Stability domains of actin genes and genomic evolution

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In eukaryotic genes the protein coding sequence is split into several fragments, the exons, separated by non-coding DNA stretches, the introns. Prokaryotes do not have introns in their genome. We report the calculations of stability domains of actin genes for various organisms in the animal, plant and fungi kingdoms. Actin genes have been chosen because they have been highly conserved during evolution. In these genes all introns were removed so as to mimic ancient genes at the time of the early eukaryotic development, i.e, before introns insertion. Common stability boundaries are found in evolutionary distant organisms, which implies that these boundaries date from the early origin of eukaryotes. In general boundaries correspond with introns positions of vertebrates and other animals actins, but not much for plants and fungi. The sharpest boundary is found in a locus where fungi, algae and animals have introns in positions separated by one nucleotide only, which identifies a hot-spot for insertion. These results suggest that some introns may have been incorporated into the genomes through a thermodynamic driven mechanism, in agreement with previous observations on human genes. They also suggest a different mechanism for introns insertion in plants and animals.

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I. INTRODUCTION

Differently from their prokaryotic counterparts, the large majority of eukaryotic genes are split. The parts of the gene which carry the genetic code from which the proteins are synthesized, the exons, are interrupted by long stretches of “junk DNA”, the introns. Much is still uncertain about introns and in general about junk DNA. There is however a clear advantage for a gene of hosting introns: different mRNAs and henceforth different proteins can be synthesized from the same gene through a mechanism known as alternative splicing (see Fig. 1). In different tissues of a multicellular organism the mRNAs are synthesized by placing the exons in different order or by skipping some of them. This produces quite similar, but not identical, proteins. Alternative splicing is responsible for the appearance of slightly different proteins, say, in brain and in liver both encoded by the same gene.

The origin of introns has triggered quite some debate in the past years. The discussion was polarized into two different viewpoints: the “introns early” [2] and the “introns late” [3] theories. The introns late viewpoint states that introns came “late” in the evolution, say after the separation between eukaryotic and prokaryotic kingdoms. Ancient genomes, like nowadays bacteria, had no introns. During evolution introns were inserted at some positions in the coding sequence of eukaryotes. Bacteria did not get introns in order to keep their genome short. According to the introns early perspective introns were already present in ancient genomes. In these genomes mini-genes were separated by junk DNA sequences. Complex genes appeared during evolution when the mini-genes were assembled together.

Although the issue is not completely settled yet, there is a widespread agreement about the fact that most of introns were inserted late in the genome, except for few which can have a very old origin [4]. The question that remain unanswered is: through which mechanism introns were inserted into the genes? Did they target some specific stretches of sequences or their insertion was a random process?

In a previous paper [3] we suggested that some introns may have targeted and got inserted in specific regions of the gene because of some physical stability proper-

![Fig. 1: Through the mechanism of alternative splicing different RNAs and thus different proteins can be obtained from the same intron-containing DNA sequence. Typically, these different RNAs are synthesized in different tissues. In the example shown here three different RNAs are formed from the same DNA sequence: exons are colored, while introns are white. (a) Exons are assembled following the same order as in the DNA sequence. (b) Exon 2 is skipped. (c) Exons 2 and 3 are in reversed order compared to the DNA sequence.](image-url)
ties of these regions. DNA is an inhomogeneous polymer: sequences richer in CG nucleotides are more stable than AT rich regions since CG pairs form three hydrogen bonds, while AT only two. Using a random set of 80 human genes we found that there is a strong correlation between intron positions and stability boundaries \[3\], to be defined more precisely in the next section.

The aim of this paper is to investigate further on this issue. We consider here a single gene, the actin, and analyze its stability on animals, plants and fungi. Although originating from a single gene in a common ancestor, actin genes have diversified during evolution. Several different actins are present in the genome of a given eukaryote. By analyzing the stability properties of genes belonging to a common family we gain insight on mechanisms of introns insertion. We will show that common stability boundaries are found in actin sequences of species belonging to different kingdoms. This implies that the boundaries observed in this and in previous work \[3\] have a very remote origin, dating back to the development of early eukaryotes and supports previous observations that stability boundaries may have influenced the insertion of at least some introns. An extensive discussion of the consequences of our findings is given in the final section of this paper.

