Comparative analysis of the base biases at the gene terminal portions in seven eukaryote genomes

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ABSTRACT

Adenine nucleotides have been found to appear preferentially in the regions after the initiation codons or before the termination codons of bacterial genes. Our previous experiments showed that AAA and AAT, the two most frequent second codons in Escherichia coli, significantly enhance translation efficiency. To determine whether such a characteristic feature of base frequencies exists in eukaryote genes, we performed a comparative analysis of the base biases at the gene terminal portions using the proteomes of seven eukaryotes. Here we show that the base appearance at the codon third positions of gene terminal regions is highly biased in eukaryote genomes, although the codon third positions are almost free from amino acid preference. The bias changes depending on its position in a gene, and is characteristic of each species. We also found that bias is most outstanding at the second codon, the codon after the initiation codon. NCN is preferred in every genome; in particular, GCG is strongly favored in human and plant genes. The presence of the bias implies that the base sequences at the second codon affect translation efficiency in eukaryotes as well as bacteria.

INTRODUCTION

In bacterial genomes there is a well-characterized sequence element called the Shine–Dalgarno (SD) sequence, which affects the initiation step of translation. The SD sequence is a polypurine stretch, such as AGGAGGU, and is found ~3–10 nt upstream of the initiation codon in bacterial genes (1). It interacts with the complementary sequence at the 3′-end of the 16S rRNA by base pairing, which promotes attachment of the 30S ribosomal subunit to the mRNA (2). Besides the SD sequence, the second codon, the codon immediately after the initiation codon, is reported to affect the efficiency of translation (3–5). Recently we examined the effect of the second codon on translation efficiency by introducing mutations in a monitor gene of Escherichia coli (4). In that paper, we showed that AAA and AAT, the two most frequent second codons in E. coli, significantly enhanced translation efficiency compared with the wild-type, whereas the effects of other codons, including CTG, the most frequent codon throughout the E. coli genome, were not significant. Statistical studies using the whole genome sequences of several bacteria revealed characteristic base biases surrounding the initiation codon or the termination codon, such as A-rich biases present in both 5′- and 3′-terminal portions of open reading frames (6). Therefore, the preference for A-rich codons at the second codon is common to several bacterial species. Some other elements, such as the downstream box (7) or CA repeats (8), are also reported to enhance translation in E. coli. Moreover, the base following the termination codon is biased in some bacterial genomes (6) and it has been shown that the identity of this base determines the efficiency of translation termination in E. coli (9).

In the case of eukaryotes, the mechanism of translation initiation is quite different from bacteria. According to the scanning model proposed by Kozak (10; for a review, see 11), first, the preinitiation complex comprising the 40S ribosomal subunit, the initiator tRNA and some initiation factors attaches to the 5′-end of the mRNA, then the complex begins scanning along the mRNA until it reaches the initiation codon. The initiation codon is recognizable when it is within the Kozak consensus sequence, GCCACCAugG (aug represents the initiation codon) (12). It has been shown that mutations affecting the A nucleotide at position –3 (three bases before the initiation codon), the most highly conserved position in the consensus sequence, strongly impair translation initiation in vivo and in vitro (11). A strong contribution of G at the position next to the initiation codon was also confirmed in experiments with several species (11). However, nothing is known about the mechanism that propels the scanning complex, and how the consensus sequence is recognized and how it functions are not yet known (11). Therefore, although the Kozak model is widely accepted, the precise mechanism of translation initiation in eukaryote cells is still unclear.

Watanabe et al. performed a statistical analysis of the base biases around the initiation and termination codons using all genes in the genome of Saccharomyces cerevisiae (6).
Following *S.cerevisiae* (13), the whole genome sequences of various eukaryotes have been published: *Caenorhabditis elegans* (14), *Drosophila melanogaster* (15), *Arabidopsis thaliana* (16), *Homo sapiens* (17) and *Schizosaccharomyces pombe* (18). The purpose of this study is to elucidate unknown sequence elements which affect translation initiation or termination in eukaryote genomes and, especially, to investigate the possibility that the second codon affects translation initiation in eukaryotes. For this purpose, we extensively examined the base biases after the initiation codon or before the termination codon in the genes of seven eukaryotes for which the complete genome sequences are available [for *Oryza sativa*, all the genes on chromosome 1 (19) were analyzed].

