

*Review Letter***Intron-dependent evolution: preferred types of exons and introns**

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Exon insertions and exon duplications, two major mechanisms of exon shuffling, are shown to involve modules that have introns of the same phase class at both their 5'- and 3'-ends. At the sites of intronic recombinations exon insertions and duplications create new introns which belong to the same phase class as the recipient introns. As a consequence of repeated exon insertions and exon duplications introns of a single phase class predominate in the resulting genes, i.e. gene assembly by exon shuffling is reflected both by this nonrandom intron phase usage and by the correlation between the domain organization of the proteins and exon-intron organization of their genes. Genes that appeared before the eukaryote-prokaryote split do not show these diagnostic signs of exon shuffling. Since ancestral introns (e.g. self-splicing introns) did not favour intronic recombination, exon shuffling may not have been significant in the early part of protein evolution.

Molecular evolution; Exon shuffling

**1. INTRODUCTION**

Soon after the discovery of split genes it was realised that recombination in introns could provide a mechanism for the shuffling of exonic sequences, and in this way produce new genes from parts of old ones [1]. According to one widely accepted view, introns are the relics of the primordial assembly of genes from pieces: introns were present in the genomes of the common ancestors of prokaryotes and eukaryotes, but prokaryotes lost their introns in a process that eliminated noninformational DNA [2]. In contrast, the selfish DNA or transposon theory of the origin of introns assumes that eukaryotes evolved from prokaryotes lacking

split genes and that in the eukaryotic lineage introns were inserted into previously intact genes [3,4]. Since the debate over the origin of introns is by no means settled, there is no a priori way of deciding whether an intron did participate in the assembly of a gene or was inserted later. This ambiguity in the interpretation of data frequently makes it difficult to reconstruct the ancestral exon-intron structure of genes and to decide whether exon-shuffling events contributed to their formation.

The evolution of plasma proteases provides one of the few examples where evidence for exon reassortments is unequivocal. The noncatalytic regions of the proteases of blood coagulation and fibrinolysis are known to consist of different combinations of kringle-, growth factor-, finger- and calcium-binding modules [5]. Structures homologous to these domains have been shown to occur in fibronectin [6,7], epidermal growth factor precur-

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sor [6,8] and LDL receptor [9], suggesting exchange of modules between different protein families. Recently we have proposed a detailed evolutionary scenario for the assembly of the non-catalytic region of proteases from these modules [5]. It was shown that the modules which had evolved outside the family of serine proteases were inserted individually between the signal-peptide domain and the zymogen-activation domain of an ancestral trypsin-like protease and subsequent duplication and exchange of modules led to the present diversity of the noncatalytic chains. It was pointed out that exon shuffling played an important role in the frequent reassortment of modules as in the genes the modules are usually separated by introns. Recent data suggest that the non-catalytic regions of the serine proteases of the complement cascade were also assembled from modules [10] in a way similar to that proposed for the proteases of blood coagulation and fibrinolysis.

The gene structures of a large number of proteins harbouring the modules of plasma proteases are already known, permitting the reconstruction of the ancestral exon-intron structure of these modules in order to learn more about the finer details of the exon-shuffling process. Analysis of the complex evolutionary history of these modules has revealed some general rules of exon shuffling.

## 2. DEFINITIONS

### 2.1. *Phase classes of introns*

Introns of protein genes were classified according to their position relative to the reading frame of the genes [11]: (i) introns present in the 5'- or 3'-noncoding regions of the primary transcripts; (ii) introns lying between the first and second nucleotides of a codon (phase 1 intron); (iii) introns lying between the second and third nucleotides of a codon (phase 2 intron); (iv) introns lying between two codons (phase 0 intron).

### 2.2. *Homology of introns*

Introns are considered homologous if they can be shown to be derived from the same ancestral intron. Since the nucleotide sequence of introns drifts rapidly, sequence comparison can reveal similarity only between introns of genes that diverged recently. Homology of some introns,

however, may be recognized simply from their position in the sequence: they lie in the same position of the aligned sequences of homologous genes or gene segments and split the reading frame in the same phase. The position of homologous introns may differ since numerous cases of alternative splicing illustrate that splice-junction sliding can insert or delete large peptide segments, shifting the position of the intron, but the original intron phase is always maintained [12-16]. Another group of alternative splicing indicates that optional insertion or skipping of exons can divide or fuse introns but the newly created introns always belong to the same phase class as their progenitor(s) [17-22].