II. THERMODYNAMIC STABILITY

When a double helical DNA in solution is brought to a sufficiently high temperature the two strands dissociate, or melt. DNA oligonucleotides of 20-30 base pairs melt at a single temperature. This temperature can be estimated using the nearest-neighbor model from which one computes Gibbs free energies, enthalpies and entropies of melting. Quite some effort has been dedicated in the past years to an accurate determination of these thermodynamic parameters (see e.g. \[8\] and references therein), due to importance of DNA melting and of the reverse transition, the DNA hybridization, in many biotechnological processes. The melting temperature depends, besides on the sequence composition, on salt concentration and pH of the solution.

If the sequences are sufficiently long, DNA melting becomes a multistep process \[8\]. Regions of the sequence which are GC-richer will melt at higher temperatures compared to GC-poorer regions. The interesting quantities to calculate in this case are multiple partial melting temperatures and at the same time one needs to determine the regions of the sequence which melt at those temperatures. In order to perform such type of calculation various statistical mechanical models have been developed \[7, 8\]. The calculations presented in this paper are based on the Meltsim algorithm \[9\], in which a DNA configuration is approximated by a sequence of non-interacting loops and helical segments according to the Poland-Scheraga model \[10, 11\]. In this approach each base pair is in two possible states either open \((\theta_i = 0)\) or closed \((\theta_i = 1)\), where \(\theta\) defines the order parameter and \(i\) is an index running over all base pairs of a sequence \((i = 1, 2 \ldots N)\). In the Meltsim algorithm recursion relations \[12\] and an approximation for the closed loops entropy \[13\] allow a rapid computation of the opening/closing probability at any given temperature for chains of several thousands base pairs.

Computations based on Poland-Scheraga model have been quite popular in the past years \[14, 15, 16, 17, 18, 19, 20\]. Yeramian et al. analyzed the genomes of \(S.\ cerevisiae\) (Yeast) \[14\] and of \(P.\ falciparum\) \[15\] and identified genes on the basis of thermodynamic signals obtained from the melting analysis. The effect of mismatches \[16\] and of disorder \[19\] on DNA melting have also been discussed. A recent study has produced the melting map of the whole human genome \[21\].

The other popular model for studies of the thermodynamics of the DNA is the Peyrard-Bishop model \[8, 22\], which has attracted quite some attention in recent years \[23, 24, 25, 26, 27, 28\]. This model is probably more accurate on shorter length scales as a configuration is identified by the distances between complementary bases and not by a simple boolean variable \((\theta = 0, 1)\) as in the Poland-Scheraga picture. However for the purposes of calculating stability properties which involve melting domains of about hundred basis the Poland-Scheraga model is good enough. Programs like Meltsim have been fine tuned to fit experimental data \[9, 29\]. Interestingly, the thermodynamic boundaries found in the Meltsim approach in a previous paper \[3\] have also been found in an analysis of the Peyrard-Bishop model \[22\]. This shows that the properties discussed here are robust and model independent.

In this paper we have used the same set of thermodynamic parameters as in Ref. \[30\]. In order to estimate the thermal stability boundaries, we proceed as follows. Starting from sufficiently low temperatures in which the whole chain is in a helical state, we increase the temperature at a constant small step \(\Delta T\) (= 0.01°C in the calculation). At each point the configuration of the chain is calculated and the boundaries between helix and coil regions recorded. To discriminate between a helical and a coiled region we calculate the average value of \(\theta_i\) at a given temperature and define the boundary as the point separating a \(\theta > 1/2\) domain from a \(\theta < 1/2\) domain.

