Alternatively and Constitutively Spliced Exons Are Subject to Different Evolutionary Forces

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There has been a controversy on whether alternatively spliced exons (ASEs) evolve faster than constitutively spliced exons (CSEs). Although it has been noted that ASEs are subject to weaker selective constraints than CSEs, so they evolve faster, there have also been studies that indicated slower evolution in ASEs than in CSEs. In this study, we retrieve more than 5,000 human-mouse orthologous exons and calculate the synonymous (KA) and nonsynonymous (KS) substitution rates in these exons. Our results show that ASEs have higher KA values and higher KA/KS ratios than CSEs, indicating faster amino acid–level evolution in ASEs. The faster evolution may in part be due to weaker selective constraints. It is also possible that the faster rate is in part due to faster functional evolution in ASEs. On the other hand, the majority of ASEs have lower KS values than CSEs. With reference to the substitution rate in introns, we show that the KA values in ASEs are close to the neutral substitution rate, whereas the synonymous substitution rate in CSEs has likely been accelerated. The elevated synonymous rate in CSEs is not related to CpG dinucleotides or low-complexity regions of protein but may be weakly related to codon usage bias. The overall trends of higher KA and lower KS in ASEs than in CSEs are also observed in human-rat and mouse-rat comparisons. Therefore, our observations hold for mammals of different molecular clocks.

Introduction

In complex organisms such as mammals, the frequency of alternative splicing (AS) is high. Genomic studies have suggested that as high as 40–60% of human genes undergo AS (Mironov, Fickett, and Gelfand 1999; Kan et al. 2001; Modrek et al. 2001; Kan, States, and Gish 2002). AS has been shown to be associated with nonsense-mediated decay (Wachtel et al. 2004; Stamm et al. 2005), programmed cell death (Wu, Tang, and Havlioglu 2003), and many other important biological processes. Some AS events were shown to be highly related to human diseases (Orban and Olah 2003; Sazani and Kole 2003; Garcia-Blanco, Baraniak, and Lasda 2004; Rossi 2004; Venables 2004). Therefore, AS has become an important topic in a variety of fields such as oncology, molecular medicine, and developmental biology.

In evolution, AS is thought to be one of the major mechanisms of increasing transcriptome complexity (Hanke et al. 1999; Modrek and Lee 2002). It allows the generation of different transcript/protein isoforms from the same genes, thus increasing functional diversity of proteome without increasing the number of genes. Several studies have suggested that AS plays a major role in genome evolution because of relatively weaker negative selection pressure (Kan, States, and Gish 2002; Boue, Letunic, and Bork 2003; Modrek and Lee 2003; Xing and Lee 2004). Because new gene functions may arise from insertion or deletion of an exon, it was suggested that alternatively spliced exons (ASEs) can accelerate gene evolution. This hypothesis has been supported by recent studies. Examples include the following: (1) a significant proportion of ASEs is species specific and not conserved between human and rodents in contrast with high conservation of orthologous gene structures between these species (Nurdinov et al. 2003); (2) AS is associated with exon gain or loss events, implying faster evolution in ASEs than in constitutively spliced exons (CSEs) (Modrek and Lee 2003); (3) ASEs tend to insert or delete complete protein domains more frequently than expected by chance, leading to increase in functional diversity (Kriventseva et al. 2003); and (4) ASEs tend to have higher KA/KS (nonsynonymous to synonymous substitution rate) ratios than CSEs as revealed from comparison of orthologous exons between human and other species (Iida and Akashi 2000; Hurst and Pel 2001; Filip and Mundy 2004; Xing and Lee 2005a, 2005b). However, it has also been suggested that ASEs evolve at a slower pace than CSEs. Indeed, ASEs have been found to be better conserved than CSEs (Sorek and Ast 2003; Sorek et al. 2004; Sugnet et al. 2004), to be under stronger selection to conserve reading frame (Resch et al. 2004), and to have fewer single-nucleotide polymorphisms (Yeo et al. 2005). It was also observed that the introns flanking ASEs are better conserved than introns flanking CSEs (Sorek and Ast 2003; Philippis, Park, and Graveley 2004), implying that ASEs are under stronger selection pressure than CSEs. In addition, Cusack and Wölfe (2005) suggested that genes undergoing AS by exon skipping were more constrained than the genome average. In view of these conflicting conclusions, we conducted an extensive analysis to compare evolutionary rates in ASEs and CSEs and to explore possible selection forces that underlie ASE evolution.