In summary, the position and even the number of homologous introns can change during evolution, but the intron phase class appears to be a more conservative trait. Homologous introns shifted in position by one of the above mechanisms are expected to coincide with gaps in the aligned sequences, but to be of identical phase.

The probability of changing intron phase by splice-junction sliding is much less than that of sliding only one of the exon-intron boundaries: such a change would assume that both splice junctions of the intron undergo simultaneous and compensatory changes of intron phase to avoid disruption of the reading frame.

## 3. BIAS IN THE CHOICE OF EXONS

A common feature of all the modules that participated in the assembly of the noncatalytic regions of the proteases of the fibrinolytic, blood coagulation and complement cascades is that phase 1 introns are found at both their 5'- and 3'-boundaries (table 1).

If we classify exons (or exon sets) with respect to the position of their 5'- and 3'-splice junctions in the reading frame we arrive at exon classes that differ markedly in their versatility in exon shuffling. Exons which have introns of the same phase class at both their ends (symmetrical exons of classes 1-1, 2-2 and 0-0) are the only ones that can be inserted into introns (of the same phase class), can undergo tandem duplication into adjacent introns, or can be deleted by intronic recombination (fig.1). 'Nonsymmetrical exons' if inserted, duplicated or deleted by intronic recombination would disrupt the reading frame. It seems thus certain that the

Table 1

Modules of plasma proteases and related proteins

	Phase class of introns at the boundaries of the module		References
	5'	3'	
<b>Growth factor module</b>			
F IX (2), F X (2), PC (2)	1	1	23-26
u-PA (1), t-PA (1)	1	1	27-29
EGFP (9), LDLR (3)	1	1	30-31
PZ (2), F VII (2)		ND	32-33
Clr(1), C9 (1), F XII (2)		ND	10,34-35
<b>Calcium-binding module</b>			
F IX (1), F X (1)	1	1	23-26
PC (1), PT (1)	1	1	23-24
<b>Kringle-module</b>			
u-PA (1), t-PA (2)	1	1	27-29
PT (2)	1	1	36
FN (2)	1	1	37
PL (5), F XII (2), BSF (2)		ND	35,38-39
<b>Finger-module</b>			
t-PA (1)	1	1	29
FN (12)	1	1	37
F XII (1)		ND	35
<b>Complement B module</b>			
IL (2)	1	1	17
Hp (2)	1	1	40
Clr (2), B (3), C2 (3)		ND	10,41-42
H (20), F XIII (10)		ND	43-44
$\beta_2$ -GP (4), C4BP (8)		ND	45-46
<b>LDL receptor module</b>			
LDLR (7)	1	1	9,30-31
C9 (1)		ND	34
<b>Fibronectin type III module</b>			
FN (15)	1	1	12-14,22,47

Abbreviations: F VII, F IX, F X, F XII, F XIII, factors VII, IX, X, XII, XIII; PC, Pz, protein C and protein Z; PT, prothrombin; PL, plasminogen; u-PA, t-PA, urokinase- and tissue-type plasminogen activator; EGFP, epidermal growth factor precursor; LDLR, LDL

frequency with which the growth factor-, kringle-, finger, complement factor B, LDL receptor and fibronectin type III modules were reshuffled and duplicated during evolution (cf. table 1) has been due to this special feature of the symmetrical exons (exon sets) that carried the ancestors of these modules.

It is noteworthy that of the three symmetrical exon classes always exons of the 1-1 class were chosen during the evolution of plasma proteases. A plausible explanation for this bias is that the phase 1 intron present between the signal-peptide and zymogen-activation domains of the ancestral protease gene was a good recipient only for exons of the 1-1 class. Since this insertion and all subsequent insertions and duplications divided and thus caused the proliferation of phase 1 introns, the 'affinity' of the noncatalytic regions for further symmetrical 1-1 exons has been increased continuously. This bias in the choice of exons eventually led to gene structures in which all inter-module introns belong to the phase 1 class.