Typical outputs of the calculations are shown in the graphs of Fig. 2, 3 and 6. In these graphs the x-axis is the temperature, while the y-axis represents the position along the sequence. For each gene we considered only the coding sequence (CDS) with all introns removed. This is the so-called complementary DNA (cDNA), which is a double stranded copy of the mRNA and can be obtained from it in laboratory through reverse transcription. For the purposes of inferring information on genome evolution we can look at cDNA as an old gene before introns were inserted. In order to avoid boundary effects, i.e. dissociation dominated by the opening of forks at the edges, we have enclosed the sequences by two stretches
of poly(G) of two hundred nucleotides each (a poly(G) sequence is a stretch of DNA composed only of nucleotides G, in this case the sequence referred to is double-stranded with one strand containing only G’s while the other strand contains only C’s). These stretches have high melting temperature, hence they dissociate well beyond the melting temperatures of the CDS. The solid thick lines in Figs. 2, 3, 4 and 6 separate the coiled from the helical regions (to the right and to the left of the curve, respectively). Due to strong cooperativity [31], the DNA melts through few sharp transitions involving the dissociation of hundred of base pairs simultaneously. Hence, only few stability domains are found in the analysis of a sequence of 1000 base pairs.

III. ACTIN

Actin proteins play a central role in eukaryotes. Actin filaments constitute the cytoskeleton of all eukaryotic cells, and are the site of interactions with many other proteins, as for instance motor proteins or actin-bundling proteins [1]. A mutation in a specific actin protein site may result in a change in its interactions with several proteins that bind near the mutated site. While the mutation can favor the interaction with one specific protein, it is likely disrupt interactions with many other proteins. Hence, in order to maintain the multiple interactions with all its partners, actin proteins have been highly conserved during evolution. Obviously there is lower conservation at the gene level compared with the conservation of amino acid sequence for the corresponding protein, as the genetic code is degenerate and multiple codons encode for the same amino acid. For actin there is roughly a 80% of sequence conservation between human and yeast (S. Cerevisiae) genes, while 95% conservation of amino acids in the proteins [1].

Genomic evolution is believed to have occurred mainly through gene duplication and mutations [1]: At a given time an error in the replication of DNA produces two
TABLE I: Table of intron positions in Actin genes for vertebrates and green plants. The label refers to the codon position following the table of Ref. [32]. The three positions surrounded by a box are those for which a stability boundary was found.

| Vertebrates | 20-3 | 43-3 | 86-3 | 123-3 | 152-1 | 206-1 | 270-1 | 330-3 | 356-3 |
|-------------|------|------|------|-------|-------|-------|-------|-------|-------|
| α_1         | x    | x    | x    | x     | x     | x     | x     | x     | x     |
| β           | x    | x    | x    | x     |       |       |       |       |       |
| γ_1         | x    | x    | x    |       | x     | x     | x     |       |       |
| α_2         | x    | x    | x    | x     | x     | x     | x     | x     | x     |
| γ_2         | x    | x    | x    |       | x     | x     | x     |       |       |

Green plants | | | | | | | | | |

FIG. 3: (Color online) Melting domains for actin genes of D. Melanogaster. The area descriptions are the same as in Fig. 2 (a,b) and C. Elegans (c). GenBank entries: (a) NM_169525 (Act87E), (b) NM_079643 (Act88F) and (c) NM_073416.

copies of a gene, which are inherited to a daughter cell. These genes further evolve separately accumulating different point mutations and thus diverging in time. As the process is repeated one obtains from a single ancestor gene a family of closely related genes. In vertebrates there are three classes of actins known as α, β and γ actins. The α actins are found in muscle cells, while the β and γ are found in non-muscle cells. Plant actins also form a large family with even more genes than in vertebrates. For instance more than 10 different actin genes have been identified in the genome of the plant Arabidopsis Thaliana.

