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Review

Are synonymous codons indeed synonymous?

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Abstract

It has long been known that the distribution and frequency of occurence of synonymous codons can vary greatly among different species, and that the abundance of isoaccepting tRNA species could also be very different. The interaction of these two factors may influence the rate and efficiency of protein synthesis and therefore synonymous mutations might influence the fitness of the organism and cannot be treated generally as 'neutral' in an evolutionary sense. These general effects of synonymous mutations, and their possible role in evolution, have been discussed in several recent papers. This review, however, will only deal with the influence of synonymous codon replacements on the expression of individual genes. It will describe the possible mechanisms of such effects and will present examples demonstrating the existence and effects of each of these mechanisms.

Keywords: isoaccepting tRNA; miRNA; mRNA half-life; mRNA secondary structure; synonymous codon; translation kinetics.

Introduction

Since the deciphering of the genetic code it has been an integral part of our textbook knowledge, that the code is degenerate, i.e., except for methionine and tryptophan, all amino acids are coded by two to six different codons, and that these codons are synonymous. Synonymous, in principle, should mean that the replacement of one with another (either by spontaneous or by artificially induced mutation) would not lead to any phenotypic or fitness change. In other words, such mutations would be strictly neutral in an evolutionary sense. However, several lines of evidence seem to indicate that this is not always true; synonymous mutations are not necessarily neutral. The evolutionary aspects of synonymous mutations have been discussed in several recent reviews (1–5).

It has long been known that the cellular concentrations of isoaccepting tRNAs can be very different in different organisms, and that this fact probably influences codon usage (6). It is generally assumed that the frequency of occurence of synonymous codons is related to the species-specific concentrations of isoaccepting tRNAs. It has also been shown that within one species (e.g., human), the level of expression of individual genes is correlated with the frequency of optimal codons (i.e., codons with the highest tRNA gene copy number) (7, 8).

Thus codon usage might be optimised in such a way that the frequency of occurrence of any particular codon for each amino acid is correlated with the abundance of the corresponding tRNA molecule. This is not always true, however. In a recent review Czech et al. (9) emphasise the importance of the precise assessment of tRNA composition and summarise the methods applicable for determining the concentrations of tRNA species. If and when such a correlation exists, an obvious consequence of this fact is that synonymous mutations could lead to a slowdown of protein synthesis. This effect is often practically exploited in biotechnology where developers intentionally change the codon usage in transgenes, thereby adapting it to the optimal codon usage of the host organism. Angov et al. (10) used known relationships between codon usage frequencies and secondary protein structure to develop an algorithm ('codon harmonisation') for identifying regions of slowly translated mRNA that are putatively associated with link/end segments. It then recommends synonymous replacement codons having usage frequencies in the heterologous expression host that are less than or equal to the usage frequencies of native codons in the native expression host. For protein regions other than these putative link/end segments it recommends synonymous substitutions with codons having usage frequencies matched as nearly as possible to the native expression system. They used this algorithm for the optimal expression of several *Plasmodium falciparum* proteins in *E. coli*.

Sometimes the opposite effect is deliberately evoked. Coleman et al. (11) reported that by introducing underrepresented synonymous codons into the gene coding for poliovirus capsid protein, a slowdown of viral protein synthesis was achieved, and the resulting poliovirus could be used as an attenuated vaccinating agent.

This factor and its possible effects have long been known. In fact the codon bias of any gene towards common codons is reflected in the Codon Adaptation Index (CAI) (12). In this review, however, I do not discuss either the general effects of species-specific synonymous codon distribution or the potentially non-neutral evolutionary role of synonymous mutations. I shall restrict myself only to the discussion of such cases where the synonymous replacement of coding triplet(s) in a particular gene leads to a specific quantitative or qualitative change in the expression of that gene.

What could be the mechanisms of such specific alterations?

1. In split genes of Eukaryotes, synonymous mutations in and around splicing sites may lead to different splicing patterns.
2. If the mutation changes the secondary or tertiary structure of the messenger, this can lead to altered efficiency of various steps (especially initiation) of the translation process.
3. Another consequence of altered messenger conformation can be the changed rate of decay (i.e., half-life) of the messenger.
4. The synonymous mutation may influence the interaction of the messenger with a regulatory micro-RNA, thereby changing the regulation of translation.
5. The synonymous mutation may directly influence the kinetics of translation and this in turn may change the folding pattern and therefore some properties of the coded protein.
6. This latter effect may lead to different secondary covalent modifications of the protein.

