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Identification of protein coding genes in genomes with statistical functions based on the circular code

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Abstract

A new statistical approach using functions based on the circular code classifies correctly more than 93% of bases in protein (coding) genes and non-coding genes of human sequences. Based on this statistical study, a research software called 'Analysis of Coding Genes' (ACG) has been developed for identifying protein genes in the genomes and for determining their frame. Furthermore, the software ACG also allows an evaluation of the length of protein genes, their position in the genome, their relative position between themselves, and the prediction of internal frames in protein genes.

Keywords: Protein coding genes; Genomes; Circular code; Statistical functions; Research software

1. Introduction

The concept of code 'without commas', introduced by Crick et al. (1957) for the protein (coding) genes, is a code readable in only one out of three frames. Such a theoretical code without commas, called circular code in the theory of codes (e.g. Béal, 1993; Berstel and Perrin, 1985), is a particular set X of trinucleotides such that a concatenation (a series) of trinucleotides of X leads to sequences that cannot be decomposed in another frame with a concatenation of trinucleotides of X.

For example, suppose that X is the following set of trinucleotides: $X = \{AAC, AAT, ACC, ATC, ATT, CAG, CTC, CTG, GAA, GAC, GAG, GAT, GCC, GGC, GGT, GTA, GTC, GTT, TAC, TTC\}$. Some trinucleotides of X are concatenated randomly, for example as follows:

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– ...CAG,GCC,TTC,AAT,ACC,ACC,CAG,GAA,GAG,GTA,ATT,ACC,AAT,GTA,AAC,TAC,TTC, ACC,ATC...

The commas between the trinucleotides show the frame of construction (reading frame in biology). Suppose now that the commas are 'lost', leading to the sequence:

– ...CAGGCCTTCAATACCACCCAGGAAGAGGTAATTACCAATGTAAACTACTTCACCA TC...

The problem is to retrieve the original frame of construction. There are three obvious possibilities:

– ...C,AGG,CCT,TCA,ATA,CCA,CCC,AGG,AAG,AGG,TAA,TTA,CCA,ATG,TAA,ACT,ACT, TCA,CCA,TC...
– ...CA,GGC,CTT,CAA,TAC,CAC,CCA,GGA,AGA,GGT,AAT,TAC,CAA,TGT,AAA,CTA,CTT, CAC,CAT,C...
– ...CAG,GCC,TTC,AAT,ACC,ACC,CAG,GAA,GAG,GTA,ATT,ACC,AAT,GTA,AAC,TAC,TTC, ACC,ATC...

If the set X of trinucleotides is a circular code, then there is an unique solution:

– ...CAG,GCC,TTC,AAT,ACC,ACC,CAG,GAA,GAG,GTA,ATT,ACC,AAT,GTA,AAC,TAC, TTC,ACC,ATC...

This unique solution is obtained by choosing a window (sufficiently large) in any position in the sequence and then verifying the belonging of the trinucleotides of the window to X:

The first decomposition proposed is rejected immediately as the first trinucleotide AGG in the window does not belong to X. The second decomposition proposed is rejected with a window of 13 nucleotides. Indeed, the first nucleotide A in the window may belong to several trinucleotides of X, e.g. GTA. The trinucleotides GGT, AAT, and

...CAGGCCTTCAATACCACCCAGGAAG[AGG,TAATTACCAATGTAAACTACTTCACCATC...
...CAGGCCTTCAATACCACCCAGGAAG[A,GGT,AAT,TAC,CAA,TGTAAACTACTTCACCATC...
...CAGGCCTTCAATACCACCCAGGAAG[AG,GTA,ATT,ACC,AAT,GTA,AAC,TAC,TTC,ACC,ATC,...

TAC following A belong to X. The next trinucleotide CAA does not belong to X as the 13th nucleotide A (from the beginning of the window) differs from the unique possibility G of CAG belonging to X. The third decomposition is the original one as all the trinucleotides in the window belong to X and the original decomposition of the sequence is deduced automatically.

Such a code was proposed by Crick et al. (1957) in order to explain how the reading of a series of nucleotides in the protein genes could code for the amino acids constituting the proteins. The two problems stressed were: why are there more trinucleotides than amino acids and how to choose the correct reading frame? Crick et al. (1957) proposed that only 20 among 64 trinucleotides code for the 20 amino acids. However, the determination of a set of 20 trinucleotides forming a circular code X depends on a great number of constraints:

i) A trinucleotide with identical nucleotides (AAA, CCC, GGG or TTT) must be excluded from such a code. Indeed, the concatenation of AAA with itself does not allow the retrieval of the reading (original) frame as there are three possible decompositions:
...AAA,AAA,AAA,...,
...A,AAA,AAA,AA...
and ...AA,AAA,AAA,A...
ii) Two trinucleotides related to circular permutation, e.g. ATC and TCA, must be excluded from such a code. Indeed, the concatenation of ATC with itself does not allow the retrieval of the reading (original) frame as there are two possible decompositions: ...ATC,ATC,ATC,... and ...A,TCA,TCA,TC...

Therefore, by excluding AAA, CCC, GGG and TTT and by gathering the 60 remaining trinucleotides in 20 classes of three trinucleotides so that, in each class, the three trinucleotides are deduced from each other by circular permutations, e.g. ATC, TCA, and CAT, a circular code, has only one trinucleotide per class and, therefore, contains at most 20 trinucleotides (maximal circular code). This trinucleotide number is identical to the amino acid number suggesting a circular code assigning one trinucleotide per amino acid.

No set of 20 trinucleotides leading to a circular code has been found at this time. Furthermore, the two discoveries that the trinucleotide TTT, an 'excluded' trinucleotide in the concept of circular code, codes for phenylalanine (Nirenberg and Matthaei, 1961) and that the protein genes are placed in the reading frame with a particular trinucleotide, namely the start trinucleotide ATG, have led to giving up the concept of circular code on the alphabet {A,C,G,T}. For several biological reasons, in particular the interaction between mRNA and tRNA, the concept of circular code has been resumed subsequently regarding the alphabet {R,Y} (R = purine = A or G, Y = pyrimidine = C or T) with two trinucleotide models for the primitive protein genes: RRY (Crick et al., 1976) and RNY (N = R or Y) (Eigen and Schuster, 1978).

Unexpectedly, a maximal circular code has been identified recently in the protein genes of both eukaryotes and prokaryotes on the alphabet {A, C, G, T} (Arquès and Michel, 1996). This circular code has been obtained by two methods:

i) by computing the occurrence frequencies of the 64 trinucleotides AAA,...,TTT in the three frames of protein genes and then, by assigning each trinucleotide to the frame associated with its highest frequency (Arquès and Michel, 1996);
ii) by computing the 12 288 (3×64^2) autocorrelation functions analysing the probability that a trinucleotide in any frame occurs any i bases N after a trinucleotide in a given frame of protein genes and then, by classifying these autocorrelation functions according to their modulo 3 periodicity for deducing a frame for each trinucleotide (Arquès and Michel, 1997a).

The maximal circular code identified is the set X_0 = {AAC,AAT,ACC,ATC,ATT, CAG,CTC,CTG,GAA,GAC, GAG,GAT,GCC, GGC,GGT,GTA,GTC,GTT,TAC,TTC} of 20 tri-

Table 1

List per frame and in lexicographical order of the trinucleotides of the complementary circular code identified in protein coding genes of eukaryotes and prokaryotes (Arquès and Michel, 1996)

T_0:	AAA	AAC	AAT	ACC	ATC	ATT	CAG	CTC	CTG	GAA	GAC	GAG	GAT	GCC	GGC	GGT	GTA	GTC	GTT	TAC	TTC	TTT
T_1:	AAG	ACA	ACG	ACT	AGC	AGG	ATA	ATG	CCA	CCC	CCG	GCG	GTG	TAG	TCA	TCC	TCG	TCT	TGC	TTA	TTG	
T_2:	AGA	AGT	CAA	CAC	CAT	CCT	CGA	CGC	CGG	CGT	CTA	CTT	GCA	GCT	GGA	GGG	TAA	TAT	TGA	TGG	TGT	

Circularity property with the three circular codes X_0, X_1, and X_2 of 20 trinucleotides identified in protein coding genes of eukaryotes and prokaryotes

X_0:	AAC	AAT	ACC	ATC	ATT	CAG	CTC	CTG	GAA	GAC	GAG	GAT	GCC	GGC	GGT	GTA	GTC	GTT	TAC	TTC
X_1:	ACA	ATA	CCA	TCA	TTA	AGC	TCC	TGC	AAG	ACG	AGG	ATG	CCG	GCG	GTG	TAG	TCG	TTG	ACT	TCT
X_2:	CAA	TAA	CAC	CAT	TAT	GCA	CCT	GCT	AGA	CGA	GGA	TGA	CGC	CGG	TGG	AGT	CGT	TGT	CTA	CTT

Complementarity property with the three circular codes X_0, X_1, and X_2 of 20 trinucleotides identified in protein coding genes of eukaryotes and prokaryotes. This property is also verified with T_0 (AAA and TTT) and T_1 and T_2 (CCC and GGG)