A. Intron positions

The Table compares the intron positions of vertebrates and land plants. A more complete table which contains 56 different intron positions for actins of different organisms can be found in Ref. [32]. In total there are 7 intron positions for vertebrates actins. These positions are labeled, following the notation of Ref. [32], by two numbers. The first number refers to the codon in the sequence and the second one (between 1 and 3) indicates where the intron is inserted in the codon. A 3 signifies that the intron is inserted after the third nucleotide of the codon, hence the intron does not break the codon. The codon numbers are given with respect to a reference sequence, which is the α actin of vertebrates. Although plants have more actin isoforms than vertebrates, somewhat quite surprisingly they have only 3 intron positions, one of which (152-1) is in common with the vertebrates lineage.

B. Melting domains for animal actins

We start with the description of melting domains in animal actins. Although Fig. 2 shows exclusively human actins, we found very similar melting profiles also in α, β and γ actins of other vertebrates as: Canis familiaris (dog), Bos Taurus (cow), Danio Rerio (zebrafish), Gallus Gallus (chicken) etc. . Hence, the conclusions drawn from the analysis of Fig. 2 are probably valid for all vertebrates.

Figure 2(a) shows the melting behavior of the human actin β (GenBank entry NM_001101). This sequence has four introns at positions 43-3, 123-3, 270-1 and 330-3 (see Table I). These positions are indicated by horizontal lines in Fig. 2(a). In this sequence melting is a three state process. First, at around 85°C the exon bounded between introns 270-1 and 330-3 melts. Next the whole CDS sequence melts except for a short fragment bounded by the intron at 43-3, which then melts only beyond 90°C. There
is a remarkable correspondence between the 43-3 position and a sharp stability boundary. Also positions 270-1 and 330-3 show a similar, although weaker, correspondence. This β actin sequence has already been analyzed in Ref. 3 (see Fig. 2 in Ref. 3). In that analysis the correspondence with the intron 330-3 was missed because different boundary conditions were used. In this work the CDS is embedded between two poly(G) stretches, so that melting inside the sequence is always through the formation of loops bounded between two helical regions. In Ref. 2 some parts of the untranslated regions bounding the CDS were included in the analysis. As no stable boundary helical regions were included, part of the melting in Ref. 2 occurred through fork openings from the boundaries. The inclusion of untranslated regions, i.e. of the original genomic neighborhood in the analysis, is probably not an optimal choice as these regions are poorly conserved during evolution. As the aim is to look for signals from ancient genomes it is better to embed the CDS between two poly(G) stretches. In this way all the sequences analyzed are treated on equal footing.

The Figs. 2(b-f) show other melting domains for homologous vertebrates actin genes. These sequences have 4 (e), 5 (b,c) and 7 (d,f) intron positions. There are three introns positions which are in common in the vertebrates actins: 43-3, 270-1 and 330-3. These are also those for which a correspondence with thermal boundary was found in the β gene of Fig. 2(a). The correspondence between thermodynamic boundaries with the introns positions 43-3 and 270-1 is also observed in the three other sequences of Fig. 2 (b,c,f). Note the very sharp signal from the 43-3 intron in the case (c). The intron at position 330-3 shows a good correspondence with stability boundaries for the sequences in Fig. 2 (b,c). A much weaker, but noticeable, correspondence is with the intron at position 206-1 in sequences (c) and (d). The stability boundary is slightly shifted from the 206-1 in sequences (d) and (f). The correspondence between intron positions and thermodynamic boundaries is absent in the sequence (d).

Another interesting feature of vertebrates actins can be seen in Fig. 2(b). This sequence is the actin α1 which hosts 5 introns in its coding region. These are marked by horizontal lines. The two remaining of the total 7 intron positions of vertebrates actins, 86-3 and 123-3, are indicated by horizontal lines. As it can be seen from Fig. 2(b), these two positions correspond to stability boundaries. The correspondence of a stability boundary with the 123-3 is also visible in (c) and (e). In the latter example the 123-3 is a nucleation site for a small loop. The sequences of Fig. 2 (d), (e) and (f) show a much weaker correspondence between intron positions and stability boundaries.

