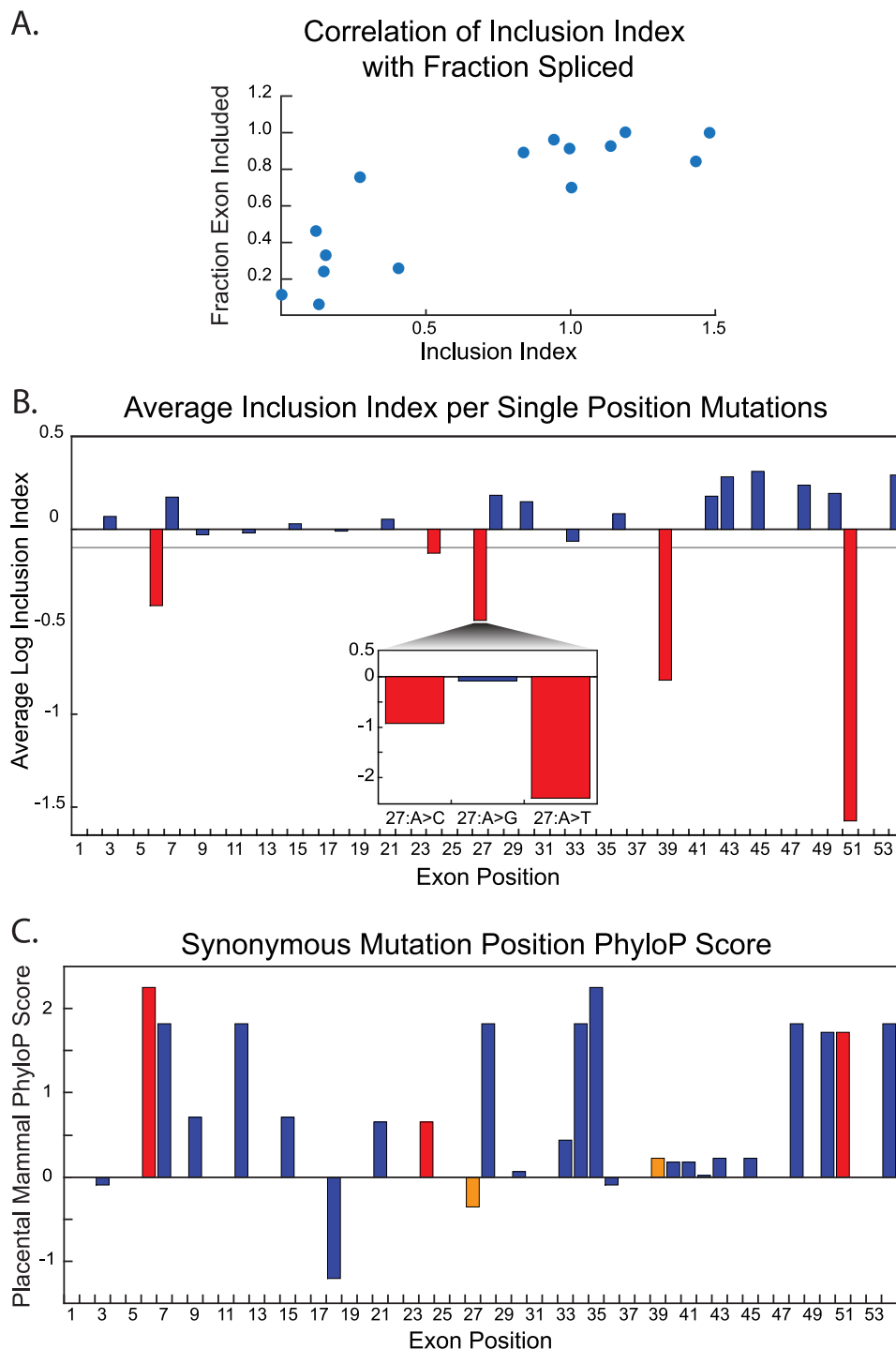
To test the hypothesis that impermissible nucleotide changes at synonymous positions correlate with evolutionary conservation, we compared the mutated positions with their conservation scores as measured by PhyloP (28, 29). PhyloP uses a 46-vertebrate species alignment to assign positive values to positions that are conserved and gives lower or negative values to positions that are rapidly evolving or less conserved positions. Presumably, if a synonymous position is important for splicing, it is expected that its nucleotide identity would be conserved. This expectation is met when evaluating nucleotides at positions 6 and 51 (Fig. 2C). Based on the PhyloP score both positions are highly conserved, which is in agreement with the hypothesis that the nucleotide identity may be evolutionarily fixed due to selective pressures for correct splicing. However, as data from mutations at positions 27 and 39 indicate, this splicing correlated conser-