Materials and Methods

Retrieval of CSEs and ASEs

Human CSEs and ASEs were available from the online database ASAP (the Alternative Splicing Annotation Project [Lee et al. 2003]) at http://www.bioinfo.mbi.ucla.edu/ASAP/. By mapping the ASAP-provided homolog table to the ASAP genomic data set or the corresponding mouse UniGene expressed sequence tag (EST) sequences (ftp://ftp.ncbi.nih.gov/repository/UniGene/, March 2005),
4,630 human CSEs and their orthologous mouse exonic sequences were extracted (Fig. 1).

Because ASEs that have been conserved between human and mouse were not available from ASAP, we BlastN aligned the human ASE plus two flanking exons against the whole mouse UniGene EST database to identify the orthologous mouse counterparts of human ASEs (Fig. 1). Mouse exons that had a ≥70% sequence identity to the full lengths of human exon queries were extracted. A total of 788 human ASEs, including 512 major-form exons, 21 minor-form exons, and 255 undetermined-form exons, were paired with their mouse orthologs. The classification of major-form (included in at least two-thirds of the EST counts), minor-form (skipped in at least two-thirds of the EST counts), and undetermined-form (in the intermediate case or ≤5 ESTs in total) exons was retrieved from ASAP. Because only a small number of minor-form exons were available, we grouped minor-form exons with undetermined-form exons to form nonmajor-form (NM) exons. In addition, orthologous human-rat and mouse-rat exon pairs, including CSEs and ASEs, were identified based on BlastN alignments between human/mouse exons and the rat UniGene database. The sequences of exons analyzed in this study are available at http://www.sinica.edu.tw/~trees/ASE_CSE/ASE_CSE.htm.

Retrieval of Pure (Constitutive) Introns

Human-mouse orthologous introns that did not overlap with any known human transcripts (the “pure” or “constitutive” introns) were extracted from the University of California, Santa Cruz (UCSC) Genome Browser at http://hgdownload.cse.ucsc.edu/ goldenPath/hg17/multiz8way/. Sequences with lower than 70% identity were excluded. The human intronic sequences in the remaining human-mouse sequence alignments were then matched to the Human Gene Index (HGI) Release 15 from the TIGR database (The Institute of Genome Research; http://www.tigr.org/tdb/tgi) using the CRASA program (Chuang et al. 2003). Introns that were matched to HGI entries were excluded because they might, in fact, be ASEs. The flanking exons of these pure introns were further examined to reconfirm that they were conserved between human and mouse. By doing so, we could be confident that the introns retrieved were true orthologous introns between human and mouse.

Computation of Divergence at Fourfold Degenerate Sites

We extracted fourfold degenerate sites from human CSEs, major-form exons, NM exons, and their mouse counterparts. To exclude the effect of CpG dinucleotides, only sites that were neither preceded by a “C” nor followed by a “G” (“non-CpG–prone sites”) were considered. The extracted sites of the three exon types from human and mouse were submitted to the BASEML program of the PAML package (Yang 1997; Yang and Nielsen 2000) for calculation of genetic distance.

Computation of \( K_A, K_S, K_A/K_S, K_r, \) and \( K_i \) Values

For the \( K_d/K_S \) ratio analysis of orthologous exon pairs, two procedures were performed: (1) detecting reading frames of human protein-coding exons by BlastX aligning these exons against the corresponding RefSeq protein sequences (ftp://ftp.ncbi.nih.gov/ genomes/H_sapiens/protein/) and (2) calculating \( K_A, K_S, \) and \( K_d/K_S \) values using the YN00 program of the PAML package (also see Fig. 1). The substitution rates of human-mouse orthologous exons (\( K_r \) values of ASEs and CSEs) and pure introns (\( K_i \) value) were measured using the TN93 model implemented in the BASEML program of the PAML package.
Different Transversion/Transition Ratios in ASEs and CSEs

Changes (while transitions tend to be synonymous changes), CSEs. Because transversions are mostly nonsynonymous

Similar, NM exons have the highest average transversion to
degenerate sites. CSEs have the high-

tions, were retrieved.