**MATERIALS AND METHODS**

**Data**

The sequences of human genes were downloaded from the RefSeq database (http://www.ncbi.nih.gov/RefSeq/). We used only curated entries with an accession prefix NM_ in RefSeq and thus our data set for human genes does not contain any genes predicted by *ab initio* computation (20). The sequences of the whole proteomes of *D.melanogaster* and *C.elegans* were obtained from BDGP database release 2 (http://www.fruitfly.org) and wormpep76 (http://www.sanger.ac.uk/Projects/C_elegans/wormpep/), respectively. The proteomes of *A.thaliana*, *S.cerevisiae*, *S.pombe* and *E.coli* were retrieved from NCBI Entrez Genomes (http://www.ncbi.nih.gov/Entrez/) (accession nos: NC_003070, NC_003071, NC_003074, NC_003075 and NC_003076 for *A.thaliana*; NC_001133–NC_001148 for *S.cerevisiae*; NC_003421, NC_003423 and NC_003424 for *S.pombe*; NC_000913 for *E.coli*). The sequences of the genes on *O.sativa* chromosome 1 were received from Takuji Sasaki (19). The following data were excluded from the gene set of each species: (i) mitochondrial and chloroplast genes; (ii) sequences containing ambiguous bases; (iii) sequences that do not start from an initiation codon or end with a termination codon; (iv) alternative transcripts except the longest one among them; (v) sequences of which the length from the initiation to the termination codon is shorter than 300 bases. Condition (v) is required because we performed analyses up to the 100th codon from the initiation and termination codons. The numbers of genes used for the analyses were: *H.sapiens*, 12 769; *D.melanogaster*, 12 580; *C.elegans*, 18 547; *A.thaliana*, 24 983; *O.sativa*, 5830; *S.cerevisiae*, 6113; *S.pombe*, 4515; *E.coli*, 3891.

**Evaluation of the biases: the G-test**

The *G*-test (21), or log-likelihood ratio test, was employed for evaluating the deviation of observed distribution of bases or codons from the expected distribution at each position in Figures 1 and 3. In this article, a position in a gene is indicated by a codon number. The initiation codon is numbered 1 and the second codon is codon 2. Codons are also numbered backward from the termination codon using negative numbers; the termination codon is referred to as codon −1 and the codon immediately before the termination codon is numbered −2. All genes for each species in our data set were aligned with the codons from 1 (the initiation codon) to 100 and from −100 to −1 (the termination codon), without gaps. The bias in codon appearance at codon position *i* (Fig. 1) is evaluated by the *G*-value, defined as

\[ G = 2 \sum_{i=1}^{n} O_i \ln(\frac{O_i}{E_i}) - (n - 1) \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i} \]

where *n* is the number of genes, *O* is the fraction of codon *x* among all codons except the termination codons at codon *i*, and *E* is the fraction of codons calculated from all of the codons in all genes for each species. The bias in base appearance at the *j*th letter (j = 1, 2 or 3) in codon position *i* (Fig. 3) is calculated similarly as

\[ G = 2 \sum_{i=1}^{n} O_i \ln(\frac{O_i}{E_i}) = 2 \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i} \]

where *O* is the fraction of nucleotide *n* (A, T, G or C) at the *j*th letter in codon *i*, and *E* is the fraction of nucleotide *n* at the *j*th letter in the entire region of all genes for a given species. It has been shown that the distribution of the *G*-values can be approximated by the χ² distribution when the sample sizes are large (21). In Figure 3, corresponding probabilities (*P*) were calculated from the *G*-values using the χ² distribution with 3 degrees of freedom. The reason we adopted *G*-values instead of the traditional χ² values, χ² = 2Σ{(O − E)²/E}, is that each term in the definition of the *G*-value directly shows that an observed value is larger or smaller than the expected one, whereas the χ² value only gives the deviation of the observed value from the expectation (6). We plotted the value *G*/2 instead of *G*, since this value is free from *N*, thus it is more useful for a comparison of biases among various species having different numbers of genes.