T_0:	AAA	AAC	AAT	ACC	ATC	CAG	CTC	GAA	GAC	GCC	GTA									
	TTT	GTT	ATT	GGT	GAT	CTG	GAG	TTC	GTC	GGC	TAC									
T_1:	AAG	ACA	ACG	ACT	AGC	AGG	ATA	ATG	CCA	CCC	CCG	GCG	GTG	TAG	TCA	TCC	TCG	TCT	TGC	TTA	TTG	
T_2:	CTT	TGT	CGT	AGT	GCT	CCT	TAT	CAT	TGG	GGG	CGG	CGC	CAC	CTA	TGA	GGA	CGA	AGA	GCA	TAA	CAA	

Three subsets of trinucleotides can be identified: $T_0 = X_0 \cup \{AAA,TTT\}$in frame 0, $T_1 = X_1 \cup \{CCC\}$ in frame 1 and $T_2 = X_2 \cup \{GGG\}$ in frame 2. The three sets X_0, X_1, and X_2 of 20 trinucleotides are maximal circular codes.

nucleotides in frame 0 of protein genes (reading frame). Furthermore, the two sets X_1 and X_2 of 20 trinucleotides identified in the frames 1 and 2, respectively, (frames 1 and 2 being the frame 0 misplaced trinucleotides in the shifted frames is equal to 24.6%. If the trinucleotides of X are concatenated randomly, for example as follows:

...GAA,GAG,GTA,GTA,ACC,AAT,GTA,CTC,TAC,TTC,ACC,ATC...
then, the trinucleotides in frame 1:
...G,AAG,AGG,TAG,TAA,CCA,ATG,TAC,TCT,ACT,TCA,CCA,TC...
and the trinucleotides in frame 2:
...GA,AGA,GGT,AGT,AAC,CAA,TGT,ACT,CTA,CTT,CAC,CAT,C...

---

shifted by one and two nucleotides respectively in the 5′–3′ direction) by these two methods, are also maximal circular codes (Table 1). These three circular codes have several important properties:

i) circularity: X_0 generates X_1 by one circular permutation and X_2 by another circular permutation (one and two circular permutations of each trinucleotide of X_0 lead to the trinucleotides of X_1 and X_2 respectively) (Table 1).
ii) complementarity: X_0 is self-complementary (ten trinucleotides of X_0 are complementary to the ten other trinucleotides of X_0) and, X_1 and X_2 are complementary to each other (the 20 trinucleotides of X_1 are complementary to the 20 trinucleotides of X_2) (Table 1). Note that this property is also verified with $T_0 = X_0 \cup \{AAA,TTT\}$, $T_1 = X_1 \cup \{CCC\}$ and $T_2 = X_2 \cup \{GGG\}$ (Table 1).
iii) rarity: the occurrence probability of X_0 is equal to 6×10^{-8}. As there are 20 classes of three trinucleotides (see above), the number of potential circular codes is $3^{20} = 3\,486\,784\,401$. The computed number of complementary circular codes with two shifted circular codes (called C^3 codes), such as X_0, is 216. Therefore, its probability is $216/3^{20} = 6 \times 10^{-8}$.
iv) flexibility: the lengths of the minimal windows to automatically retrieve the frames 0, 1, and 2 with the three circular codes X_0, X_1, and X_2 respectively, are all equal to 13 nucleotides and represent the largest window length among the 216 C^3 codes. The frequency of belong mainly to X_1 and X_2, respectively. A few trinucleotides are misplaced in the shifted frames. With this example, in frame 1, nine trinucleotides belong to X_1, one trinucleotide (TAC) to X_0 and one trinucleotide (TAA) to X_2. In frame 2, eight trinucleotides belong to X_2, two trinucleotides (GGT, AAC) to X_0 and one trinucleotide (ACT) to X_1. By computing exactly, the average frequencies of misplaced trinucleotides in frame 1 are 11.9 for X_0 and 12.7% for X_2. In frame 2, the average frequencies of misplaced trinucleotides are 11.9 for X_0 and 12.7% for X_1. The complementarity property explains on the one hand that the frequency equality of X_0 in frames 1 and 2 and on the other hand, the frequency equality of X_2 in frame 1 and X_1 in frame 2. The sum of percentages of misplaced trinucleotides in frame 1 (X_0 and X_2) is equal to the sum of percentages of misplaced trinucleotides in frame 2 (X_0 and X_1) and is equal to 24.6%. This value is close to the highest frequency (27.9%) of misplaced trinucleotides among the 216 C^3 codes. The four types of nucleotides occur in the three trinucleotide sites with the three circular codes X_0, X_1, and X_2 (Table 1).
v) evolutionary: an evolutionary analytical model at three parameters (p, q, t) based on an independent mixing of the 20 trinucleotides of X_0 with equiprobability (1/20) followed by $t \approx 4$ substitutions per trinucleotide according to the proportions $p \approx 0.1$, $q \approx 0.1$ and $r = 1 - p - q \approx 0.8$ in the three trinucleotide sites,

respectively, retrieves the frequencies of X_0, X_1, and X_2 observed in the three frames of protein genes.

The proof that X_0, X_1, and X_2 are circular codes, the detailed explanation of the properties (i–iv) and the different biological consequences, in particular on the two-letter genetic alphabets, the genetic code and the amino acid frequencies in proteins, are given in Arquès and Michel (1996, 1997a). The property (v) is described in Arquès et al. (1998, 1999).

Note: a non-complementary circular code has been identified recently in the mitochondrial protein genes (Arquès and Michel, 1997b).

As the circular code is a strong structural property of protein genes, different statistical functions based on the circular code are investigated in this paper in order to discriminate between coding and non-coding genes. Indeed, the sets of 20 trinucleotides based on a circular code, i.e. the 216 C^3 codes and in particular X_0, X_1, and X_2, have a lesser number of misplaced trinucleotides in the shifted frames compared with the vast majority of sets without particular property. This low number implies that the three circular codes X_0, X_1, and X_2 can clearly be associated with the three frames 0, 1, and 2, respectively, (detailed in method).

After having validated this statistical approach with the human sequences from the EMBL database, research software has been developed for identifying protein genes in genomes and for determining their frame. Furthermore, this software also allows an evaluation of the length of protein genes, their position in the genome, their relative position between themselves, and the prediction of internal frames. These possibilities are presented with five examples taken from human chromosomes: a large protein gene, a complementary protein gene, a series of five exons, a protein gene with four internal frames, and a possible coding region in the human DNA sequence. An example with a prokaryotic genome is also given.

2. Method

2.1. Introduction

The method developed is based on a strong structural property of protein genes, i.e. the circular code, and in particular its properties of circularity and complementarity. This method differs from the classical methods, such as the codon usage methods and the HMM methods, at least for the following reasons:

i) The circular code is observed in protein genes of eukaryotes as well as of prokaryotes and is not found in the non-coding genes (Arquès et al., 1998). Therefore, a method based on this circular code can be applied independently of the type of eukaryotic/prokaryotic organism under investigation. In contrast, the codon usage methods use codon frequencies that depend on the species and the functional classes of protein genes (see e.g. Karlin et al., 1998).

Table 2
A few examples taken from the Table 1 of Arquès and Michel (1996) showing that the codons GTC and GTT belonging to X_0 occur in frame 0 with lower frequencies compared with the codons ATG belonging to X_1 and CAA belonging to X_2, etc.

Codon in frame 0	Frequency (%)	Codon in frame 1	Frequency (%)	Codon in frame 2	Frequency (%)
ATG	2.31	ATG	3.08	ATG	0.57
CAA	1.65	CAA	1.55	CAA	3.71
CCA	1.66	CCA	2.91	CCA	2.04
GGA	1.76	GGA	1.27	GGA	3.49
GTC	1.60	GTC	0.81	GTC	1.05
GTT	1.55	GTT	0.75	GTT	1.35
TCC	1.63	TCC	1.85	TCC	1.40

ii) The circular code X_0 (respectively, X_1 and X_2) contains the 20 codons having a preferential occurrence in the frame 0 (respectively, 1 and 2). It is important to stress that the set X_0, for example, does not necessarily represent the common codons in frame 0, i.e. the 20 codons having the highest frequencies in frame 0 (see a few examples in Table 2).

iii) The 216 C^3 codes have a low number of misplaced trinucleotides in the shifted frames, 27.9% in the worst case and 24.6% for X_0. This number is close to $2/3 \approx 66.6\%$ with the vast majority of trinucleotide sets without particular property. Indeed, by excluding AAA, CCC, GGG, and TTT, there is one chance out of three to observe, for example, a codon of X_1 in frame 1, i.e. two chances out of three to observe a codon of X_0 or X_2 in frame 1. In summary, the method developed according to the circular code allows to associate clearly the three sets of trinucleotides X_0, X_1, and X_2 with the three frames 0, 1 and 2 respectively of protein genes.

iv) The complementarity property of these three sets X_0, X_1, and X_2 is used for identifying protein genes on the direct strand but also on the complementary strand (see the definition of the four functions below).

v) The method developed is based on the global probabilities of X_0, X_1, and X_2 and not on the individual codon probabilities that are used in the codon usage methods.