We detected protein domains using the InterProScan
package and the INTERPRO resource (Mulder et al. 2005;
ac.uk/InterProScan/index.html and ftp://ftp.ebi.ac.uk/pub/
databases/interpro/prscan/, respectively). The globular do-

cency, shows the same tendencies as

Table 1 shows the basic features of the exons retrieved
in this study. It is apparent that lengths, average sequence
identities, and GC contents differ only slightly from each
other among the three exon types. The same comment
applies to the percentages of CpG dinucleotides and their
derivatives (TpG and CpA). However, for GC contents at
fourfold degenerate sites and substitution rates at non-
CpG–prone fourfold degenerate sites, CSEs have the high-
est values, while NM exons have the lowest, but the reverse
trend is observed for effective number of codons. Mean-
while, the percentage of exons with length divisible by three
is the highest in NM exons and the lowest in CSEs. Simi-
larly, NM exons have the highest average transversion to
transition ratio, followed by major-form exons and then by
CSEs. Because transversions are mostly nonsynonymous changes
(while transitions tend to be synonymous changes), different
transversion/transition ratios in ASEs and CSEs
can be associated with different \( K_A \) and \( K_S \) values in these
exons. Indeed, our results show that the largest average and
median \( K_A \) values occur in NM exons, followed by major-
form exons, and then by CSEs, whereas the reverse is true for
the \( K_S \) values (Fig. 2A and B and Table 1). Interestingly,
despite the fact that the median (average) \( K_S \) value is lower in
ASEs than in CSEs, the median (average) \( K_A \) is higher in
ASEs. Note that the differences of \( K_A \), \( K_S \), and \( K_A/K_S \) be-
tween CSEs and ASEs are all significant, while the differences
between major-form and NM exons are not (Table 2).

It is possible that selection of data sets may affect our
results. For example, if the ASEs retrieved happen to be
biased toward fast-evolving exons, it is then not surprising
to observe higher \( K_A \) values in ASEs than in CSEs. To address
this possibility, we retrieved human-rat orthologous ASEs and
CSEs for the same evolutionary analysis. As shown in
Table 3, substitution rates derived from human-rat ortho-
logous exons have similar trends as observed in the
human-mouse comparison. That is, ASEs have higher me-
dian \( K_A \) values and higher \( K_A/K_S \) ratios but lower median \( K_S \) values than CSEs. The probability of observing the same
trends from two biased data sets appears to be small. There-
fore, our results are very likely unbiased. Furthermore, be-
cause rodents have a faster molecular clock than primates
(Li 1997; Nekrutkenko, Chung, and Li 2003), comparison of
human-mouse (or human-rat) orthologous exons might
yield tendencies that would not hold in comparisons of spe-
cies with similar molecular clocks. However, an analysis
using orthologous exons from mouse and rat, which have
similar molecular clocks, shows the same tendencies as
above. Therefore, the trends of higher \( K_A \) and lower \( K_S \) values
in ASEs than in CSEs are not affected by species sele-
ction or different molecular clocks. Our estimated
median \( K_A/K_S \) ratio between mouse and rat is lower than
that reported in the rat genome analysis (Gibbs et al.
2004). Note that mouse-rat homologous genes were used to
derive \( K_A/K_S \) ratios in the rat genome analysis, whereas

### Table 1

| Basic Properties and Evolutionary Features (\( K_A, K_A, K_S, \) and \( K_A/K_S \) values) of the Retrieved Human-Mouse Orthologous Exons |
|------------------|-----------------|-------------------|
|                  | CSEs            | Major             | Nonmajor          |
| Number of exons analyzed | 4,630           | 512               | 276               |
| Median length (mean) (bp)  | 115 (124)       | 111 (117)         | 110 (119)         |
| Average sequence identity (%) | 88.35          | 89.07             | 89.22             |
| GC content (%)          | 51.09           | 51.09             | 50.41             |
| Average CpG, TpG, and CpA dinucleotides content (%) | 19.18           | 18.97             | 18.64             |
| GC content at fourfold degenerate sites (%) | 57.54           | 55.56             | 54.70             |
| Substitution rate at non-CpG–prone fourfold degenerate sites (%) | 0.394           | 0.344             | 0.314             |
| Effective number of codons | 53.7           | 54.6              | 54.8              |
| Percentage of exon length divisible by 3 (%) | 37.75           | 42.19             | 48.91             |
| Average transversion/transformation ratio | 0.475           | 0.518             | 0.564             |
| Median \( K_A \) value (mean) | 0.130 (0.132)  | 0.122 (0.123)     | 0.118 (0.120)     |
| Median \( K_A \) value (mean) | 0.026 (0.040)  | 0.033 (0.046)     | 0.043 (0.062)     |
| Median \( K_A \) value (mean) | 0.588 (0.873)  | 0.468 (0.632)     | 0.447 (0.605)     |
| Median \( K_A/K_S \) ratio (mean) | 0.037 (0.091)  | 0.096 (0.144)     | 0.098 (0.181)     |

