Challenges in Elucidating 5' mRNA Sequences: Causes, Consequences, and Solutions for Accurate Protein Sequencing (Review)

Alessia Bianchi, Elena Fabbri, Francesca Zanardi, Daniela Morandi, Giulia Rizzo

1 Department of Biomedical Sciences, Unit of Molecular Biology, University of Ferrara, I-44121 Ferrara; 2 Department of Biological and Environmental Sciences, University of Perugia, I-06123 Perugia, Italy

Abstract. The known difficulty in obtaining the actual full length, complete sequence of a messenger RNA (mRNA) may lead to the erroneous determination of its coding sequence at the 5' region (5' end mRNA artifact), and consequently to the wrong assignment of the translation start codon, leading to the inaccurate prediction of the encoded polypeptide at its amino terminus. Among the known human genes whose study was affected by this artifact, we can include disco interacting protein 2 homolog A (*DIP2A*; *KIAA0184*), Down syndrome critical region 1 (*DSCR1*), SON DNA binding protein (*SON*), trefoil factor 3 (*TFF3*) and URB1 ribosome biogenesis 1 homolog (*URB1*; *KIAA0539*) on chromosome 21, as well as receptor for activated C kinase 1 (*RACK1*, also known as *GNB2L1*), glutaminyl-tRNA synthetase (*QARS*) and tyrosyl-DNA phosphodiesterase 2 (*TDP2*) along with another 474 loci, including interleukin 16 (*IL16*). In this review, we discuss the causes of this issue, its quantitative incidence in biomedical research, the consequences in biology and medicine, and the possible solutions for obtaining the actual amino acid sequence of proteins in the post-genomics era.

1. Introduction

Since the late 1990s, the availability of public, large databases containing growing information about genes, gene products (RNAs and proteins), genomes and molecular functions has radically changed the traditional approach to gene discovery and characterization. Combining the deposited data about informational molecules (1,2) obtained from multiple species is a straightforward method to gain rapid knowledge about the structure of an organism's genes and gene products, which in turn may be used to obtain clues as to the function of each individual gene. While this possibility has allowed the generation of an amount of data incomparable to what was obtained by classic molecular biology methods used in the pre-genomic era (3), the fact that the quality and degree of the information available for an individual gene may tend to decrease is less evident. For example, if we consider the characterization of the messenger RNA (mRNA) expressed by a human locus, through the 1980s and 1990s it was typical to obtain accurate information about the total mRNA size and tissue distribution by northern blot analysis and about the transcription initiation sites by S1 nuclease mapping, primer extension and run-off assays (4). In later years, mRNA full-length sequences were obtained by tailored experiments designed for polymerase chain reaction (PCR) amplification of DNA complementary to RNA (cDNA) ends [rapid amplification of cDNA ends (RACE)], alternative splicing information by cDNA *in vivo* and *in vitro* cloning of individual RNA isoforms, and protein sequences by *in vitro* translation and polypeptide biochemical analysis. Indeed, genes were usually studied on a one-by-one basis, and there was the possibility to cross-check data made available through different methods (5). An example would be the comparison of the mRNA length deduced from northern blotting (taking into account the polyadenylated tail) and the one of the isolated cDNA (6), or the comparison of the molecular weight of a known protein (7) and the one of the polypeptide predicted to be encoded by the open reading frame (ORF)/coding sequence (CDS) of its relative cDNA.

New large-scale methods cannot always reach the resolution of previous ones; therefore, while they set a new standard in the methods used in genetics, more detailed analysis aimed at characterizing each individual gene remains necessary in order to avoid incomplete or erroneous knowledge of the gene structure and function. However, the genome-scale information has been in turn invaluable in effectively directing further investigations needed for each genomic locus using classical methods. This has been shown in particular for the human genome, by a large corpus of millions of short sequences (a few hundred base pairs in length) which has been derived by partial, single-pass sequencing of the cDNA clones from RNA of specific tissues (8). These have accumulated in the expressed sequence tag (EST) database since its creation >20 years ago (9). A variety of EST-based methods (10,11) were then used for the rapid *in silico* cloning of genes (12), determining differential gene expression (13), characterizing alternative forms of transcripts derived from alternative splicing (14,15), and defining at least one complete ORF (16) for each mRNA. This last point is a well-known issue in molecular biology and genomics, with relevant consequences for the prediction of the gene product structure and function, and will be analyzed in detail in this review.