vation is not typical in our dataset (Fig. 2C). Several of the synonymous mutation positions that had significant fitness index changes exhibit very low PhyloP scores. We conclude that the PhyloP score at a given wobble position does not directly predict the influence of the nucleotide on pre-mRNA splicing.

One potential explanation for the lack of a direct correlation between the PhyloP score and the fitness index value at synonymous positions is the fact that the exon/intron architecture may vary between species (Fig. 4A). As such, the evolutionary pressures to maintain a sequence for a particular splicing pattern can be different between species, eliminating positional conservation restrictions imposed by splicing. Our analysis clearly shows that different nucleotide changes at the same position can range from neutral to high impact, demonstrating that some nucleotide changes are permissible whereas others are not (Fig. 2B, *inset*, and Table 1). As PhyloP only measures the frequency of a nucleotide substitution irrespective of patterns or trends of mutations at a specific position, it is expected that in some cases the conservation score cannot adequately reflect splicing pressures. To circumvent these PhyloP limitations we filtered species alignments based on the requirement of similar intron/exon architecture around *SMN1* exon 7. Using the Ensembl! genomic alignment tool, we hand filtered species alignments based on *SMN* exon 7 architectural conservation. This filtering approach reduced the number of aligned species to 17, demonstrating that even among the more closely related placental vertebrates large differences within the exon/intron architecture exist. The filtered alignments were then evaluated for the presence or absence of synonymous mutations that were introduced by our high-throughput approach (Fig. 4B). Interestingly, none of the synonymous mutations that caused significant decreases in the fitness index were found in this alignment data, whereas synonymous mutants that do not decrease the fitness index value were admissible (Fig. 4B). This perfect correlation between admissible/inadmissible synonymous mutations and their fitness index values suggests that the exon/intron architecture filtered conservation evaluation at synonymous positions can be utilized to determine exon posi-



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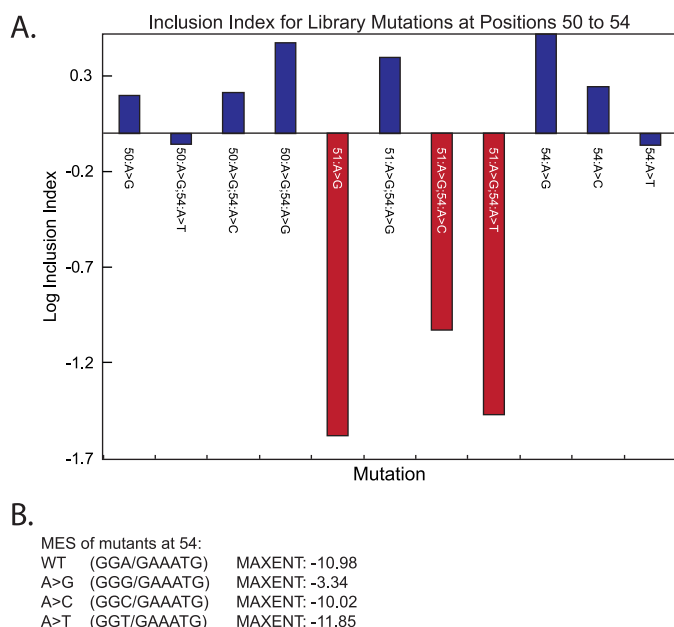