*Because large \( K_A \) values and \( K_A/K_S \) ratios may be methodologically suspect, \( K_A \) values >10 and \( K_A/K_S \) ratios >10 were not considered (Hilfier et al. 2004) when we calculated their average values.*
only well-annotated exons are used in this study. That is, only exons that are well defined to be alternatively or constitutively spliced are included in this study. Inclusion of less well-annotated exons, predicted genes, or fast-evolving genes may result in elevated estimates of $\frac{K_A}{K_S}$ ratios. Notwithstanding the difference in $\frac{K_A}{K_S}$ ratio estimates between different studies, the overall trend of higher $\frac{K_A}{K_S}$ ratios in ASEs than in CSEs holds well in human-rodent and mouse-rat comparisons, in agreement with previous studies (Xing and Lee 2005).

Our estimates of $K_A$ values are smaller than those observed in previous studies (Waterston et al. 2002). The reason is that a large portion (>60%) of exons (including CSEs and ASEs) analyzed in this study encode for protein domains (data not shown). Regions with domains tend to have lower $K_A$ values than regions not containing domains (Waterston et al. 2002). It is emphasized that the exons analyzed in this study are well-annotated ASEs and CSEs. We recognize that a large number of exons are not included in this study because they are not well annotated and cannot be reliably classified into ASEs and CSEs.

### Substitutions at Synonymous Sites

There are two possible explanations for a lower synonymous rate in ASEs than in CSEs: CSEs and ASEs have different mutation rates or they are under different selection pressures. The first explanation has the intriguing implication that the mutation rate varies among regions of a gene. However, this scenario requires a mechanism to distinguish CSEs from ASEs for different mutation rates to occur. Because the GC content, length, and Homo-Mus sequence identity of the two exon types are highly similar (Table 1) and because ASEs and CSEs are located in the same genomic region, it is likely that ASEs and CSEs mutate at similar rates. Therefore, we suggest that different selection pressures have operated on ASEs and CSEs, leading to different $K_S$ values in the two exon types. Under this scenario, the synonymous rate is either reduced in ASEs or elevated in CSEs.

To determine which of the two possibilities is more probable, we retrieved ~110,000 human-mouse orthologous introns that do not include any ASE or EST match (i.e., pure or constitutive introns) from the UCSC Genome Browser and calculated the substitution rates (see Materials and Methods).

![Figure 2](https://academic.oup.com/mbe/article-abstract/23/3/675/1110436)

**Fig. 2.**—The cumulative distributions of human-mouse orthologous CSEs, ASEs (major-form exons), and ASEs (NM exons) are plotted against (A) the $K_A$ values, (B) the $K_S$ values, and (C) the $K_A/K_S$ ratios.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>CSEs versus Major-Form ASEs</th>
<th>CSEs versus NM ASEs</th>
<th>Major-Form Versus NM ASEs</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_e$</td>
<td>$P &lt; 0.002$</td>
<td>$P &lt; 0.002$</td>
<td>$P &gt; 0.1$</td>
</tr>
<tr>
<td>$K_A$</td>
<td>$P &lt; 0.006$</td>
<td>$P &lt; 0.002$</td>
<td>$P &gt; 0.1$</td>
</tr>
<tr>
<td>$K_S$</td>
<td>$P &lt; 1 \times 10^{-5}$</td>
<td>$P &lt; 1 \times 10^{-6}$</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>$K_A/K_S$</td>
<td>$P &lt; 1 \times 10^{-6}$</td>
<td>$P &lt; 1 \times 10^{-6}$</td>
<td>$P &gt; 0.05$</td>
</tr>
</tbody>
</table>