In the following sections I shall try to summarise the available evidence for the existence of each of these possible mechanisms.

Splicing

As the vast majority of eukaryotic messengers undergo splicing before translation, leaving only a fraction of the primary product of transcription in the final messenger RNA (these remaining sequences are called exons), it is obvious that the determinants of this splicing process also influence the expression of the corresponding genes. Thus synonymous mutations at the splice sites can lead to aberrant splicing. Synonymous mutations might also create new 'cryptic' splice sites. Moreover, it is also known that exonic sites not at the splice junction can increase (splicing enhancers) or decrease (splicing silencers) the rate of splicing. Splicing modulators are oligomeric motifs that recruit splicosomal proteins to facilitate splice site recognition.

In the case of several monogenic human diseases it has long been known that the mutant phenotype could be the result of aberrant splicing, exon skipping, or changed concentrations of alternative splice products. The underlying mutation is not necessarily missense or nonsense, it could in principle be synonymous. For instance, in the case of cystic fibrosis transmembrane regulator protein (*CFTR*) it has been shown that one quarter of synonymous mutations lead to exon skipping, resulting in the production of inactive protein (13). Two reviews (2, 14) summarise in tabulated form those references that report synonymous mutations associated with aberrant splicing, thus leading to various human diseases, for example optic atrophy (15), McArdle disease (16), X-linked hydrocephalus (17), thrombasthenia (18), glycine encephalopathy (19), gangliosidosis (20), Marfan syndrome (21), phenylketonuria (22) and others. Another review (23) summarises the mechanisms by which alternative splicing is controlled, and discusses the possible role of synonymous mutations. As already mentioned, the mutation does not necessarily affect the splice site itself. There are exonic splicing silencers and enhancers and synonymous mutations at these sites can also lead to aberrant splicing (24) (Figure 1).

Effect of messenger structure on translation efficiency

Perhaps the most convincing evidence for the differential effect of synonymous codons on gene expression is the thorough study of Kudla et al. (25). These authors constructed a synthetic library of 154 genes containing a variety of synonymous codons, coding for the same green fluorescent protein (GFP). When expressed in *Escherichia coli*, the GFP levels varied 250-fold. The authors then calculated for each GFP construct the minimum free energy associated with the secondary structure of the entire mRNA or specific regions of it. As for the entire RNA this value was not correlated with the efficiency, but for the first third of the mRNA it was, they concluded that the effect of this folding on the rate of initiation was responsible for a decisive part of this wide variation (Figure 2).

In a similar study (26) the expression level of two commercially valuable proteins in *Escherichia coli* was investigated by using synthetic genes containing synonymous codons. The expression levels achieved varied between undetectable and 30% of total cellular protein. The optimal codons were those that were read by tRNAs most highly charged under amino acid starvation conditions, and not those that are most abundant in highly expressed *Escherichia coli* proteins.

The most likely target of this effect is the initiation process. De Smit and van Duin (27) have shown that a single nucleotide change in the initiation region that turns the mismatch in a helix into a match might decrease protein expression by a factor of 500.

The important role of the initiation region is emphasised in the work of Golterman et al. (28). These authors have shown that by targeting a synonymous codon library to the 5′ coding sequence (near the AUG initiation codon) in a bacterial

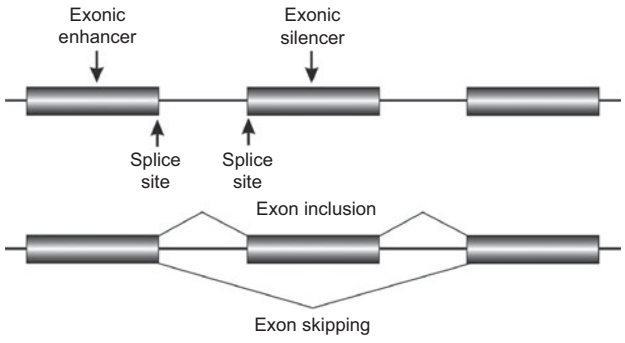

Figure 1 Possible sites where synonymous replacement might influence splicing, and some of their possible effects.