#### 4. BIAS IN THE SELECTION OF PARTNERS FOR EXCHANGE OF EXONS

The evolutionary significance of exon shuffling mainly lies in its ability to exchange exons between nonhomologous genes. Illegitimate intronic recombination between unrelated genes may be aided by the middle repetitive sequences present throughout the genome (including introns) since alignment of these sequences can favour the encounter of unrelated genes and exchange of their exons [1]. The evolutionary history of plasma proteases, however, indicates that exon shuffling does not mean a random sampling of the total exon pool of the genome. In many cases it was clear that exon exchange occurred between genes that

receptor; FN, fibronectin; BSF, bovine seminal fluid protein PDC-109; Hp, haptoglobin; IL, interleukin 2 receptor; C1r, B, C2, H, C9, complement components C1r, B, C2, H and C9; C4BP, complement C4b binding protein;  $\beta_2$ -GP,  $\beta_2$ -glycoprotein I. The numbers in parentheses following the abbreviations of proteins indicate the number of internal repeats of the given module in that protein. For definition of phase class of introns see text. ND indicates that the exon-intron structure of the genes has not yet been determined

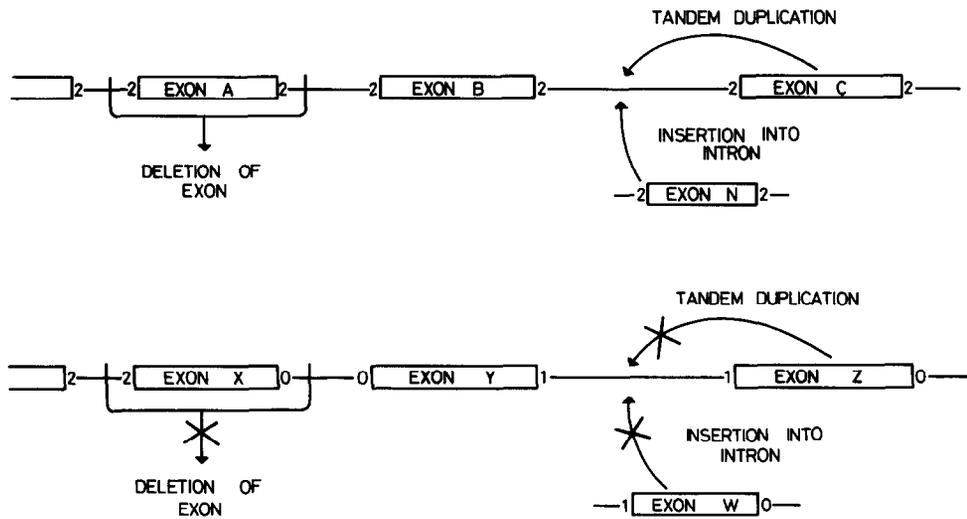


Fig.1. Intron phase and exon shuffling. Only exons that have introns of the same phase class at their 5'- and 3'-ends can be inserted, deleted or duplicated by intronic recombination without disrupting the reading frame. The numbers indicate the phase class of splice junctions.

already shared some homologous exon sequences [5]. The explanation for this bias in the choice of partners for exon exchange is that the preexisting homology of genes increases the chances of their alignment and thus favours further exchange of exons. Since this implies a saltatory effect whereby exchange between members of two gene families will lead to more exchanges between these gene families, exon shuffling among members of a selected group of gene families is strongly preferred. The genes of the various plasma proteases, epidermal growth factor precursor, LDL receptor, fibronectin, complement C4b-binding protein,  $\beta_2$ -glycoprotein I, complement C9, complement factor H and factor XIII, were obviously assembled from a common pool of 1-1 exons by promiscuous exon shuffling within this group (table 1). It is tempting to assume that clusters of gene families (clans of gene families) sharing symmetrical exons of the 2-2 or 0-0 type will also become evident in the future.