### Table 3

**Evolutionary Features of Human-Rat and Mouse-Rat Orthologous Exons**

<table>
<thead>
<tr>
<th></th>
<th>CSEs</th>
<th>Major</th>
<th>Nonmajor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human-rat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of exons analyzed</td>
<td>622</td>
<td>117</td>
<td>68</td>
</tr>
<tr>
<td>Median $K_A$ value</td>
<td>0.021</td>
<td>0.034</td>
<td>0.039</td>
</tr>
<tr>
<td>Median $K_S$ value</td>
<td>0.623</td>
<td>0.486</td>
<td>0.437</td>
</tr>
<tr>
<td>Median $K_A/K_S$ ratio</td>
<td>0.030</td>
<td>0.061</td>
<td>0.082</td>
</tr>
<tr>
<td>Mouse-rat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of exons analyzed</td>
<td>3,774</td>
<td>382</td>
<td>176</td>
</tr>
<tr>
<td>Median $K_A$ value</td>
<td>0.007</td>
<td>0.014</td>
<td>0.019</td>
</tr>
<tr>
<td>Median $K_S$ value</td>
<td>0.192</td>
<td>0.181</td>
<td>0.179</td>
</tr>
<tr>
<td>Median $K_A/K_S$ ratio</td>
<td>0.016</td>
<td>0.070</td>
<td>0.092</td>
</tr>
</tbody>
</table>
Many ASEs Are Under Strong Selection Pressure

Despite the tendency of fast evolution in ASEs, as high as ~30% of ASEs have a small $K_A$ (~0.02), and the distribution curves of the three exon types are barely distinguishable for the 30% of exons (Fig. 2A). A similar trend is also observed in the cumulative distributions of $K_{A}\!/K_{S}$ ratios (Fig. 2C), with the three curves diverging from each other at ~35% cumulative proportion (or $K_{A}\!/K_{S} = 0.05$). These observations indicate that a significant fraction of ASEs have very low rates of evolutionary changes and are under strong negative selection. It is likely that these ASEs may be alternative conserved exons (i.e., ASEs that are conserved between the compared species [Yeo et al. 2005]) and may have important biological functions so that nonsynonymous base substitutions are repressed in these exons. Note that the exons analyzed in this study are conserved between humans and mice. Therefore, these exons may be more conserved than newly gained (or species specific) ASEs (Cusack and Wolfe 2005). Newly added exons may be under positive selection (or relaxed negative selection) and develop new functions. Overall, our results reveal that more than 60% of the three exon types have different $K_A$ and $K_{A}\!/K_{S}$ ratios (Fig. 2A and C), indicating that the majority of CSEs, major-form exons and NM exons are under different selection pressures, which may have been caused by changes of protein-level selection pressure, translational selections, and selections at silent sites (Iida and Akashi 2000; Hurst and Pal 2001).

Discussion

Our results indicate that ASEs have lower synonymous rates but higher nonsynonymous rates than CSEs. The elevated $K_{A}\!/K_{S}$ ratio in ASEs implies that ASEs may have contributed more to protein diversity than CSEs during mammalian evolution. Our results also show that the synonymous substitution rates in ASEs are close to those of pure introns. We therefore suggest that the synonymous rates in ASEs are close to neutral rates. Although introns are known to contain sequences that are subject to functional constraint (Nobrega et al. 2003, 2004; Rastegar et al. 2004; Ovcharenko et al. 2005), it has been estimated that intronic sequences that are under selection pressure occupy only a small fraction of introns (Keightley and Gaffney 2003). Moreover, previous studies indicated that introns were, in general, subject to less selection pressure than exons (Li 1997). In other words, evolutionary rates of introns are closer to neutral than those of exons. Therefore, it should be reasonable to infer that synonymous rates in ASEs are close to neutral rates, while those in CSEs are accelerated. Furthermore, with a large number of pure introns (~110,000) analyzed, our estimates of substitution rates of human-mouse orthologous pure introns, which are similar to the results from previous studies (Castresana 2002), should be representative. Our conclusion is in agreement with the previous observation of a higher synonymous substitution rate in coding exons than the substitution rate in introns (Moriyama and Powell 1996; Cargill et al. 1999; Halushka et al. 1999; Zwick, Cutler, and Chakravarti 2000; Subramanian and Kumar 2003). Because ASEs have a dual role of exon and intron, it is expected that the synonymous substitution rate of ASEs falls in between the substitution rates of CSEs and introns. Moreover, it has been suggested that introns flanking ASEs are more conserved than those flanking CSEs (Sorek and Ast 2003; Sorek et al. 2004; Sugnet et al. 2004). Although we suggest that the synonymous substitution rate in CSEs is accelerated, there has not been direct evidence to relate the $K_{S}$ values in CSEs and the nucleotide substitution rates in introns flanking CSEs. Because exons and introns are under different selection pressures and have different mechanisms of evolution, it is likely that CSEs and their flanking introns evolve at different paces.