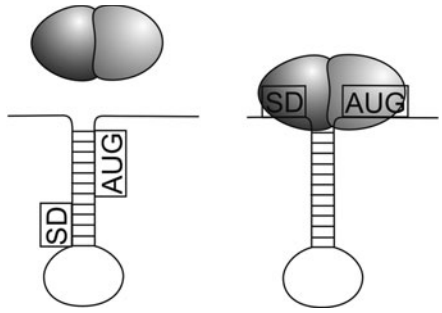

Figure 2 Effect of mRNA secondary structure on translation initiation in bacteria. Double-stranded structure involving the initiation codon (AUG) and the Shine-Dalgarno (SD) sequence usually inhibits translation. Initiation is uninhibited if the structured element occurs between these two, or downstream of the initiation codon.

expression system, the level of the coded protein (GFP) varied 300-fold.

A specific example of the significant influence of synonymous codons on gene expression is the case of human catechol-O-methyltransferase (COMT). This is a very important enzyme. Its activity affects pain perception, cognitive function and affective mood. Its coding gene contains four SNPs, one in the promoter region, one nonsynonymous SNP and two synonymous SNPs in the coding region. It has been demonstrated that the largest phenotypically observable differences between the naturally existing haplotypes are due to the synonymous SNPs that influence the secondary structure of the messenger. The most stable structure was associated with the lowest protein level and enzymatic activity. When the stable stem-loop structure was eliminated with site-directed mutagenesis, the higher level of the COMT protein was restored (29, 30).

An interesting example has been presented by Bartoszewski et al. (31). These authors studied a disease-causing short deletion mutation in the *CFTR* gene. This deletion removes the last C of an isoleucine-coding triplet and the first two Ts of a phenylalanine-coding triplet. This leads to the loss of a phenylalanine from the protein and a synonymous codon change for isoleucine (ATT instead of ATC). The consequence of these changes is a 'misfolded' messenger RNA and a decreased rate of translation. If the isoleucine is coded by the naturally occurring (ATC) and the neighbouring phenylalanine by TTC (instead of TTT), the messenger structure is similar to that of the wild-type, and the protein level is even higher than in the wild-type. Thus they conclude that although the deletion mutation leads to a one-amino acid shorter protein, its severity is mainly due to the 'misfolding' of the messenger due to the synonymous codon change.

Effect of messenger stability/half-life

In the case of another important protein that plays a role in human neurobiology, dopamine receptor D2, synonymous codons probably influence the level of the coded protein by changing the rate of decay (half-life) of the corresponding messenger (32) (Figure 3).

Duan and Antezana (33) presented experimental evidence for their claim that increasing the frequencey of UA dinucleotides in a messenger by synonymous replacements leads to faster decay of the messenger.

In accordance with these experimental results, Chamary and Hurst (34), in a theoretical paper, present evidence for the hypothesis that synonymous mutations are under selective pressure, because by changing the secondary structure of messengers they also change their stability. This explains the observed preference for cytosin at four-fold degenerate sites.

Capon et al. (35) have shown that a synonymous replacement in the gene coding for human corneodesmosin (CDSN) leads to two-fold higher stability of the messenger and this is associated with increased susceptibility to psoriasis.

Effect of mRNA interaction with miRNA

For several years the micro-RNA-mediated effect of synonymous codon replacement on gene expression has only been a theoretical possibility. A bioinformatic analysis of this prediction tried to assess its role, but the calculated possible effect was small enough to be ignored (36). However, a very convincing case has recently been reported by Brest et al. (37). The starting point of the work of these authors was the observation that a synonymous variant (CTG vs. TTG, both coding for leucine) within the *IRGM* coding region, in perfect linkage disequilibrium with a 20 kb deletion upstream of *IRGM*, has been strongly associated with Crohn's disease in individuals of European descent (38). They first analysed *in silico* the binding of miRNAs to the different forms of IRGM mRNA, and found that the ability to bind to the risk haplotype mRNA is lost for miR-196A and miR-196B. They then confirmed this prediction experimentally by finding that the synonymous C/T polymorphism of *IRGM* is located within the 'seed' region, where mRNA-miRNA forms a complex within RISC (RNA-induced silencing complex), which is important for mRNA regulation (Figure 4).

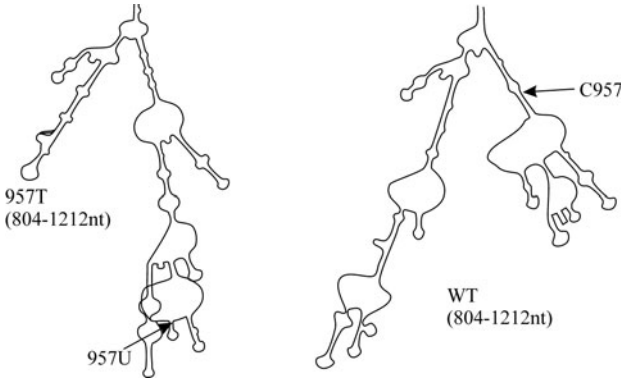

Figure 3 Predicted partial (between nucleotides 804–1212) schematic structures of two haplotypes of the dopamine receptor mRNA differing only at one synonymous SNP (at 957).