**FIGURE 2. Analysis of library mutants with a single mutation.** *A*, correlation between fitness index values derived from the high-throughput analysis and exon inclusion levels of individual mutants as determined by PCR analysis. *B*, average fitness index values for library mutants with mutations at a single hexamer position. The gray line represents the significance cutoff. Mutations that caused a significant decrease in the fitness index value are highlighted in red. All other single mutant positions are in blue. The inset is an example of the nucleotide-specific mutational effects observed. Specific mutations at a given position can display large differences in the fitness index as illustrated by position 27. *C*, conservation scores at synonymous mutation positions across *SMN1* exon 7 based on placental mammal PhyloP scores. Bars in red denote high conservation at the same position where mutations cause significant decreases in the fitness index. Bars in orange show the positions where conservation is low and where mutations cause significant decreases in the fitness index.

tions important for efficient splicing, at least in the context of *SMN* exon 7.

**Hexamer Library Mutations**—Mutations of more than one nucleotide position within a hexamer appear to either rescue or to exacerbate the fitness index changes seen in the single mutant variations (Fig. 5 and Table 1). By themselves, muta-

tions 24:A>G, and 27:A>C/T cause significant decreases in the fitness index value, but surrounding positions (21, 28, and 30) do not (Fig. 5A). When mutated in combination, synergistic decreases in the fitness index are observed, even in the case of the mutation 24:A>G with 27:A>G (not significant on its own, but significant with position 24). Conversely, when paired with



**FIGURE 3. Analysis of library mutants at the 5' splice site of *SMN1* exon 7/intron 7.** *A*, fitness index values for mutants at the last positions of exon 7. Mutations that caused a significant decrease in the fitness index value are highlighted in red. All other mutations are in blue. *B*, maximum entropy scores for the wild-type (WT) 5' splice site and the mutations at position 54, which is the last exonic nucleotide. Greater maximum entropy score correlate with more efficient splice site usage. The slash in the sequences denotes the exon-intron junction.

mutations at positions 28, 29, or 30, the affects of position 27 are decreased, although not always to the point of being not significant (except in the case of 27:A>C, 30:G>A where there was no appreciable change). These results suggest that neighboring mutations within the context of a hexamer can influence exon inclusion in multiple ways. These data and others (Figs. 3 and 5) suggest that specific internal sequences in the exon are vital for optimal exon inclusion in a hexamer specific context.

The extensive amount of study devoted to the *SMN1* gene allowed multiple built-in controls for our analysis by correlating changes in regions that are known to be important binding sites for splicing regulatory elements with changes observed in the fitness index (Fig. 5C) (4, 13, 24–27, 30–32). At position 3, none of the 3 possible single silent mutations alter the fitness index appreciably (Fig. 5B). However, when combined with the C to T mutation at position 6 exon skipping is exasperated, consistent with the notion that the binding site for splicing regulators are disrupted or that more stable RNA secondary structures are formed (4, 13, 24, 25, 27, 30–32). The same synergy is also observed when mutations in positions 6 and 9 are combined. However, if position 7 is mutated along with positions 6 or 9, the reduction in exon inclusion is no longer significant. This result reproduces the finding that the A to C mutation at position 7 rescues the C to T mutation at position 6 either through inhibiting the binding of Sam68, or by preventing the binding of another inhibitory protein (33, 34). Similar examples of synergistic or compensatory mutations are observed to occur at the Tra2- $\beta$ 1 (SRSF10) binding site (positions 18–28), at the 3' inhibitory secondary structure (positions 33–42), and at the 5' splice site (21, 23, 32, 35) (Figs. 2, 3, and 5, Table 1). These mutations all involve sequence-specific com-

pensatory mutations that can relieve the decrease in the fitness index caused by other mutations.

When multiple mutations within a hexamer are analyzed using the intron/exon architecture filtered alignment method described above, none of the mutations that cause negative changes in the fitness index were represented in organisms with similar intron/exon architecture. The alignment showed 11 possible synonymous mutations, all of which appeared in our analysis as non-significant changes to the fitness index (Fig. 4B). This observation supports the conclusion that splicing-detrimental hexamer mutants are selected against due to splicing-related sequence constraints.