2.2. Definition of statistical functions

Let t be a trinucleotide in the set {AAA, ..., TTT} (64 trinucleotides). Let F be a population with $m(F)$ sequences S. Each sequence S has a base length $l(S)$. Let w_i be a window of n trinucleotides starting at the base position i, $i = 1, \ldots, l(S)-3n+1$, in a sequence S of F, i.e. $w_i = t_1 \ldots t_n$ where t_j is the jth trinucleotide in the window w_i. Let T_g, $g \in \{0, 1, 2\}$, be the three subsets of trinucleotides constituting the three circular codes in the protein coding genes of eukaryotes and prokaryotes, T_0 in the open reading frame (frame 0) and, T_1 and T_2, in the shifted frames 1 and 2, respectively (Table 1). In a given window w_i, the function

$$\delta_g(t_j) = \begin{cases} 1 & \text{if } t_j \in T_g \\ 0 & \text{if } t_j \notin t_g \end{cases}$$

determines whether or not if the trinucleotide t_j at the position j in w_i belongs to T_g with $g \in \{0, 1, 2\}$. Next, the occurrence frequency $P(T_g, w_i)$ of a subset T_g in w_i, is $P(T_g, w_i) = \Sigma_{j=1}^{n} \delta_g(t_j)/n$ where n is the total number of trinucleotides in the window w_i.

Several statistical functions based on the properties of the circular code, are defined:

$$F_1(i) = P(T_0, w_i) \quad (1)$$

$$F_2(i) = P(T_0, w_i) - P(T_2, w_i) \quad (2)$$

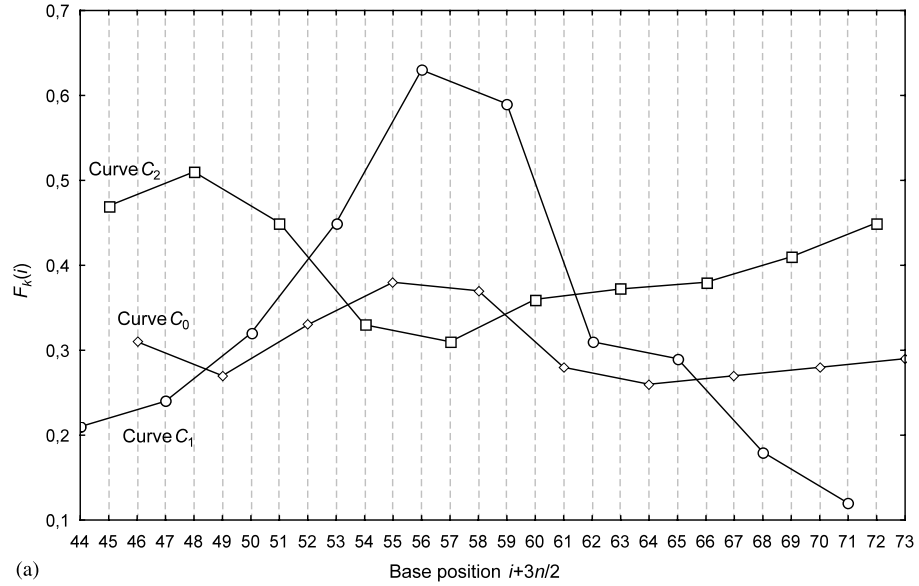
$$F_3(i) = \frac{(21/22)2P(T_0, w_i)}{(P(T_1, w_i) + P(T_2, w_i))} \quad (3)$$

$$F_4(i) = \sum_{j=0}^{2} P(T_j, w_{i+j}) \quad (4)$$

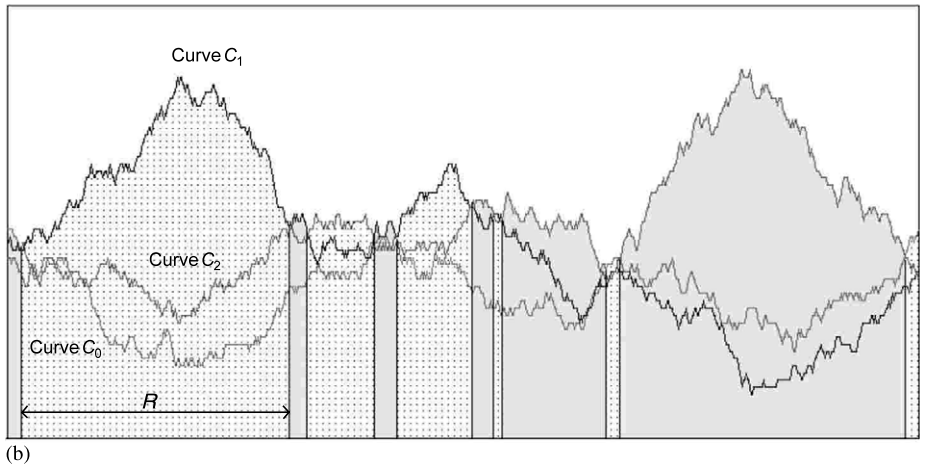
These four statistical functions use different properties of the circular code, in particular the properties of circularity and complementarity.

The function F_1 is the simplest, and is based on the circular code X_0 (extended to T_0) in each window w_i. In a protein gene, $F_1(i)$ associated with the reading frame of the sequence (i.e. w_i in reading frame and, therefore, w_{i+1} and w_{i+2} in the shifted frames 1 and 2, respectively) is in general greater than $F_1(i+1)$ and $F_1(i+2)$ as the occurrence probability of T_0 is by definition maximum in the reading frame (see point (ii) of Section 2.1 explaining the misplaced trinucleotides).

The function F_2 considers the two circular codes X_0 and X_2 (extended to T_0 and T_2). The probability difference $P(T_0, w_i) - P(T_2, w_i)$ is maximum among the 18 possible probability differences in the 3 frames. Indeed, the average probabilities of T_0, T_1, and T_2 in the frame 0 (respectively, 1, 2) of protein genes are 49% (respectively, 26.5%, 32%), 28.5% (respectively, 43%, 23%), and 22.5% (respectively, 30.5%, 45%) (Arquès et al., 1998). By consequence, the max-



(a)



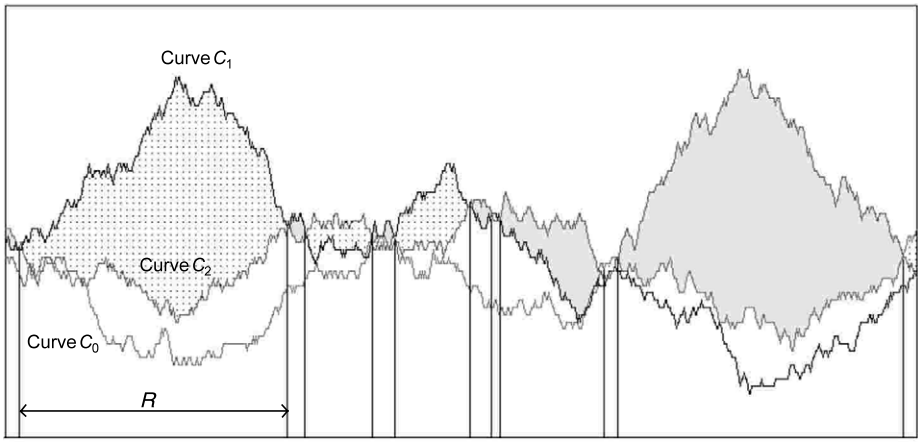
(b)

Fig. 1. (a) Representation of a function F_k by three curves modulo 3. By convention, the curve C_0 (respectively, C_1, C_2) joins the points in base position 1 (respectively, 2, 0) modulo 3 and, therefore, is related to the base position in frame 0 (respectively, 1, 2) determined from the beginning of the sequence. (b) Representation of the discrete sums $\Sigma_{c\in R}F_k^M(c)$ in different ranges R by surfaces. For a given range, the surface is associated with the highest curve. (c) Representation of the discrete sums $\Sigma_{c\in R}F_k^D(c)$ in different ranges R by surfaces. For a given range, the surface is associated with the difference between the two highest curves.

imum probability difference in frame 0 (respectively, 1, 2) is 26.5% with $\mathrm{Prob}(T_0)-\mathrm{Prob}(T_2)$ (respectively, 16.5% with $\mathrm{Prob}(T_1)-\mathrm{Prob}(T_0)$, 22% with $\mathrm{Prob}(T_2)-\mathrm{Prob}(T_1)$).

The functions F_3 and F_4 are based on the three circular codes X_0, X_1, and X_2 (extended to T_0, T_1, and T_2). The function F_3 tests a ratio that is maximum in the reading frame. The functions F_1, F_2, and F_3 favor the circular code X_0 characterising the reading frame, while the function F_4 considers the three circular codes in their three associated frames.

Finally, as T_0 is self-complementary, and as T_1 and T_2 are complementary to each other, the four



(c)

Fig. 1 (*Continued*)

functions have a similar behaviour on both strands.

These statistical functions are represented by curves as follows. As the value of $F_k(i)$ is a mean value computed from the bases of the window w_i, i.e. the bases i, $i+1$, …, $i+3n-1$, the point associated with $F_k(i)$ is represented graphically in the abscissa by the base position $i+\lfloor 3n/2 \rfloor$ (greatest integer less or equal) and in the ordinate by $F_k(i)$. As the protein coding genes have three frames, a function F_k is represented by three curves where the points are joined modulo 3: by convention, the curve C_j, $j=0$, 1, 2, joins the points associated with $F_k(i)$ with $i=(j+1)$ mod 3, i.e. the curve C_0 (respectively, C_1, C_2) joins the points in base position 1 (respectively, 2, 0) modulo 3 (Fig. 1a) and, therefore, is related to the base position in frame 0 (respectively, 1, 2) determined from the beginning of the sequence.

The four functions defined above are extended to the trinucleotide concept as follows: the maximum of a given previous function F_k in a series of three successive bases and the difference between the maximum and the second highest of a given previous function F_k in a series of three successive bases.