**Evaluation of the biases: the Z-test**

We also performed the *Z*-test to evaluate the deviation of observed appearance of a particular base, amino acid and codon from the expected appearance, shown in Figure 2 and Tables S1 and S2, available as Supplementary Material. The biased second codons

\[ Z = \frac{1}{\sqrt{N}} \sum_{i=1}^{n} \frac{(O_i - E_i)}{E_i} \]

where *N* is the number of genes, *O* is the fraction of a particular base, amino acid or codon at a given position and *E* is the fraction of a particular base, amino acid or codon in the entire region of all genes for each species.

**RESULTS**

**The biased second codons**

In order to survey sequence biases at each position of a gene, we first examined biases in codon appearance using genes in the genomes of seven eukaryotes and *E.coli* (Fig. 1). We found that the second codon is the most biased among all the positions in a gene for every eukaryote examined, as well as *E.coli*, with the exception of *D.melanogaster*, for which the bias at codon −2 is slightly higher than at codon 2. The difference in the biases between the second codon and other positions is more apparent for most eukaryotes than for *E.coli*.

We then performed more detailed analyses of the second codon in the appearance of bases, amino acids and codons (Fig. 2). Figure 2 shows that the patterns of biases at the second codon in eukaryote genes are different from that in *E.coli* genes. The base at each letter in the second codon is biased for A in *E.coli* genes (Fig. 2A, rightmost column), as previously reported (4). In contrast, we found that C is highly preferentially used at the second letter in the second codon for every eukaryote examined in this study. The fraction of C at this position is more than 10% greater than the expectation for
every species (Table S1). It was also found that G is highly preferred at the first and the third letters in the second codon for humans, \textit{A.thaliana} and \textit{O.sativa}. In agreement with these findings, the most frequent and statistically biased second codon for these three species is GCG encoding an alanine (Fig. 2C). In fact, the appearance of GCG at the second codon is more than 10 times larger than the expectation for humans and \textit{A.thaliana}; the fraction of GCG among all 61 codons is only 0.75 and 0.86% in the entire region of all genes for humans and \textit{A.thaliana}, respectively, while it appears at 7.94 and 8.98%, respectively, at the second codon. For \textit{D.melanogaster}, \textit{C.elegans}, \textit{S.cerevisiae} and \textit{S.pombe}, serine-encoding codons are favored as the second codon, although their biases are not outstanding compared with humans or plants.

**Position-dependent base biases**

We also examined biases in base appearance near the termini of genes, besides the second codon. In order to avoid the effect of amino acid preference, we investigated the bias at the third letter in each codon position (Fig. 3). The biases were evaluated by the \(G\)-values, which are known to be approximated by the \(\chi^2\) distribution when the sample sizes are large (21). We found that base appearance is also highly biased in positions other than the second codon. The patterns of the biases are different from species to species and the bias gradually changes depending on its position. For example, the following features are observed for human genes (Fig. 3 and Table S2). First, as mentioned above, the third letter in the second codon is highly biased for G, the fraction being 9% higher than the expectation. Second, GC-rich biases are found in codons 3 to ~60 (\(P < 0.1\%\)), with an intriguing peak at around codon 12. Third, weak but significant A-rich biases are found in the codons from around ~15 to ~3. Fourth, the third letter in codon ~2 is highly C-rich. \textit{Arabidopsis thaliana} and \textit{O.sativa} genes also show strong biases in base appearance (Fig. 3 and Table S2).
DISCUSSION

In this article, we found that the base appearance at the codon third position is highly biased at gene terminal positions in eukaryotes. As shown in Tables S1 and S2, the difference from expectation is as much as 10%. The bias changes gradually depending on its position and generates a species-specific pattern. Because the codon third letters hardly affect the amino acid sequence, this observation cannot be explained by biases in amino acid appearance due to, for example, uneven representation of protein families in each proteome. Such biases in base appearance near the initiation or termination codon are possible signals for controlling translation initiation or termination.