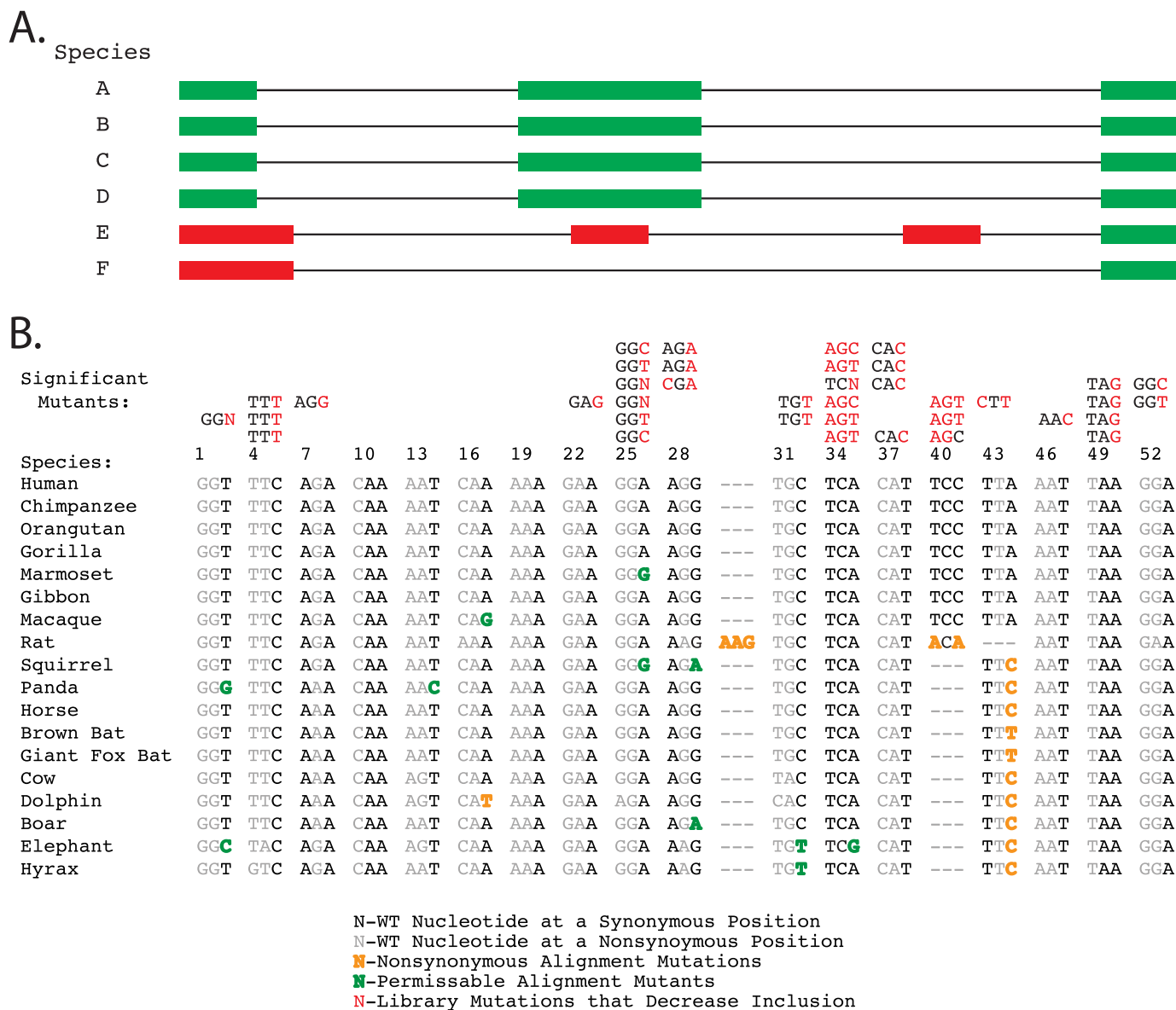
**Analysis of Synonymous SNPs**—The filtered phylogenetic comparison approach may allow the identification of genetic mutations that have a high probability to alter pre-mRNA splicing. To test this hypothesis we applied the modified alignment analysis (Fig. 4A) to evaluate whether disease-associated synonymous SNPs are likely to be associated with inducing changes in pre-mRNA splicing. Forty human disease-associated synonymous SNPs and 40 randomly selected synonymous SNPs from the Ensembl! Perl API were combined as a representation of genomic SNPs in coding regions (20, 36). The genomic sequence within a  $\pm 5$  nt window around the SNP was compared with the homologous sequence of aligned organisms containing similar exon/intron architecture. This sequence window was analyzed for the occurrence of the human SNP nucleotide and surrounding nucleotide changes (supplemental Table S1).