Let $\tilde{t}_c$ be the cth trinucleotide in the genome sequence, i.e. constituted by the three bases in position i such that $c=\lceil i/3 \rceil$ (smallest integer greater or equal). Then,

$$F_k^M(c) = \max_{j=0,1,2} F_k(3c-j) \tag{5}$$

$$F_k^D(c) = F_k(3c-j_0) - F_k(3c-j_1) \tag{6}$$

so that j_0, j_1, $j_2 \in \{0, 1, 2\}$ are defined by the inequality $F_k(3c-j_0) \geq F_k(3c-j_1) \geq F_k(3c-j_2)$.

Note: The two functions F_k^M and F_k^D always have values greater than or equal to 0.

The statistical significance of the two functions F_k^M and F_k^D is evaluated according to the parameter s based on the discrete sum (called 'surface') of the values of a function in a given range R of trinucleotides. The function F_k^M (respectively, F_k^D) identifies the three bases of the trinucleotide $\tilde{t}_c$ as coding bases if $\Sigma_{r\in R} F_k^M(r) > s$ (respectively, $\Sigma_{r\in R} F_k^D(r) > s$) where R is the greatest range containing c such that $\forall r \in R$, $\max_{j=0,1,2} F_k(3r-j) = F_k(3r-j_0)$ where $j_0 \in \{0, 1, 2\}$ is constant. In order to visualise this concept, these two discrete sums $\Sigma_{r\in R} F_k^M(r)$ (respectively, $\Sigma_{r\in R} F_k^D(r)$) are represented in the Fig. 1b (respectively, Fig. 1c).

In summary, eight functions are analysed with the parameter s, with F_k^M and F_k^D by varying k between 1 and 4. This statistical method is the main scientific part of the research software that is presented below.

2.3. Development of a research software called ACG

The main functionalities of the research software called ACG are the statistical analyses of

different functions based on the circular code in sequence populations, the identification of protein genes in genomes, and the determination of their frame. Furthermore, several patterns of protein genes can be evaluated: their length, their position in the genome, their relative position between themselves, and the presence of internal frames. Several examples of these possibilities are given in the Section 3.

The software is written with three units: a sequence analysis unit, a statistical function unit and an interface unit.

The sequence analysis unit reads the sequences and computes the occurrence frequency $P(T_g, w_i)$ in a window according to the algorithm described below. This unit calls the statistical function unit for computing a chosen function F_k^M and F_k^D. Precisely, the four functions F_k and their trinucleotide evaluation F_k^M or F_k^D are implemented in this statistical function unit, which allows statistical numerical results on a sequence population F (eventually on one sequence). The interface unit allows the choice of different statistical parameters: the EMBL sequence file (population F or sequence S), the statistical function F_k^M or F_k^D, the window length n in trinucleotides, and the statistical surface parameter s. It also has a graphical functionality for displaying the graphical curves: the start base position in the sequence, the curve display window length in bases, the left/right scroll of a curve allowing to display a curve again, and a coloured curve associated with the frame for a direct interpretation. The curve display window can be printed on a broad range of printing devices. The statistical numerical results are stored in text files.

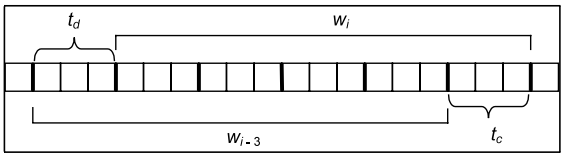


Fig. 2. Computation of the three occurrence frequencies $P(T_g, w_i)$ in the window w_i from the window w_{i-3}. The two windows w_i and w_{i-3} differ only from one trinucleotide. The 'destroyed trinucleotide' t_d is the trinucleotide belonging to w_{i-3} but not to w_i, i.e. the trinucleotide from the position $i-3$ to $i-1$. Similarly, the 'constructed trinucleotide' t_c is the trinucleotide belonging to w_i but not to w_{i-3}, i.e. the last trinucleotide in w_i that starts in position $i+3(n-1)$ and ends in position $i+3n-1$.

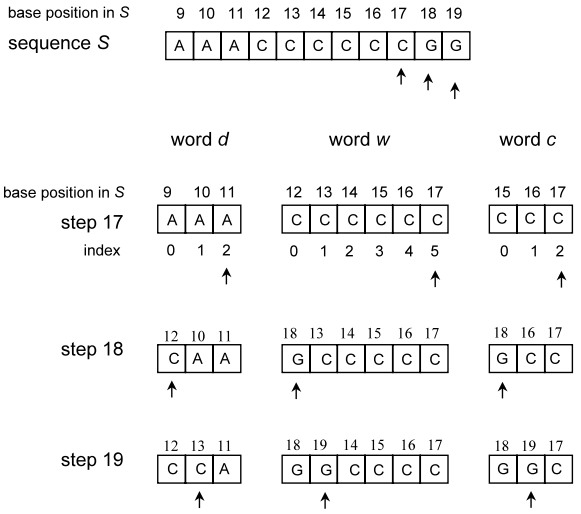


Fig. 3. Example of a progress of the algorithm. This example is applied with a subsequence of S between the base positions 9 and 19 and with a base length of the window w equal to $3n=6$. At the step $k=17$ treating the 17th base in S, the window w_i is in position $i=k-3n+1=17-6+1=12$ and contains then the bases w = CCCCCC. The 'destroyed trinucleotide' t_d is the trinucleotide starting at the position $i=12-3=9$: the word d contains d = AAA. The 'constructed trinucleotide' t_c is the trinucleotide starting at the position $i+3(n-1)=12+3=15$: the word c contains $c=$ CCC. At this step $w=w_{12}$, $d=t_d$ and $c=t_c$. At the step $k=18$ treating the 18th base in the sequence, the base in the position (k mod $3n$) = 0 in the word w is moved in the position (k mod 3) = 0 in the word d. Therefore, $d=$ CAA. Then, the 18th base in the sequence is read and placed at the position (k mod 3) = 0 in the word c and at the position (k mod $3n$) = 0 in the word w. Therefore, $c=$ GCC and $w=$ GCCCCC. At this step, $w \neq w_{13}$, $d \neq t_d$ and $c \neq t_c$. The words w, d and c can be obtained by circular permutations from w_i, t_d and t_c, respectively. This process is reiterated at the step 19.

This structure in units easily allows modifications and extensions of the software ACG. ACG has been developed to be interactive and user-friendly. ACG is written in Pascal Delphi and implemented on IBM compatible microcomputers. It can be used without any computer knowledge.

The algorithm for computing the occurrence frequency $P(T_g, w_i)$ is constructed such that the different bases in each sequence are read only one time.

A window w_i of n trinucleotides runs from the first ($i=1$) base to the base position $i=l(S)-3n+$

1 in the sequence. At each step of the algorithm, a base is read in the sequence and treated.

From the base position $k=1$ to $k=3n+2$, the algorithm computes the values $P(T_g, w_i)$ for $g=0$, 1, 2 and $i=1$, 2, 3.

From the base position $k=3n+3$ to $l(S)$, the algorithm computes for $i=k-3n+1$, the values $P(T_g, w_i)$ for $g=0$, 1, 2, i.e. from $i=4$ to $l(S)-3n+1$. The value $P(T_g, w_i)$ is deduced from $P(T_g, w_{i-3})$. Indeed, the two windows w_i and w_{i-3} differ only from one trinucleotide (Fig. 2). Let the 'destroyed trinucleotide' t_d be the trinucleotide belonging to w_{i-3} but not to w_i, i.e. the trinucleotide from the position $i-3$ to $i-1$. Similarly, let the 'constructed trinucleotide' t_c be the trinucleotide belonging to w_i but not to w_{i-3}, i.e. the last trinucleotide in w_i that starts in position $i+3(n-1)$ and ends in position $i+3n-1$ (Fig. 2). Suppose that $t_d \in T_g$ and $t_c \in T_{g'}$. If $g \neq g'$, then $P(T_g, w_i) = P(T_g, w_{i-3})-1/n$ and $P(T_{g'}, w_i) = P(T_{g'}, w_{i-3})+1/n$. If $g=g'$, then $P(T_{g''}, w_i) = P(T_{g''}, w_{i-3})$ for $g''=0$, 1, 2.

This algorithm is implemented with three words indexed from 0: two words d and c of length three associated with the destroyed and constructed trinucleotides respectively, and w of length $3n$, with the current window. At the step treating the kth base in the sequence, the base in the position k modulo $3n$ in the word w is moved in the position k modulo 3 in the word d. Then, the kth base in the sequence is read and placed at the position k modulo 3 in the word c and at the position k modulo $3n$ in the word w. In this way, the three words contain correctly the series of bases of the sequence which is read only one time.