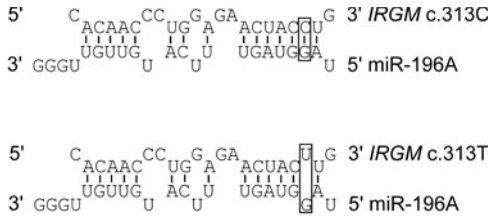

Figure 4 Interaction of the two different haplotypes of IRGM mRNA with miR-196A.

Moreover, transfection with miR-196 in cell culture led to decreased IRGM expression. They have also shown that in the intestinal epithelia of patients with Crohn's disease, miR-196 is overexpressed.

Effect of translation kinetics on protein structure

It has long been known that protein secondary structure motifs are somehow correlated with structural elements in the messenger RNA (39, 40), but it was unclear what the mechanistic explanation of this phenomenon could be.

A thought-provoking review by Komar (41) summarises the evidence suggesting that translational pausing at rare codons might influence the folding of specific segments of the nascent protein. This hypothesis was also put forward recently by other authors (42–44). Zhang et al. (42) studied the folding efficiency of the *E. coli* multidomain protein SufI and found that clustered codons that pair to low-abundance tRNA isoacceptors can form slow-translating regions in the mRNA and cause transient ribosomal arrest. Synonymous substitutions to codons with highly abundant tRNAs accelerate translation. These authors propose that local discontinuous translation temporally separates the translation of segments of the peptide chain and thereby influences co-translational folding (Figure 5).

Recently several individually analysed cases have been reported, showing the role of this kinetic effect. Cortazzo et al. (45) demonstrated that the solubility of the EgFABP1 protein was markedly reduced by a synonymous mutation when expressed in *Escherichia coli.*

An interesting case is that of the multidrug resistance (*MDR1*) gene. The most common haplotype of this gene contains three polymorphic sites 1236C>T (rs 1128503), 2677G>T (rs 2032582) and 3435C>T (rs 1045642), of which the first and the last are synonymous. As Kimchi-Sarfaty et al. (46) have shown, the synonymous polymorphism at 3435 in two haplotypes of this gene leads to the synthesis of a P-glycoprotein (P-gp) product with altered drug and inhibitor interactions. The authors hypothesise that the rare synonymous codon at this site slows down the process of translation, thereby causing a changed folding and insertion of the protein into the membrane. This explains the altered substrate and inhibitor interaction sites. It must be mentioned that in an earlier paper Wang et al. (47) attributed this effect to changed mRNA stability, but Kimchi-Sarfaty et al. claim

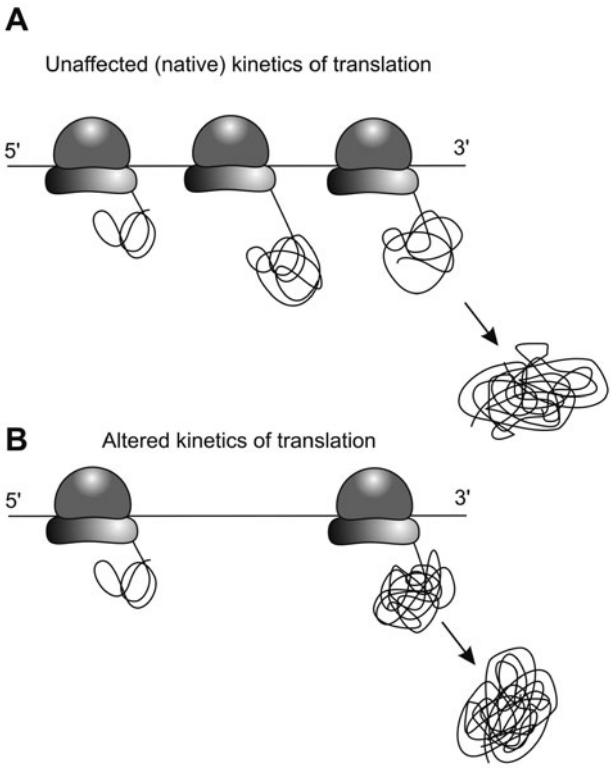

Figure 5 Different co-translational folding pathways. (A) Unaffected kinetics of translation results in native protein conformation. (B) Ribosomal stalling results in protein trapped in altered conformation.

that the levels of mRNA were the same in the two haplotypes. Fung and Gottesman (48) after discussing very thoroughly the possible mechanism of action of this polymorphism also conclude that the most probable cause is ribosome stalling. In two recent review articles (49, 50) the possible consequences of such polymorphisms for pharmacogenomics and cancer therapy are discussed.