##### 5. NONRANDOM INTRON PHASE USAGE OF GENES ASSEMBLED BY EXON SHUFFLING

Since the dominance of a single intron phase class in the noncatalytic chains of plasma proteases

and related proteins was shown to be a necessary consequence of their evolution by exon shuffling, nonrandom intron phase usage may be a diagnostic sign of gene assembly by exon recruitment. A survey of the sequences of genes of various protein families revealed numerous other cases where intron phase usage deviates significantly from random. Genes with predominantly phase 0 introns include those of type III collagen,  $\beta$ -casein and the precursor of growth hormone, i.e. proteins which have been suggested previously to have evolved by exon duplications and/or exon recruitment [48-50]. Only phase 2 introns are found in the coding region of the preproglucagon gene, the individual exons code for glucagon and for two glucagon-like peptides, indicating that internal triplication of a 2-2 exon has occurred during the evolution of this gene [51,52]. Phase 1 introns dominate in all members of the immunoglobulin supergene family: immunoglobulins [53], major histocompatibility antigens [54], Thy-1 glycoprotein [55],  $\beta_2$ -microglobulin [56], T cell antigen receptor [57-59], consistent with the importance of exon shuffling in the evolution of these genes. A single intron phase class dominates in the genes of elastin [60], the rod region of myosin heavy chain [61], troponin I [62] and interleukin 3 [63] raising the possibility that these genes also evolved by exon recruitment.

## 6. PREFERRED TYPES OF INTRONS

In addition to the cases listed above, exon shuffling has been claimed to have played a role in the formation of the genes of phosphoglycerate kinase [64], alcohol dehydrogenase [65–68], pyruvate kinase [69], glyceraldehyde-3-phosphate dehydrogenase [70,71], triosephosphate isomerase [72,73], dihydrofolate reductase and serine protease catalytic chains [74]. The present intron pattern of these genes, however, does not show the regularities in intron phase usage expected of genes that were assembled by exon shuffling. In the case of the genes of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase even detailed evolutionary pathways have been proposed assuming specific exon-duplication and exon-insertion events [64,70,71]. The suggested events, however, do not harmonize with the simple rules of phase compatibility of splice junctions and the exons that were assumed to have participated in exon insertion and exon duplication do not belong to the symmetrical exon classes. Moreover, in most cases there is no clear correlation between the position of the introns and the domains or structural motifs of these proteins [75] so neither the intron pattern nor the intron phase usage seems to support the contention that the genes were formed via recombination in these introns. A possible explanation is that the original intron pattern has been obscured by deletion and insertion of introns during the long evolutionary history of these proteins. Another possibility, however, is that exon shuffling did not play a role in the formation of these genes.

Considering the fact that most of the latter genes were formed before the eukaryote-prokaryote split, it seems justified to assume that the introns and splicing systems existing at that time (if they existed at all) differed from those characteristic of eukaryotic nuclear protein genes. Recent evidence suggests that a continuous evolutionary line leads from the archaic self-splicing introns via protein assisted self-splicing introns (where proteins help fold the RNA into the active conformation) to the splicing system of eukaryotic nuclear protein genes [76]. This implies that the most ancestral proteins either evolved without intronic recombinations or had to be assembled via recombination in such ancestral introns. The mechanism of the excision

of introns, however, has important implications for the efficiency of intronic recombinations. In the case of self-splicing introns where the intron plays an essential role in its own removal, a large portion of the intron sequence is involved in self-complementary interactions important for forming the three-dimensional structure possessing splicing activity [77]. The need to preserve self-splicing activity obviously places severe restrictions on intronic recombinations: recombination is accepted only if the recombinant ends up with a full set of the essential base-paired stems. Furthermore, since complementary sequences are not conserved, recombination even within homologous stems does not guarantee that the hybrid is able to form a perfect self-complementary stem. It is easy to see that such an intron system would lack the essence of exon shuffling, as the latter assumes that intronic recombination will result in hybrid introns that are spliced as efficiently as their progenitor introns.

It seems thus likely that exon shuffling had a career of its own. In the most ancestral organisms, even if they had self-splicing introns, exon shuffling could not contribute significantly to evolution, and therefore it seems anachronistic to expect that the most ancestral proteins were assembled in this way.

Exon shuffling came to full bloom with the evolution of introns whose role in their own excision became negligible as compared to external factors as we now see in the case of the splicing system operating on the introns of eukaryotic nuclear protein genes. The nonessential parts of these introns could accommodate large segments of 'junk' DNA and middle repetitive sequences, increasing the chances of intronic recombination. If we consider that exon shuffling provides an efficient way of proliferating the introns used in exon shuffling we may argue that this might have been one of the driving forces for the evolution of the splicing system in the direction of introns that can achieve this: introns that were most suitable for exon shuffling were also the ones that were the most prolific.

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