Example of computation (Fig. 3): The subsequence of S that is analysed comprises the base positions between 9 and 19. The base length of the window w is chosen as $3n=6$. The proposed computation starts at the step $k=17$, treating the 17th base in S. The window w_i is in position $i=k-3n+1=17-6+1=12$ and then contains the bases w = CCCCCC. The 'destroyed trinucleotide' t_d is the trinucleotide starting at the position $i=12-3=9$: the word d contains $d=$AAA. The 'constructed trinucleotide' t_c is the trinucleotide starting at the position $i+3(n-1)=12+3=15$: the word c contains $c=$CCC. Note that at this step $w=w_{12}$, $d=t_d$, and $c=t_c$. At the step $k=18$ treating the 18th base in the sequence, the base in the position $(k \bmod 3n)=0$ in the word w is moved in the position $(k \bmod 3)=0$ in the word d. Therefore, $d=$CAA. Then, the 18th base in the sequence is read and placed at the position $(k \bmod 3)=0$ in the word c and at the position $(k \bmod 3n)=0$ in the word w. Therefore, $c=$GCC and $w=$GCCCCC. Note that at this step, $w \neq w_{13}$, $d \neq t_d$, and $c \neq t_c$. The words w, d, and c can be obtained by circular permutations from w_i, t_d, and t_c, respectively. This method avoids base shifting in the words. This process is reiterated at the next steps (see the Fig. 3 for the step 19).

2.4. Data acquisition

The gene population F used for the statistical analysis is made of all human sequences (84 222 sequences, 303 124 560 bases) obtained from release 57 (December 1998) of the EMBL Nucleotide Sequence Data Library. This large population leads to stable frequencies for the different functions analysed (law of large numbers). Therefore, these functions can be compared in order to identify the most interesting. The protein coding genes are extracted according to the keyword CDS without discarding particular sequences. In this population, 9.4% of bases are annotated as coding. After the validation of the statistical approach, the research software ACG has been developed for identifying protein genes and used with the human chromosomes (Sanger Centre, March 1999).

3. Results

3.1. Statistical results

The different functions are evaluated with the software ACG according to the classical parameter Simple Matching Coefficient (SMC) (Burset and Guigó, 1996), which considers the proportion of bases (according to the EMBL release) identified correctly by the function. Let $\Sigma_{s \in F}\, l(S) = n_F$ be the total number of bases in the gene population F. Let True Positives (TP) (respectively, True Nega-

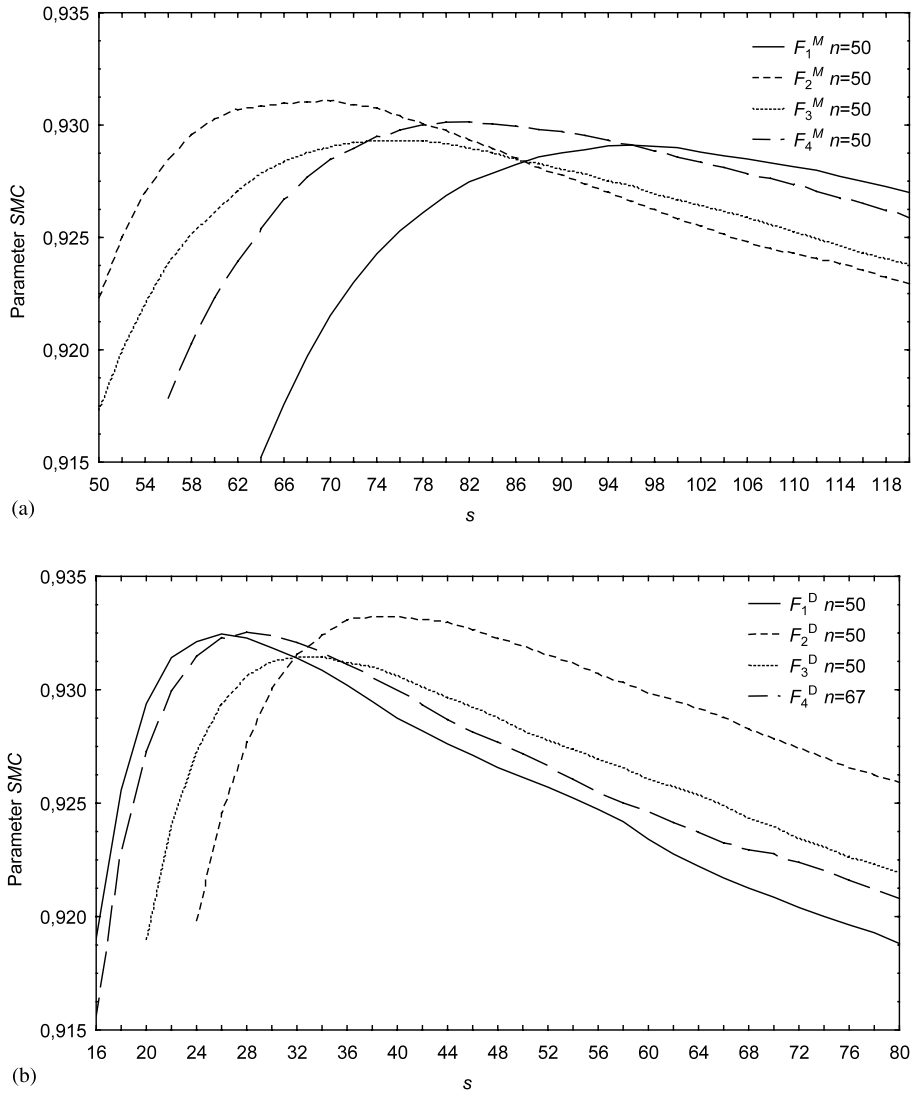


Fig. 4. (a) Statistical results giving the proportion SMC of bases identified correctly by the four functions F_k^M. The functions F_k^M are evaluated by varying the parameter surface s between 6 and 130 with a step of 2 and the window length n between 33 and 169 trinucleotides with a step of 17 trinucleotides. The maximum value of the proportion SMC is given with a function F_k^M by varying s for a given n. The four maxima of the four functions F_k^M are all less than the four maxima of the four functions F_k^D (see Fig. 4b). (b) Statistical results giving the proportion SMC of bases identified correctly by the four functions F_k^D evaluated by the parameter s. The functions F_k^D are evaluated by varying the parameter surface s between 6 and 130 with a step of 2 and the window length n between 33 and 169 trinucleotides with a step of 17 trinucleotides. The maximum value of the proportion SMC is given with a function F_k^D by varying s for a given n. The parameter SMC is maximum with the function F_2^D with $n = 50$ and $s = 38$ and equal to 93.32% of bases identified correctly.

tives (TN)) be the total number of bases identified as coding (respectively, non-coding) bases by a function (defined above) in the coding (respectively, non-coding) genes in the gene population F. The coefficient SMC is then defined as SMC = (TP+TN)/n_F (Burset and Guigó, 1996).

The eight functions F_k^M and F_k^D defined above are analysed with the coefficient SMC. These

functions are evaluated with the parameter surface s between 6 and 130 with a step of 2. They are calculated with a window length n varying between 33 and 169 trinucleotides with a step of 17 trinucleotides. For each function, a maximum value of the coefficient SMC is obtained for given values of s and n. The eight curves associated with the eight maximum values of the eight functions are represented in Fig. 4a and b by varying s for a given n. Fig. 4a (respectively 4b) gives the four curves F_k^M (respectively, F_k^D).

The Fig. 4a and b show that the coefficient SMC is maximum with the function F_2^D with $n = 50$ and $s = 38$, which identifies 93.32% of bases correctly.

For the function giving the maximum value of the coefficient SMC (F_2^D with $n = 50$ and $s = 38$), four other classical measures are computed, Sn, Sp, Sp′, and CC, as follows (Burset and Guigó, 1996). Let False Positives (FP) (respectively, False Negatives (FN)) be the total number of bases identified as coding (respectively, non-coding) bases by a function (defined above) in the non-coding (respectively, coding) genes in the gene population F. Note: $TP+TN+FP+FN = n_F$.

The definitions and results of these four measures are:

i) The Sensitivity Sn is the proportion of coding bases identified correctly by the function:

$$Sn = \frac{TP}{TP + FN} = 39.75\%$$

ii) The Specificity (Sp) is the proportion of non-coding bases identified correctly by the function:

$$Sp = \frac{TN}{TN + FP} = 98.88\%$$

iii) Another definition of the Specificity Sp′ is the proportion of coding bases among the bases identified as coding by the function:

$$Sp' = \frac{TP}{TP + FP} = 78.71\%$$

iv) The correlation coefficient CC is a measure of global accuracy where the value 1.00 corresponds to a perfect prediction and where the value 0.0 is expected for a random prediction:

$$CC = \frac{(TP \times TN) - (FN \times FP)}{((TP + FN)(TN + FP)(TP + FP)(TN + FN))^{1/2}} = 0.53$$