Fig. 3 shows the melting curves for Drosophila Melanogaster (a,b), the fruit fly, and Caenorhabditis Elegans (c), a worm. The Drosophila actins have at most one intron in the coding region either in 15-1 or 310-1. These positions differ from the vertebrates positions discussed so far. The sequence shown in Fig. 3 (a) has no introns. The melting analysis however reveals few stability boundaries close to the positions 43-3, 86-3, 270-1 and 330-3, which are the introns position of vertebrates actins. The 43-3 and 86-3 are particularly sharp. The next Drosophila sequence (b) with one intron at position 310-1 show a stability boundary close to 270-1 and a weaker one close to 330-3. Compared to the case in (a), in this sequence the signals from 43-3 and 86-3 have been lost. However a sharp boundary has appeared close to the vertebrates intron 123-3. The C. Elegans sequence of Fig. 3(c) has two introns at “new” positions 65-1 and 325-2. As in the previous examples one observes boundaries close to the 43-3, 123-3, 270-1 and 330-3 positions.

C. Melting domains for plant actins

Figure 4 shows the melting domains for actin genes of the green plant Arabidopsis Thaliana. The introns positions of actins sequences of higher plants are highly conserved (see Table I), which indicates that these introns date back to the early evolution of land plants. In 3 out of the 9 Arabidopsis sequences shown Fig. 4 (a, d, f) we find a correspondence of a thermal boundary and an intron at 152-1. This is the intron which is in common with vertebrates (see Table I). As in the Drosophila and C. Elegans sequences (Fig. 3) in general stability boundaries tend to be found at vertebrates positions 43-3, 86-3, 270-1 and 330-3. In few cases the correspondence is very striking, as in Fig. 4 (d).

In order to corroborate these findings we extended the analysis to other plants. We considered 12 additional actins from Nicotiana Tabacum (tobacco, GenBank X63603), Oryza Sativa (rice, GenBank X15862, X15863, X15864, X15865), Glycerine Max (soyabean, GenBank J01298, V00450), Solanum Tuberosum (potato, GenBank X55749, X55750, X55751, X55752) and Striga Asiatica (GenBank U68461, U68462). With the 9 sequences from A. Thaliana we have in total 21 plant actin genes. For each sequence the melting curves were calculated and then averaged. The result is shown in Fig. 5. In the graph the x and y axes are reversed compared to the Figures 1. The x-axis is the codon position, and the temperature, now in the y axis, is ordered as increasing from top to bottom. As reference four of the most commonly found introns positions for actin genes are indicated as vertical lines. The averaging introduces some smoothening, but it confirms the existence of a sharp stability boundary close to the 43-3 position. Two weaker boundaries are found close to the positions 86-3 and 270-1.

D. Melting domains for fungi actins

To conclude the analysis of the stability behavior of actin genes we consider now fungi. Fig. 6 shows the melt-
FIG. 4: (Color online) Melting domains for actin sequences of the *A. Thaliana*. The area descriptions are the same as in Fig. 2. GenBank entries: (a) M20016 (AAc1), (b) NM_180280 (ACT2), (c) U39480 (ACT3), (d) U27980 (ACT4), (e) U27811 (ACT7), (f) U42007 (ACT8), (g) U27981 (ACT11), (h) U27982 (ACT12) and (i) U37281 (actin 2).