We showed that codon appearance is most biased at the second codon among all positions for almost all eukaryotes we examined (Fig. 1), which is consistent with the idea that the second codon affects translation efficiency in eukaryotes. Codon appearance can be affected by amino acid preference, as we discuss below, however, the third letter in the second codon, which is almost free from amino acid preference, is also highly biased for every species (see Fig. 3 and Table S1).

Figure 2. Biases at the second codon in the appearance of (A) bases, (B) amino acids and (C) codons for seven eukaryote species and E.coli. In (A), the fractions of the bases A (red), T (yellow), G (blue) and C (green) at the first (left), second (middle) and third (right) letters in codons are shown. In each rectangle, the left half represents the fraction of each base at the second codon and the right half represents the fraction of each base in the entire regions of all genes for a given species. In (B), the fraction of each amino acid at the second codon (left) and that in the entire region of all genes for each species (right) are shown. The height of each letter is proportional to the fraction of the amino acid represented by that one letter code. In (C), the fraction of each codon at the second codon (left) and that in the entire region of all genes (right) are shown. The height of each triplet is proportional to the fraction of the codon represented by that triplet. In (B) and (C), the characters are drawn in the order of their fractions from the bottom to the top. The amino acid or codon colored red is the one having the largest Z-value among all 20 amino acids or 61 codons, respectively, showing the most statistically biased amino acid or codon at the second codon for each species. Hsap, H.sapiens; Dmel, D.melanogaster; Cele, C.elegans; Atha, A.thaliana; Osat, O.sativa; Scer, S.cerevisiae; Spom, S.pombe; Ecol, E.coli.
Figure 3. Biases in base appearance at the third letter in each codon position for seven eukaryote species. In the upper graph, the biases are shown by the $G$-values divided by the gene number $N$ (see Materials and Methods). Black and light gray bars correspond to probabilities smaller and larger than 0.1% (i.e. $G$-value = 16.27), respectively. Note that the threshold value of $G/N$ corresponding to $P = 0.1\%$ is different from species to species, because $N$ is species-dependent. In the lower graph, the values of the terms corresponding to each base, A (red), T (yellow), G (blue) or C (green), in the definition of the $G$-value are shown (see Materials and Methods). Positive and negative values are shown in the upper and lower parts of the graph, respectively, without any overlap. The total of four values depicted by four colored bars in the lower graph is equal to the $G$-value in the upper graph at each position. The initiation and the termination codons are omitted.
The bias for G at the third letter in the second codon for humans, *A.thaliana* and *O.sativa* is outstanding. Among four alanine-encoding codons, GCN, the codon GCG is the most minor (10.6%) in human genes; in contrast, the fraction of GCG among the four codons is 35.9% at the second codon. This bias is common to all amino acids: we found that 12 out of 13 non-degenerate G-ending codons are used more frequently than expectation at this position in human genes with statistical significance (*P* < 5%); the only exception is the codon CGG encoding an arginine, but another G-ending codon for arginine, AGG, is highly preferentially used at this position (data not shown).

The eukaryote species we examined can be divided into two groups from the viewpoint of bias at the second codon: for humans, *A.thaliana* and *O.sativa*, GCG is greatly favored as the second codon; in contrast, for *D.melanogaster*, *C.elegans*, *S.cerevisiae* and *S.pombe*, serine-encoding codons appear frequently at this position. This observation is partially inconsistent with the Kozak consensus sequence, GCC-ACCatgG, because G is not necessarily favored as the first letter in the second codon for the species in the latter group, e.g. for *C.elegans* the fraction of G at the position (28.3%) is lower than expectation (29.5%) (Table S1). This inconsistency can be explained by the fact that the compiled genes used to obtain the consensus sequence were biased for mammals (12).