3.2. Applications of the research software ACG with the human chromosomes

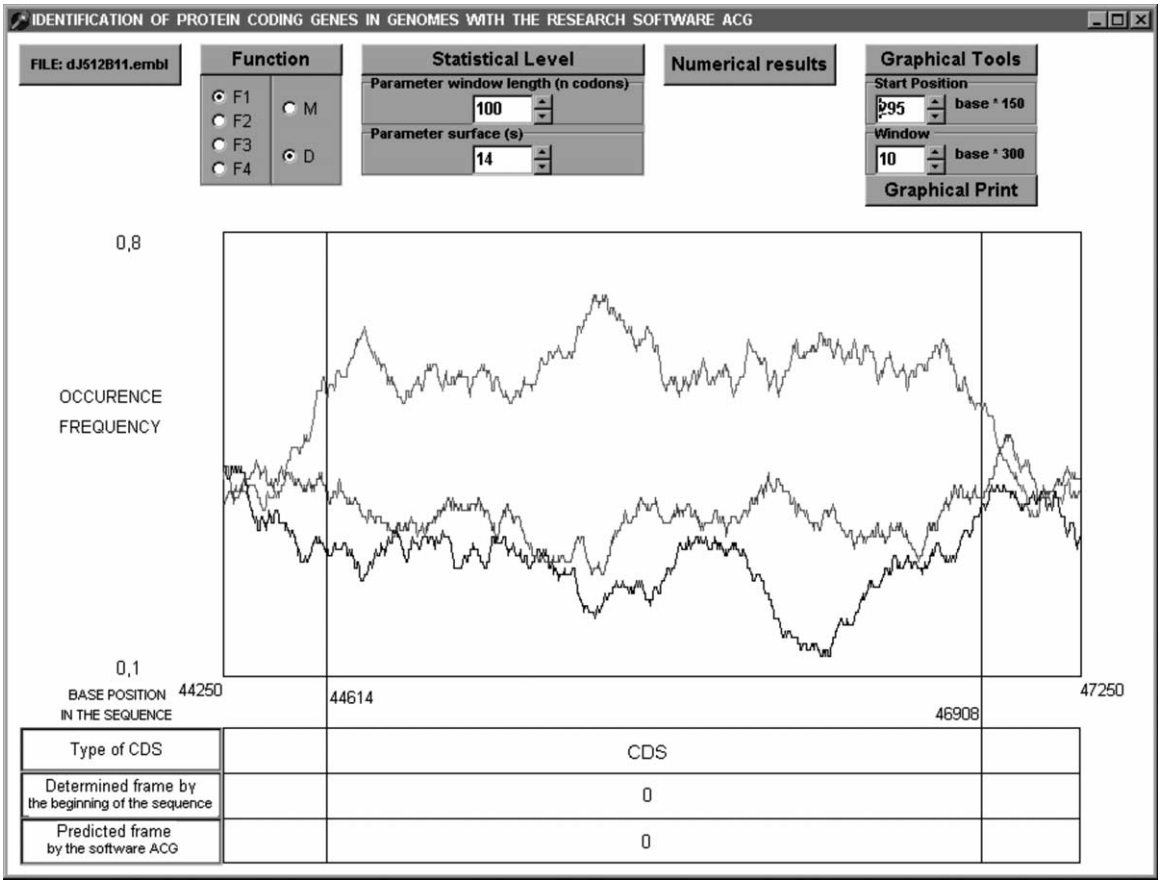
3.2.1. Three examples leading to classical results

The research software ACG identifies protein genes and their frames as follows:

> The identification of a protein gene (called CDS according to the EMBL syntax) results from a curve that is significantly greater than the two others that lead to a large surface s (notion introduced in Section 2). The existence of a top curve is justified by the fact that the associated function F_k is based on the circular code, which is a strong property of the protein genes (see Section 1). The intersection of the two highest curves allows for predicting a beginning and end regions of protein genes.
> The identification of a frame of a protein gene is deduced from the frame of the top curve determined from the beginning of the sequence (see the Section 2).

Three examples of identification of protein genes (CDS) listed in the EMBL human chromosomes with the software ACG, are given.

Fig. 5a identifies a large CDS (2295 bases in the human DNA sequence from clone 512B11 on chromosome 6p24–25). Indeed, the curve becomes greater than the two others. Note that in a random sequence, e.g. a sequence generated with the four bases with equiprobability, leads to three similar horizontal curves. These three curves are gathered together before the beginning of the CDS (5′

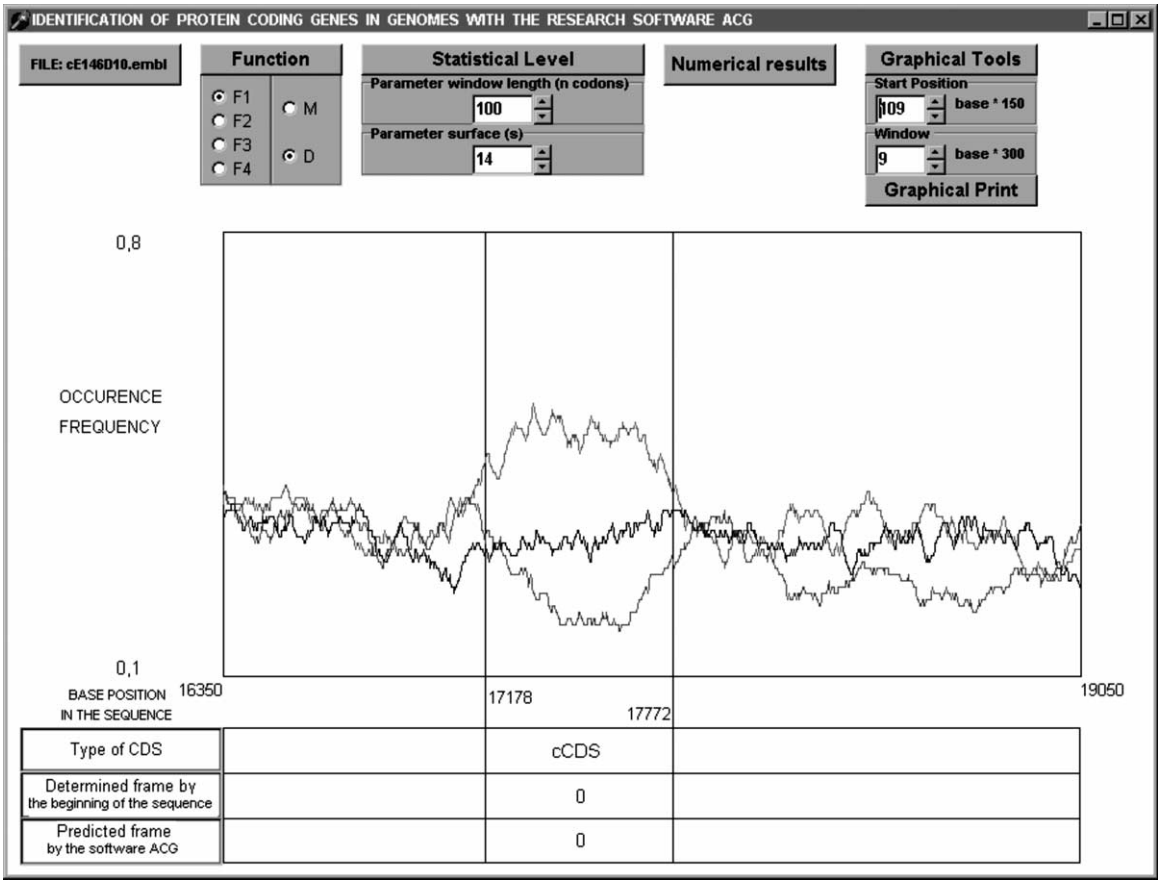


(a)

Fig. 5. (a) Identification of a protein coding gene (CDS) and its frame by the software ACG. The CDS chosen as an example, starts at the base 44 614 and ends at the base 46 908 in the human DNA sequence from clone 512B11 on chromosome 6p24–25. (b) Identification of a complementary protein coding gene (cCDS) and its frame by the software ACG. The cCDS chosen as an example, starts at the complementary base 17 772 and ends at the complementary base 17 178 in the human DNA sequence from clone E146D10 on chromosome 22. (c) Identification of several protein coding genes (CDS) and their frames by the software ACG. This example is observed in the human DNA sequence from cosmid B2046 on chromosome 6.

regions) and after the end of the CDS (3′ regions), close to random sequences. As the top curve is C_0, the predicted frame of the CDS from the beginning of the sequence, is 0. This is in agreement with the EMBL frame (frame $(44\,614-1) \bmod 3 = 0$).

Fig. 5b identifies a complementary protein coding gene (cCDS in the human DNA sequence from clone E146D10 on chromosome 22) of middle size (595 bases). The cCDS chosen as an example, starts at the complementary base 17 772 and ends at the complementary base 17 178. As the top curve is C_0, the predicted frame of the cCDS is 0. The circular code T_0, being self-complementary, a function of F_k based on T_0, leads to the same results on the DNA complementary strand. Therefore, the association of a frame with a curve, and in particular the top curve, is identical with the CDS and the cCDS. The predicted frame 0 agrees with the EMBL frame. The determination of the frame, from the beginning of the sequence, of a cCDS starting at the complementary base position j and ending at the complementary base position k



(b)

Fig. 5 (*Continued*)

is obtained with the value j modulo 3. The EMBL frame with this example is then 0, as $17\,772 \bmod 3 = 0$.