ing curves for the budding yeast *Saccharomyces Cerevisiae* (a), for *Neurospora Crassa* (b) and for *Candida Albicans* (c). In general the number of introns and their positions are highly variable in fungi actin genes: their number vary from 0 to 7 and the positions are most likely concentrated in the region before the 50th codon. This can be seen also in the sequences of Fig. 6 two of which have one intron and one has four. All introns are found before the codon 45. The melting behavior shown in Fig. 6 resembles that of the previous cases. A sharp stability boundary close to the 43-3 position and some weaker ones appearing close to positions 270-1 and 330-3 in the case (b). This correlation is absent in the case (a).
2 is present in the single-copy actin genes of the fungi Cyanophora Paradoxa. At position 44-1 there is an intron in the alga Chondrus crispus. Shifted of a single nucleotide at position 44-1 there is an intron in the alga Cyanophora Paradoxa. An intron at position 44-2 is present in the single-copy actin genes of the fungi Thermomyces lanuginosa, Aspergillus Niger, Neurospora Crassa (see Fig. 4b) and Trichoderma Reesei. The only other positions with introns separated by a single nucleotide are at 34-1 and 34-2, which however are only found in some Fungi. Hence the 43-3 is a unique site in the actin genes, which we can refer to as an “hotspot” for introns insertion.

E. A “hotspot” for introns insertion

In Ref. [32] the full table of introns positions in actin genes gives 56 different positions. There is an interesting remark concerning the position 43-3, which is shared by all vertebrates actins. Position 43-3 is relatively common in animals, but it is also the sole intron in the actin gene of the red alga Chondrus crispus [32]. Shifted of a single nucleotide at position 44-1 there is an intron in the alga Cyanophora Paradoxa [32]. An intron at position 44-2 is present in the single-copy actin genes of the fungi Thermomyces lanuginosa, Aspergillus Niger, Neurospora Crassa (see Fig. 4b) and Trichoderma Reesei. The only other positions with introns separated by a single nucleotide are at 34-1 and 34-2, which however are only found in some Fungi. Hence the 43-3 is a unique site in the actin genes, which we can refer to as an “hotspot” for introns insertion.

IV. DISCUSSION

DNA sequences, which are hundred of base pairs long, tend to melt through a series of separate temperature steps. Each step consists of the melting of a region of few hundred of base pairs. By following the melting process over a wide temperature interval one can thus identify separate melting domains, i.e. parts of the sequence which dissociate at different temperatures. The domain boundaries are points in which the sequence tend to form in a relatively wide temperature interval a stable Y-conformation separating a double helix from a coiled region (see Fig. 7).

A previous study of about 80 human genes from which introns are removed and exons linked together revealed that stability domain boundaries tend to be localized at the end of exons. This correspondence was found for about 35% of the exons analyzed. The correlation was found to be stronger for a class of so-called housekeeping genes, i.e. those genes involved in the basic cellular processes. These genes are expressed in all tissues and have been more conserved during evolution. Actin is in fact an example of a housekeeping gene. If one accepts an “introns late” viewpoint, the correlation between intron positions and stability boundaries suggests that some introns were inserted into genes at the ends of the melting domains in a process driven by thermodynamics. Such a process is illustrated in Fig. 7(i): an intronless fragment of a gene has naturally parts which are richer and poorer of CG nucleotides. When the two strands partially separate they may form a Y configuration the end of a less-stable domain and the beginning of a more stable one. Introns may have targeted these fork locations.

Another possibility that may have explained the correlation between thermodynamic boundaries and introns positions observed in Ref. [32] is schematically shown in Fig. 7(ii). Originally the insertion site does not possess a thermodynamic boundary, so the intron is inserted through a process which does not depend on thermodynamics. Once the insertion has taken place and the two exons are separated by an intron stretch. Mutations may have biased the CG content on the two exons so that their thermodynamic boundary originated after the intron insertion. However, this scheme is at odds with the results presented in this paper. We have indeed shown that boundaries in conserved positions are found in actin family genes where no introns are present close to those positions. For instance, in many actins of plants, fungi and animals there is a sharp stability boundary at the position 43-3 in sequences which have no intron at that position. Hence being found in plants, animals and fungi sequences, the stability boundary at 43-3 is rather a property of an intronless ancestor actin gene. The same is true for stability boundaries found in other intronless positions as for instance the 86-3 and 270-1.