On the other hand, previous studies indicated that synonymous changes were not neutral (Hurst and Pal 2001; Pagani, Raponi, and Baralle 2005) and that regulatory signals might reside in silent sites (Fairbrother et al. 2004a, 2004b; Wang et al. 2004). These observations may not hold for ASEs because it is possible that exonic splicing enhancers are more abundant in CSEs than in ASEs. Although
a previous study suggested that ASEs had more potential regulatory sequences than CSEs (Itoh, Washio, and Tomita 2004), these sequences have not been experimentally validated. In addition, the exact contents of regulatory sequences in exons remain unclear, making it difficult to infer the influences of these sequences on overall evolutionary rates. At any rate, given the approximate neutrality of substitutions in pure introns, we may tentatively conclude that the synonymous substitution rates in ASEs are close to neutral rates.

It has been suggested that synonymous substitution rates in ASEs are lower than those in CSEs due to selection for conserved AS regulatory signals (Baek and Green 2005; Xing and Lee 2005a). However, the higher nonsynonymous substitution rates in ASEs appear to be inconsistent with the hypothesis of regulatory signal conservation in ASEs. Because the numbers of nonsynonymous sites are, in general, larger than those of synonymous sites, it seems more probable that regulatory signals fall in nonsynonymous sites than in synonymous sites. Therefore, we suggest that the lower $K_s$ values in ASEs than in CSEs result from accelerated synonymous substitution rates in CSEs.

It is not clear why the synonymous rate is elevated in CSEs. A previous study indicated that GC-ending codons are more abundant in CSEs than in ASEs (Iida and Akashi 2000), implying different codon usage patterns between these two exon types. We used the DnaSP program (J. Rozas and R. Rozas 1999) to estimate codon usage bias in human-mouse orthologous exons. Our results showed that CSEs have a slightly smaller effective number of codons and higher GC contents at fourfold degenerate sites than ASEs. Because the differences are not very large, codon usage bias may account for just part of the difference in $K_s$ values between ASEs and CSEs. Another possible cause of the substitution rate difference is different contents of highly mutable CpG dinucleotides. However, our results show that the contents of CpG dinucleotides are similar in CSEs and ASEs (Table 1). Moreover, our analysis on non-CpG-prone fourfold degenerate sites indicates that CSEs indeed have a higher substitution rate at these sites than ASEs (Table 1), supporting the view that the elevation in the CSE synonymous rate is not related to CpG dinucleotides.

Because the ASEs analyzed in this study are conserved in the genomes of humans and mice, it is very likely that they were derived from the common ancestor of humans and rodents. In other words, these ASEs either were already alternatively spliced in the common ancestor or they changed from CSEs to ASEs after the human-rodent divergence. It is likely that these ASEs are subject to weaker functional constraint than CSEs because they are usually not involved in protein domains, as revealed in previous studies (Kriventseva et al. 2003; Xing, Xu, and Lee 2003; Cline et al. 2004; Yeo et al. 2005). Therefore, ASEs may be allowed to evolve and develop new functions without disrupting the original protein structures. From this viewpoint, positive selection may have played a significant role in the evolution of these ASEs.

In summary, our study suggests that ASEs and CSEs are subject to different evolutionary forces. The elevated nonsynonymous substitution rate in ASEs may have contributed to functional divergence in mammalian evolution.

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**Literature Cited**


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