As for the biases at the first and the second letters in the second codon, we cannot distinguish the requirement for translational efficiency from a functional constraint for specific amino acids. A bias for C at the second letter in the second codon has already been reported for *S.cerevisiae*, and was explained by the specificity of the enzyme methionine aminopeptidase (MetAP) (6). It is known that MetAP is responsible for cleaving the initiator methionine from nascent proteins only when the penultimate residue is one of the seven smallest amino acids, glycine, alanine, serine, threonine, proline, valine or cysteine (22), and this specificity is widely conserved in prokaryotes and eukaryotes (23). Four of these amino acids are encoded by NCN codons. Therefore, the requirement for methionine removal would make the base at this position biased for C.

The strong effect of G at the first letter in the second codon on the efficiency of translation initiation has been shown in experiments with mammals and plants, however, the effect is small in *S.cerevisiae* (11). This is totally consistent with our observations. Our observation further suggests that the nucleotide G at the third letter also contributes to translation. Kozak reported that the third letter in the second codon does not affect recognition of the initiation codon in a rabbit reticulocyte translation system (24). However, this is not necessarily inconsistent with our suggestion, for the following reasons: first, Kozak’s experiments were performed under suboptimal conditions in which leaky scanning should occur; second, she conducted the experiments for a limited number of codons, such as CCN, which is not preferred as the second codon. In the case of *E.coli*, it has been experimentally shown that all of the three bases in the second codon influence translation efficiency (4,5). Therefore, it is plausible that the nucleotide at the third (and possibly the second) letter, as well as the first letter, affects translation initiation in some eukaryotes, such as human and plants. In addition, quite strong biases observed at codon –2 preceding the termination codon (Fig. 1) imply that the bases at this position could affect translation termination.

We found that base appearance at the third letter is also significantly biased in positions other than the second codon. Then what is the cause of such position-dependent base biases? For human genes, strong GC-rich biases were found until codon ~60 (Fig. 3). This finding can be plausibly explained in the following way. It is known that CpG dinucleotides are under-represented in vertebrate genomes, but there are small stretches of DNA having the expected frequency of CpG, called CpG islands, which are thought to be related to the control of gene expression. For almost all housekeeping genes and many tissue-specific genes, associated CpG islands are found at the 5’-ends of the gene (25). Therefore, the 5’-terminal portions of genes are expected to be relatively GC-rich. However, GC- or C-rich biases are also observed for *D.melanogaster*, *A.thaliana* and *O.sativa* in regions beyond codon 100 (Fig. 3). The presence of CpG islands in the genomes of these species has not been reported, thus, the cause of these biases is elusive. The strong base biases in this region imply that such species would have unknown mechanisms to control gene expression.

We detected A-rich biases in exactly the same regions, codons 3–7, in the genes of *D.melanogaster*, *A.thaliana*, *S.cerevisiae* and *S.pombe* (Fig. 3). In human or *O.sativa* genes, codons 3–7 are relatively A-rich compared with surrounding regions, because the negative bias against A fades in these positions, whereas the negative bias against T does not (Fig. 3). Several codons preceding the termination codon are also biased for A in the genes of humans, *D.melanogaster* and *O.sativa* (Fig. 3). Because A-rich biases in both termini of genes are also observed in bacteria (6), there could exist some general mechanism to explain this feature. As an explanation for the preference for A near the initiation codon in *E.coli*, Eyre-Walker and Bulmer proposed selection against the formation of secondary structures in mRNA, which would interfere with binding of the ribosome near the start of a gene (26), although it does not explain the bias preceding the termination codon. The patterns of position-dependent base biases in eukaryotes seem to be decomposed into several distinct biases, implying that there are different types of sequence elements which control gene translation in eukaryote genomes.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at NAR Online.

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