The SNPs were divided into two categories based on the appearance of the evaluated SNP: neutral SNPs and putative splice altering SNPs. There were also non-conserved SNPs that had fewer than 5 organisms in the species alignment after the exon/intron architecture filtering. These cases were not considered further due to limited alignment information. Neutral SNPs were defined as SNPs that were found in the alignment one or more times without additional nucleotide changes in the surrounding sequence. The presence of sequences identical to such human SNPs in other species suggests that they are not selected against and, thus, are likely to be splicing neutral. Putative splice altering SNPs were defined as SNP sequences that did not occur in the alignment or only occurred in conjunction with other mutations in the surrounding hexamer sequence. As was argued by our experimental analysis, such a representation of human SNP sequence across species suggests that nucleotide changes at this position may alter exon inclusion.

In 15 of 80 SNPs there were fewer than 5 organisms that maintained intron/exon architecture conservation (Table 3). Another 36 alignments had multiple occurrences of the human SNP sequence without mutations in the surrounding hexamers, suggesting that these synonymous position mutations were not selected against (Table 3). In 10 of 80 cases the synonymous SNP was not observed in any aligned organism, providing circumstantial evidence that the SNP may alter splicing efficiencies, *i.e.* the SNP is selected against for that particular intron/exon architecture. In 19 cases the SNP mutation was observed in the aligned species, however, only in conjunction with additional nucleotide changes within the 5-nucleotide flanking window (Table 3). Thus, 45% of the SNPs surveyed are potentially



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**FIGURE 4. Sequence alignment of *SMN1* exon 7 with 17 animals with similar intron/exon architecture.** *A*, a graphical representation of the method used to filter species alignments based on conservation of intron/exon architecture. Species E and F would not be included in an architecture-filtered alignment. *B*, the alignment of similar mammals that pass the intron/exon architecture filter to human *SMN1* exon 7. The mutations that caused significant decreases in the fitness index value are listed above their position in the alignment with the specific hexamer mutations in red. The alignment below the hexamers shows the silent mutation positions that diverge from the human sequence. The mutations that did not cause changes in the fitness index value are highlighted in green. The color-coding used to differentiate the effects of the observed mutants is shown in the key on the figure bottom. The category “nonsynonymous alignment mutations” refers to positions in the multispecies alignment where observed nucleotide substitutions would be considered nonsynonymous mutations in the human exon.

splice altering SNPs. These observations suggest that in many cases of sequence evolution, nucleotide changes within the hexamer context are driven by the need to uphold splicing efficiency.

To determine whether synonymous SNPs in coding exons exhibit unique selection pressures, we prepared a dataset of 162 SNPs from noncoding exons or from single exon genes (supplemental Table S1). These exons should either only be under evolutionary selection for correct splicing or, in the case of single exon genes, should only be under selection for optimal protein coding. Because of their lack of protein coding constraints, noncoding exons would be expected to have more freedom to mutate nucleotides throughout the exon, not just in silent posi-

tions. For these exons it is expected that the selection on synonymous substitution positions would be reduced. Single exon genes, on the other hand, should have a different selective pressure on their synonymous mutation positions that are strictly based on protein coding within the reading frame. We analyzed these 162 control SNPs in the same manner as described above and found that single exon and non-coding genes had a significantly lower number of putative splice altering SNPs at synonymous positions, as expected from the relaxation of evolutionary pressures (Table 3). We conclude that applying the exon/intron architecture filtered approach of phylogeny aids in the identification of exonic sequence that may be necessary for exon inclusion.