In the case of the *CFTR* deletion mutation discussed in the chapter on splicing (13), the authors also hypothesise that the consequence of the 'misfolding' of the messenger is a 'misfolding' of the protein as well because the folding of the protein is cotranslational.

Effect of translation kinetics on protein modification

A very interesting case of this effect has recently been reported. In mammalian cells β-actin and γ-actin are 98% identical in amino acid sequence, but their DNA nucleotide sequence identity is only 88%, that is at several places the two genes contain different codons, coding for identical amino acids (synonymous codons). The N-terminal methionine of both proteins is removed immediately after synthesis and the penultimate acidic amino acid (aspartic acid in β-actin and glutamic acid in γ-actin) becomes arginylated. However, *in vivo* only arginylated β-actin is present stably in the cells,

arginylated γ-actin is rapidly degraded. As Zhang et al. (51) have shown, the reason is that the rate of translation of the two messengers is very different, that of γ-actin being much slower. This slower translation results in the exposure of a normally hidden lysine residue for ubiquinylation and this in turn, after arginylation, leads to the rapid degradation of the γ-actin (Figure 6).

Expert opinion and outlook

The answer to the question in the title of this review must undoubtedly be 'no'. Synonymous codons are synonymous only in the semantic sense; they are not biologically equivalent. An earlier review discussing the apparent evolutionary non-neutrality of codon bias, and speculating about the underlying mechanisms, used the expression 'anecdotal evidence', because the number of cases where the operation of such mechanisms could be experimentally demonstrated was very low. Here I have tried to enumerate all the possible mechanisms and presented examples for the operation of each of these mechanisms. The already fairly large number of these cases obviously represents only the tip of the iceberg. It can safely be predicted that these numbers will grow significantly in the future. It would be a much more difficult task to estimate the relative weight of each of these mechanisms in biological evolution. The summary effect of all these 'synonymous' mechanisms will obviously be very small in comparison to the role of all other types of mutations. The aim of this review was to show that, however small, the effect of such mutations is not zero; such effects exist and they must be reckoned with.

The most dramatic effects of synonymous codon replacement were demonstrated in *in vitro* experimental systems, using synthetic genes or messengers (25, 26) where synonymous replacements could produce several hundredfold differences in the expression levels of the coded proteins. These results are obviously very important for biotechnology and they already have been, and will continue to be, applied in several practical projects. Such large effects, however, are unlikely to occur *in vivo*. They are probably eliminated by purifying selection.

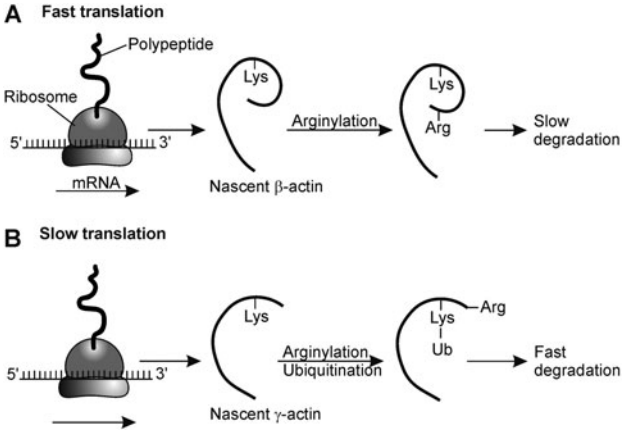

Figure 6 Different secondary modification of β and γ actin.
(A) Fast translation of β-actin allows only arginylation. (B) Slow translation of γ-actin allows both arginylation and ubiquination, leading to rapid degradation.

Among the *in vivo* examples demonstrating the effects of synonymous codon replacements, the largest number of reported cases belongs to the group described in the first chapter of this review, i.e., the splicing anomalies. This fact is probably due to a research bias, as the study of monogenic human diseases represents such an important segment of medical molecular biology research that this alone can explain the relatively large number of reported cases of synonymous codon replacement as a cause of anomalous splicing.