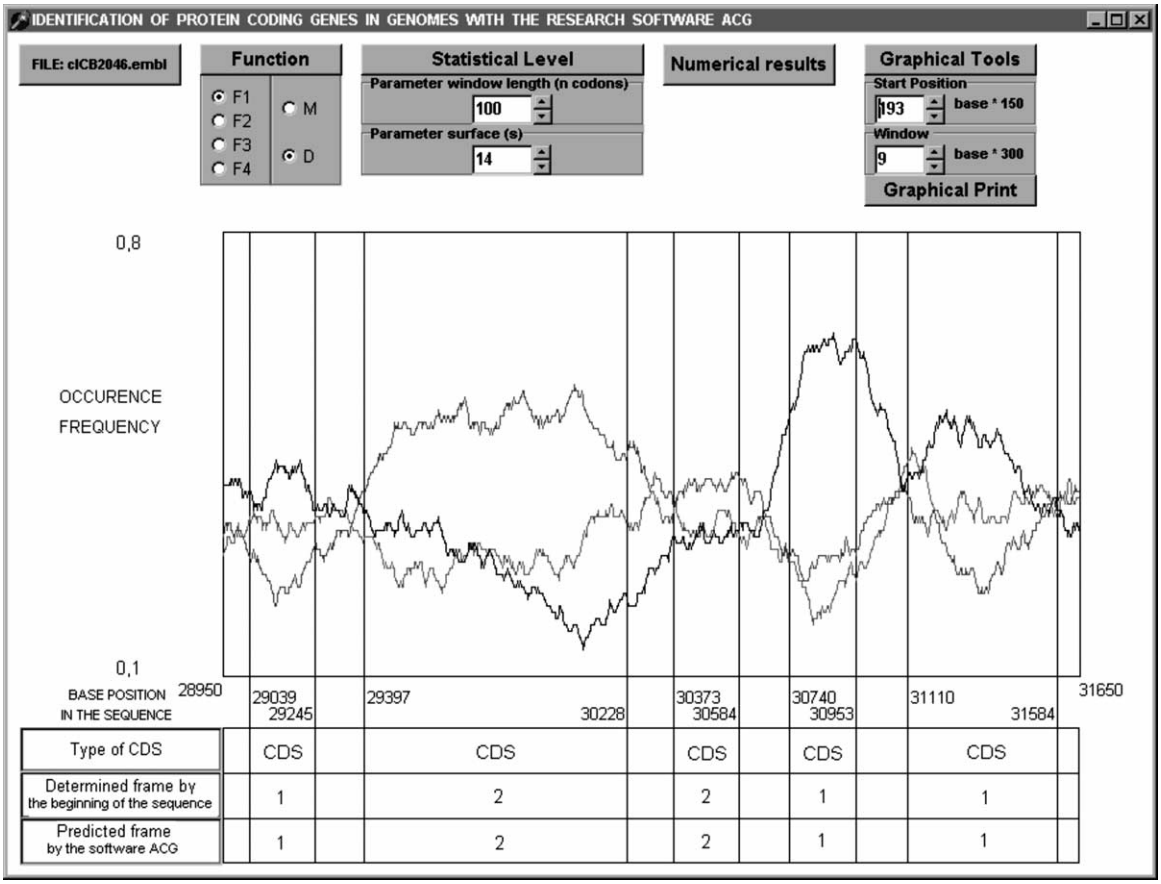
Fig. 5c identifies a series of five CDS (exons in the human DNA sequence from cosmid B2046 on chromosome 6), three among them have a small size (about 200 bases). Their frames determined by the software ACG are 1, 2, 2, 1, and 1 respectively. All the predicted frames agree with the frames deduced from the EMBL data:

the frame of the first CDS is equal to 1 as $(29\,039-1) \bmod 3 = 1$;
the frame of the second CDS is equal to 2 as $((29\,397-1)+1-(29\,245-1)-1) \quad \bmod 3 = 2$ (this expression is obtained from the beginning of the CDS, i.e. 29 397, the frame of the previous CDS, i.e. 1, and the end of the previous CDS, i.e. 29 245);
the frame of the third CDS is equal to 2 as $((30\,373-1)+2-(30\,228-1)-1) \bmod 3 = 2$;
the frame of the fourth CDS is equal to 1 $((30\,740-1)+2-(30\,584-1)-1) \bmod 3 = 1$;
the frame of the 5th CDS is equal to 1 $((31\,110-1)+1-(30\,953-1)-1) \bmod 3 = 1$.

Fig. 5c gives an analysis of the relative positions of the different exons between themselves.

3.2.2. *Two examples leading to unexpected results*

Two unexpected results obtained in the EMBL human chromosomes with the software ACG, are given: the prediction of internal frames in the



(c)

Fig. 5 (*Continued*)

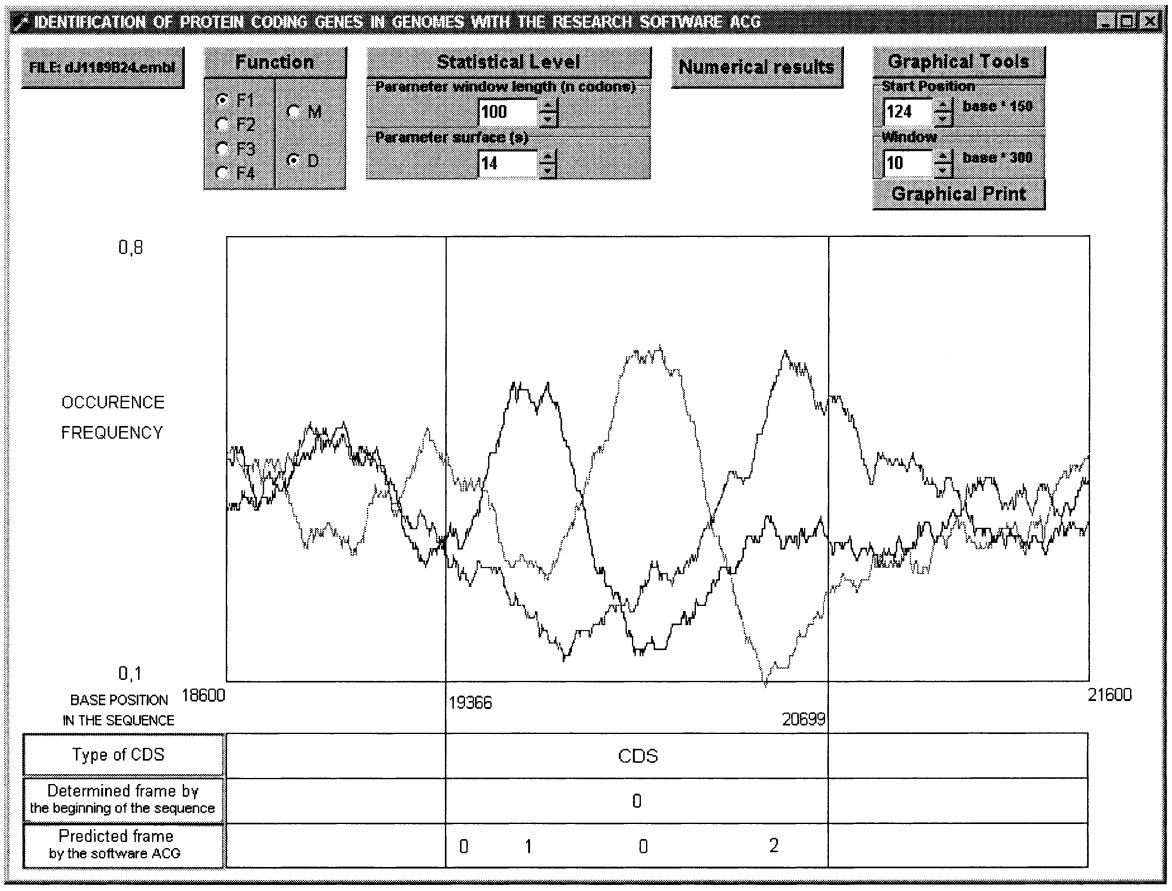
protein genes and the prediction of a coding region in the genomes.

Fig. 6a predicts internal frames in a CDS (1334 bases in the human DNA sequence from clone 1189B24 on chromosome Xq25–26.3). Indeed, there are three intersections of the two first highest curves, which are associated with four assumed internal frames 0, 1, 0 and 2. The frameshift of 1 (respectively, 2) base can be associated with 1 (respectively, 2) base insertion (modulo 3) or 2 (respectively, 1) base deletions (modulo 3). The internal frames can also be explained with the concatenation of coding regions whose lengths are not all multiple of 3. This CDS is mentioned as pseudogene in the EMBL file.

Fig. 6b predicts a coding region in the human DNA sequence from clone 1048E9 on chromosome 22q11.2–12.2 and its frame 2. This region is associated with the primary transcript starting at 18 411 and ending at 18 946. Surprisingly, the predicted frame by the software ACG is equal to the EMBL frame $(18\,411-1) \bmod 3 = 2$.