**TABLE 3**

**Summary of SNPs analyzed by filtering phylogenetic alignments for similar intron/exon architecture**

“Splice altering SNPs” represent SNPs that did not occur in the alignment of organisms with similar intron/exon architecture or, if they occurred, only occurred alongside other mutations within a 6-mer (possible compensatory mutations). “Neutral SNPs” were SNPs that had SNP occurrences without mutations in the surrounding hexamer. The classes of coding exons and exons derived from non-coding genes or single exon genes’ exons were found to be independent by a  $\chi^2$  test ( $p < 0.05$ ). The SNP identities for all groups are listed in supplemental Table S1.

Data set	Splice altering SNPs	Neutral SNPs
Disease associated and random synonymous SNPs	45% (29)	55% (36)
Randomly selected noncoding exonic or single exon SNPs	30% (34)	70% (80)

$p < 0.05$  between the two SNP datasets

prove invaluable in the determination of molecular causes of genetic disease. Our method using splicing centric filtered alignments helps to assign the importance of a particular SNP in changing pre-mRNA splicing.

*Functional Filtering of Alignments to Enrich for Splicing Related Information*—Previous discovery and analysis of splicing regulatory elements and splice altering SNPs relied on conservation data. Our analysis suggests that the limitations of this approach will cause important splicing regulatory positions to be missed. An initial comparison between our mutation outcomes and PhyloP conservation scores did not support the notion that fixation of synonymous exon positions are driven by splicing constraints. In part, this observation is explained by the fact that PhyloP scoring is not able to predict the nucleotide-specific alterations in exon inclusion that occurred in our dataset. Our analysis shows that the use of sequence identity analysis instead of divergence from a specific nucleotide is important when evaluating splicing. Limiting our comparative alignment to similar intron/exon architectures presumably enriched our search space for organisms that regulate exons in a similar fashion, a notion supported by our SNP analysis, which demonstrated that up to 45% of exonic synonymous SNPs are likely to be splice-altering SNPs.

Here, we evaluated the influence of nucleotide identities at synonymous positions on the overall splicing efficiency within the context of the 54-nt long SMN exon 7. We chose this exon mainly because its disease association sparked extensive studies that identified many exonic positions as regulator binding sites that have been shown to be important for splicing, thus providing a knowledge base essential to explain any observed fitness index differences (4, 13, 24–27, 30–32). At 54 nucleotides in length, SMN exon 7 is significantly smaller than the average length of a human exon (~120 nt). Thus, it is possible that the density of splicing regulatory sites embedded within SMN exon 7 is greater than for human exons of average size. Given these considerations, it is possible that average sized human exons may be exposed to different purifying selection pressures.

Synonymous mutations can alter the ability of an mRNA to code for protein through creation of regulatory defects. They have been shown to alter protein-folding abilities, to change RNA stability, and to induce exon loss (8, 41, 44). These observations suggest that selective pressures to maintain efficient splicing work in tandem with selective pressures to uphold all aspects of the genetic code. In our attempt to uncouple these influences we evaluated the influence of synonymous mutations on pre-mRNA splicing. It is also appreciated that a codon usage bias exists. Lower organisms experience a correlation between codon usage and tRNA copy number, suggesting an

optimization for particular codons for efficient translation (45). Interestingly, codon usage in mammals does not correlate with tRNA abundance or tRNA gene copy number, an observation distinctly different from other taxa (8, 45). This deviation, along with this work, suggest that whereas codon bias may influence the final sequence of an exon, it is part of a larger evolutionary picture that includes influences from other processes, such as pre-mRNA splicing, to properly produce functional proteins.

*Author Contributions*—Experimental design was conceived by K. J. H. and W. F. M. Cloning and construction of plasmids was carried out by L. L. and W. H. Library prep and data analysis was done by W. F. M. and A. G. Manuscript preparation was completed by W. F. M. and K. J. H.

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