We further speculate on the “introns early” perspective, i.e. the possibility that introns were already present in early genomes and were selectively lost by some species. Our findings then imply that these introns would have separated early exons (or mini-genes as they are also referred to) with different stability properties, as shown in the scheme of Fig. 7(iii). Although possible, this scenario seems to be in contradiction with most of the recent phylogenetics based studies which favor an introns late theory.

In conclusion, our work supports a mechanism given in Fig. 7(i), i.e. a thermodynamic driven introns insertion. This does not necessarily mean that the actual insertion process took place through an equilibrium transition with a temperature rise to 80°C. First of all the melting temperature depends also on other salt concentration and pH of the environment. Moreover, the boundaries found in the melting analysis, should manifest themselves also under nonequilibrium conditions. Y-configurations as those

![Diagram](image-url)
FIG. 6: (Color online) Melting domains for actin sequences of Fungi. The area descriptions are the same as in Fig. 2. (a) *Saccharomyces Cerevisiae* (yeast) (b) *Neurospora Crassa* and (c) *Candida Albicans*. GenBank entries: (a) X61502 (Act2), (b) U78026 and (c) X16377 (act1).

FIG. 7: Possible scenarios of gene evolutions. (I), (II) introns late and (III) introns early perspectives. The scheme (I) is the insertion driven by thermodynamics, supported by the analysis of stability regions reported in this paper. The scheme (II) suggests the appearance of a thermal boundary after intron insertion, a scenario not supported by the results presented in this paper. Finally the scheme (III) takes into account the introns early perspective in which the ancient exons had already different stability properties.

shown in Fig. 7 can also be generated by mechanical unzipping of DNA [33]. Quite remarkable is the fact that the sharpest boundary in actin genes (43-3) is also the locus in which introns insertion has been the most active in evolutionary distant organisms. As we have pointed out introns have also been found at positions 44-1 and 44-2. This fact is in agreement with an idea of an insertion driven by thermodynamics. As the boundary is particularly sharp the mechanism of Fig. 7(I) can have occurred independently on three different families of actin genes.

The correlation between stability boundaries and introns position in particularly sharp in several sequences analyzed, but in few cases is absent. We believe that this is due to mutations having erased the correlation from the ancient gene sequence. Although actin is highly conserved as a protein, there is no selective pressure against synonymous mutations which do not modify the aminiacid sequence. Such mutations are known to have occurred at a roughly constant rate in all genes of a given organisms [1]. Two genes of the same family in the same organism have evolved separately and mutations may have accumulated at higher/lower rates in different parts of the sequence: in some genes the mutations may have erased the ancient stability boundaries.

The problem of introns evolution has been widely debated in the biological literature (for a recent review of the state of the art see Ref. [34]). Even within the introns late perspective there is no general consensus on the mechanism of insertion and several possibile cases have been analyzed. For instance Ref. [34] reports 5 differ-
ent models of introns insertion. Most of these models in general discuss the mechanism of insertion without suggesting in which position of the sequence the insertion would have occurred. One exception is the protosplice site model [35] which suggests a bias towards a specific insertion sequence (C/A)AG(G/A) (here C/A denotes a site which can possess either a nucleotide C or A), referred to as the protosplice sites. This insertion would have lead to a structure (C/A)AG-intron-(G/A). The protosplice model and other models for introns insertions are only partially supported by the analysis of genomic data [34]. Unfortunately, the genomes nowadays investigated have been heavily reshaped by hundreds of millions of years of evolution and are quite different from genomes of early eucaryotes. Hence the answer to the question of introns origin is not an easy one. Indeed although introns were discovered 30 years ago, there still an open debate on this issue. Moreover, evolution may have taken place through complex and diversified pathways so it is not unlikely that different mechanisms of insertion have coexisted. Certainly the possibility that also the physical and thermodynamical stability of the double helix has played a role offers new insights and stimulates further research in this field.

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