4. Discussion

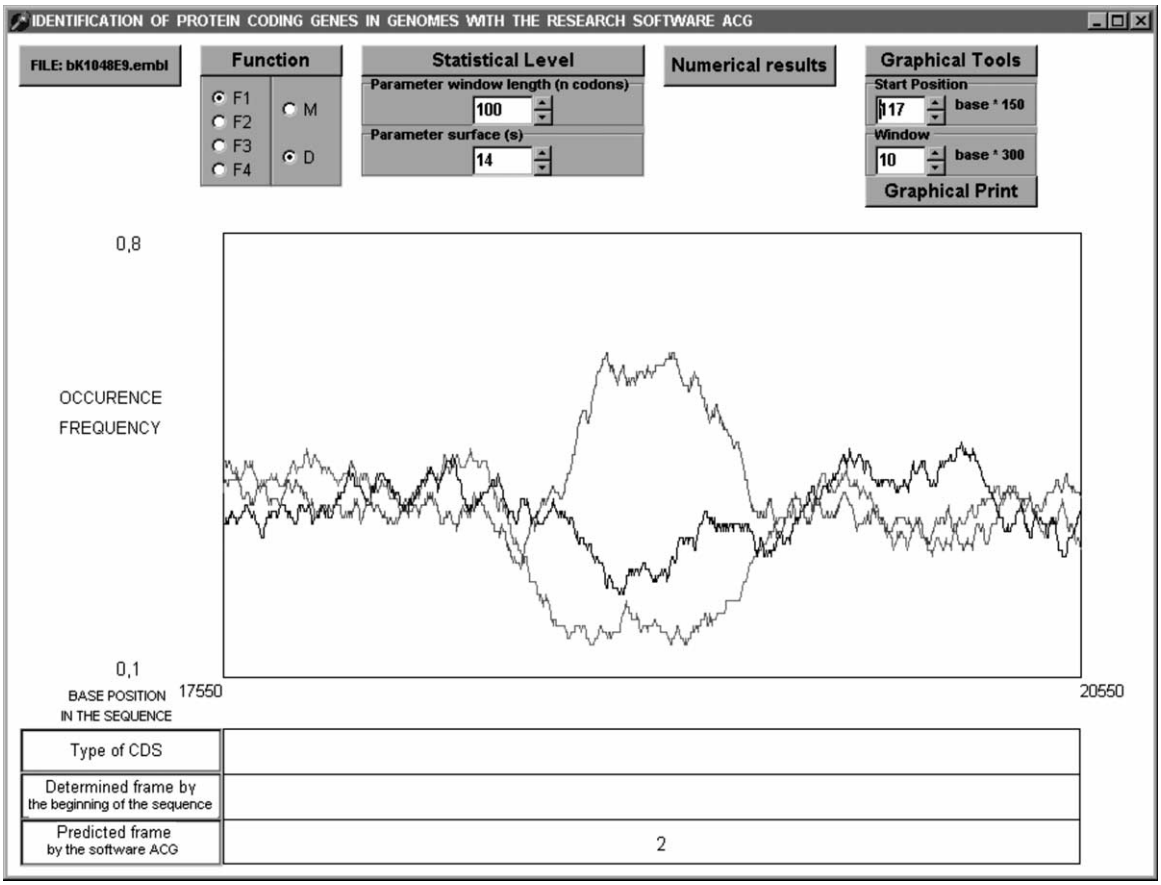
A new statistical approach using functions based on the circular code identifies 93.32% (coefficient SMC) of bases in the human sequences correctly, i.e. classifies the bases in coding and



(a)

Fig. 6. (a) Prediction of internal frames in a protein coding gene (CDS) and their internal frames by the software ACG. This example is observed in the human DNA sequence from clone 1189B24 on chromosome Xq25–26.3. (b) Prediction of a coding region in the human DNA sequence from clone 1048E9 on chromosome 22q11.2–12.2.

non-coding genes correctly. This approach has been evaluated with the coefficient SMC, which represents a good compromise between the Sensitivity Sn of coding bases identified correctly (39.75%) and the Specificity Sp of non-coding bases identified correctly (98.88%) (in the population studied, 90.6% bases are non-coding). These frequencies are retrieved by varying the size of the sequence population, e.g. by eliminating the short sequences (data not shown). Indeed, a large quantity of data used for the computation leads to stable values. The parameter s (surface) used for evaluating the statistical significance is a concept extending the natural and simplest parameter v based on the value of a function for a given base position. The statistical results obtained with this parameter v lead to a coefficient SMC that is significantly low than 93.32% (data not shown). The choice of the coefficient SMC for evaluating this new statistical approach in order to identify protein genes in genomes is confirmed by the Correlation Coefficient (CC) whose maximum (0.53) is also reached with the function F_2^D with $n = 50$ and $s = 38$. Note that if all bases



(b)

Fig. 6 (*Continued*)

are predicted as non-coding then CC is equal to 0.

The main purpose of this paper is to propose a completely new approach for identifying protein coding genes in genomes by using a gene model based on the circular code. Therefore, the method developed allows the global location of regions that are coding for proteins or not. The start and end of the coding region can be predicted by the intersection of the two highest curves. Obviously, the exact location of the boundaries can be improved in the future by analysing in detail the start regions and the end regions of coding genes by considering, for example, the start codon ATG, the stop codons TAA, TAG, and TGA, the splicing sites, the TATA box, etc. It can also be associated with other methods for identifying protein genes in genomes, such as the codon usage methods, the methods based on the hidden Markov model (HMM), etc. (e.g. Shulman et al., 1981; Shepherd, 1981; Staden and McLachlan, 1982; Fickett, 1982; Smith et al., 1983; Blaisdell, 1983; Staden, 1984; Borodovsky and Mc Ininch, 1993; Krogh et al., 1994; Burge and Karlin, 1997; Lukashin and Borodovsky, 1998; Salzberg et al., 1998; Pavy et al., 1999; Shmatkov et al., 1999, etc.). However, it should be stressed that the method in its actual state gives interesting results. Indeed, the Correlation Coefficient (CC) is equal to 0.53 with a data set containing 303 124 560 bases without discarding particular sequences. Obviously, these values become significantly better

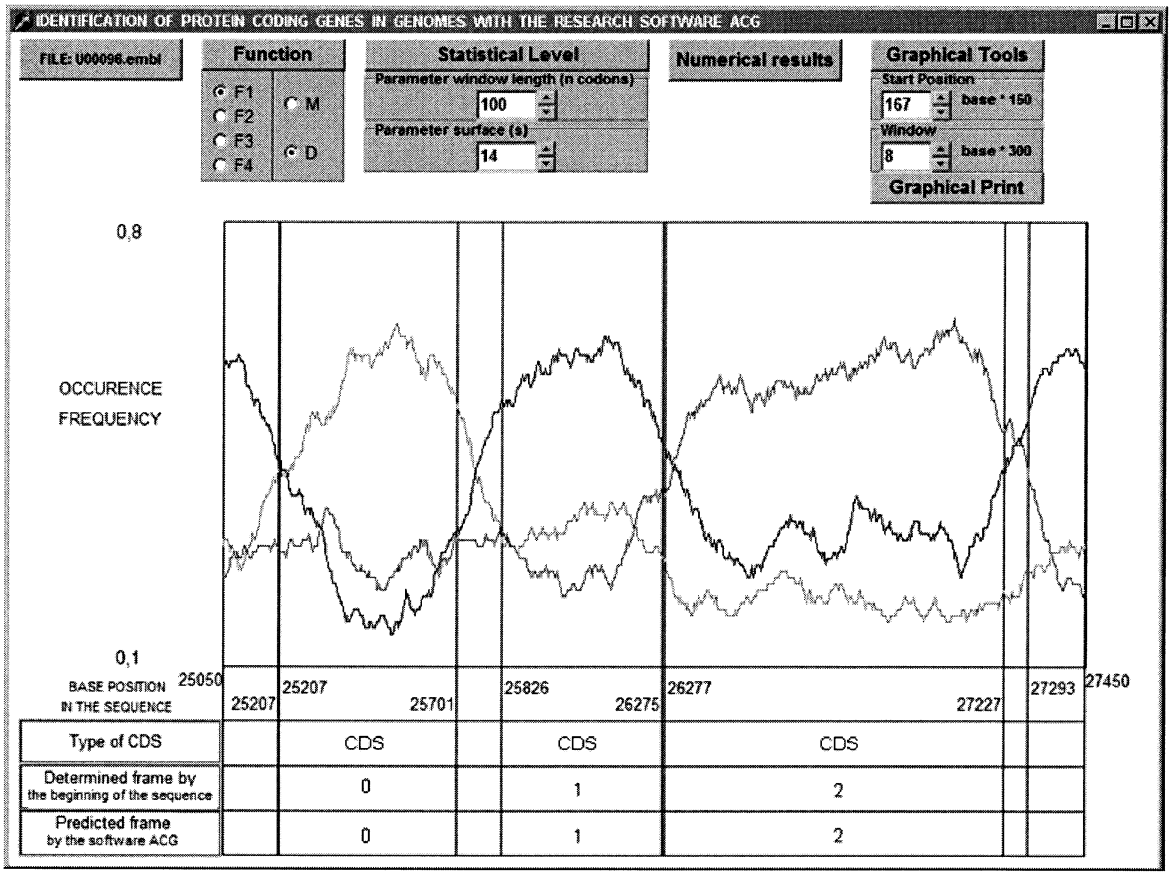


Fig. 7. Identification of several protein coding genes (CDS) and their frames by the software ACG. This example is observed in the *E. coli* K-12 MG1655 complete genome.

if there is a data selection before the statistical analysis, e.g. by discarding particular sequences: the sequences for which the exact location of the protein genes is determined ambiguously, the sequences encoding pseudogenes, etc. For example, the removal of pseudogenes (784 274 bases representing about 2.92% of the gene population studied) increases the coefficient SMC from 93.32 to 93.53%.

In summary, the research software ACG using functions based on the circular code, constitutes a new approach for identifying protein genes in genomes and for determining their frame. As it is based on the circular code of protein genes of both eukaryotes and prokaryotes, it can be applied independently of the type of eukaryotic/prokaryotic organism under investigation. An example of the use of the software ACG on a prokaryotic organism (*Escherichia coli*) is presented in Fig. 7. Furthermore, it also allows an evaluation of the length of protein genes, their position in the genome, their relative position between themselves, and the prediction of internal frames. It can be used without prerequisite knowledge: interactivity, graphical tools, possibilities of varying the parameters (the function, the length of the window, the surface level), etc. As the user-friendly software ACG is based on a new concept (circular code), the genomes can easily be investigated for obtaining new results in this research field.

Acknowledgements

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