In the last decade the polygenic, rather than the monogenic, human diseases have been the focus of medical molecular biology research. The GWA studies try to discover the associations of different SNPs (and CNVs) with the occurrence of various multigenic diseases and their clinical outcomes. In principle such associations might also involve synonymous codons, and very likely effects of this type will eventually be discovered. So far, however, this reviewer has failed to find any such association described in the literature. This failure could be explained by the facts that: 1) 80% of SNPs lie outside protein-coding genes; 2) The average gene contains only four SNPs, thus the chance of any of these being a synonymous replacement is very small; 3) In many GWA studies only the affected chromosomal loci are identified, not the genes, and even if they are, sometimes the protein sequences are not known; 4) Some GWA studies deliberately exclude synonymous codons from the analysis.

At this point it might be added that in one case where defined genes were known to be involved in a multigenic disease (the *BRCA1* and *BRCA2* genes in breast cancer), the possible role of synonymous codons was investigated. In *BRCA1* no such variants were identified. In *BRCA2* two synonymous variants were found but in a large case-control study no association with breast cancer risk was found (52).

Obviously it is much harder to find *in vivo* evidence for altered lifetime or altered translational efficiency of individual messengers caused by synonymous codon replacement. Very probably a large number of such cases in nonhuman organisms remains unreported in the literature (such unpublished observations in the author's laboratory led him to write this review).

The possible role of micro-RNA as a causative agent of altered gene expression resulting from synonymous codon replacement has so far been experimentally shown in only a single case. However, it seems to be a safe prediction that in the future many new examples of this phenomenon will be found and reported.

Of the six mechanisms discussed in this review, the first (splicing anomalies) results in altered messenger and therefore also altered protein structure. Three mechanisms (altered mRNA tertiary structure, altered mRNA lifetime, miRNA) cause only expression differences. The last two mechanisms, however, lead to altered protein structure,

despite the identical amino acid sequence, and therefore they represent an apparent contradiction of one of the basic tenets of molecular biology, Anfinsen's sequence hypothesis. It must be emphasised that the contradiction is only apparent. According to this principle, the primary sequence of a polypeptide chain unambiguously determines its final structure, under a given set of conditions. These conditions are obviously not identical in cases when the translation proceeds with more or less uniform speed along the polypeptide chain, and when the ribosome stalls at certain codons, thus leaving time for the already finished part of the chain to fold into a stable structure and influencing the folding pattern of the rest of the chain.

Finally, one could ask whether the six mechanisms discussed in this review represent the only possibilities by which synonymous replacements could change the rate of expression or the product of individual genes. Of course this question cannot be answered yet with any degree of certainty. Two new possibilities must, however, be mentioned. In a recent paper Li et al. (53) found widespread sequence differences between the human transcriptome and the corresponding DNA sequences. They claimed that these differences were non-random, and their extent far exceeded that expected on the basis of known RNA-editing mechanisms. These results still require confirmation and the clarification of the responsible mechanisms. If they prove to be correct, then obviously the problem of the possible role of synonymous codon replacements will require a thorough re-evaluation.

Another recent paper (54) claims that in the case of a particular messenger RNA (coding for X-box-binding-protein 1) translational pausing is required for the efficient targeting (into the membrane) of the messenger. The authors raise the possibility that translational pausing may be used for unexpectedly diverse cellular processes in mammalian cells. An implicit corollary of this statement would be that synonymus replacements could in principle be responsible for the alterations of these processes.

Highlights

This review summarises the available evidence demonstrating the effect of several possible mechanisms by which synonymous codon replacements could lead to altered expression of individual genes.

1. In split genes, synonymous mutations in and around splicing sites or in splicing enhancers or silencers often lead to different splicing patterns which in turn may change the structure or the quantity of the coded protein.
2. If the mutation changes the secondary or tertiary structure of the messenger, this can lead to altered efficiency of various steps (especially initiation) of the translation process.
3. Another consequence of altered messenger conformation can be the changed rate of decay (i.e., half-life) of the messenger, again leading to altered expression of the coded protein.
4. The synonymous mutation may influence the interaction of the messenger with a regulatory micro-RNA, thereby changing the regulation of translation.
5. The synonymous mutation can directly influence the kinetics of translation and this in turn may change the folding pattern and therefore some properties of the coded protein.
6. In one reported case this latter effect led to different secondary covalent modification of the coded protein.

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