2. The 5' end mRNA artifact

According to the classic molecular biology central dogma, the final effector of the genetic information is the protein (a chain of amino acids) encoded from a given gene; thus it is crucial to know the basic, primary structure of the protein (its amino acid sequence). A landmark in this field was the sequencing of the two amino acid chains composing human insulin by Sanger (17). Polypeptide sequencing has the advantage of determining the natural primary structure of the polypeptide chain, and in particular the actual first amino acid of the sequence, thanks to the ability of fluorodinitrobenzene to react with the N-terminal amino group at one end of the chain. Key subsequent advancements were the recognition that, due to the colinearity of nucleic acids and proteins and to the mechanisms of mRNA

translation, amino terminal amino acids are encoded by the 5' end of the mRNA (18). Therefore, when Sanger *et al* proposed a new effective method to sequence DNA (19), it became evident that it was much more convenient to sequence the nucleic acids rather than the proteins, and that the amino acid sequence of gene products could be conveniently deduced from the nucleotide sequence of the relative cloned cDNA. This change of experimental paradigm led to 'reverse genetics' (20), the passage from nucleic acid sequences to their functions rather than the contrary as in classic genetics and has had the fundamental consequence that actually, since the late 1970s, the vast majority of protein sequences were no longer directly determined, but were predicted following sequencing of the relative cDNAs according to rules for recognition of the start codon (first-AUG rule, optimal sequence context) and the genetic code (21).

While this advancement greatly sped up the pace of the availability of protein sequences, it should be kept in mind that all standard experimental methods for the cloning of cDNA are affected by a potential inability to effectively clone the 5' region of mRNA in its completeness (22). This is due to the reverse transcriptase (RT) failure to extend first-strand cDNA along the full length of the mRNA template toward its 5' end (22) (Fig. 1), an operation whose success depends on the natural processivity of the enzyme, as well as its quality, the integrity of the RNA, the secondary structures assumed by the 5' region of the mRNA hampering the RT progression and the reaction conditions (23).

It should be highlighted here that, due to the intrinsic functional mechanisms of the polymerases able to generate DNA copies of mRNAs, cDNA is typically obtained through a primer starting polymerization from the 3' region of the mRNA [e.g., a poly(dT) oligonucleotide pairing with the poly(dA) tail present in the vast majority of mRNAs]. This implies that a cDNA collection is by definition enriched in the 3' regions of the mRNAs, and consequently it is expected that the prediction of the amino acid sequence at the carboxy terminus of the gene product is more accurate than the one at the amino terminus. This problem was recognized early on, in the publication of the first sequenced human cDNA, the one for the β chain of hemoglobin in 1977 when the 5'-untranslated region (UTR) was the last region to be reported in December (24) following previous descriptions of 3'-UTR in April (25) and CDS in July (26): 'cloning cDNA has proven to be a most valuable technique for sequencing mRNA (27,28). During the construction of double-stranded cDNA, however, a considerable number of 5'-non-coding region sequences are lost. The independent sequencing of this region will therefore be a necessary step to complete our knowledge of the primary structure of any mRNA' (24); Okayama and Berg clearly wrote in 1982: 'obtaining cloned cDNAs with complete 5'-UTR and protein-coding sequences is rare, particularly if the mRNA codes for a large protein. Although such truncated cDNAs are still useful as hybridization probes, they cannot direct the synthesis of complete proteins after their introduction into bacterial or mammalian cells via appropriate expression vectors' (23).

A flourishing of reports in the 1980s presented the determination of the often called 'cDNA full-length sequence' for many human genes. For the reasons discussed, the concept of the 'full-length sequence' becomes *de facto* equivalent to the one of 'completeness of mRNA sequence at its 5' end' and remains an open issue in molecular biology as cDNA incompletely representing the 5' end of the relative mRNA may lead to the incorrect assignment of the first AUG codon. In these cases, should an additional upstream AUG - in frame with the previously determined one - have been identified in a more complete mRNA 5' end, it would have been considered the actual translation start codon, thus extending the predicted amino terminus sequence of the product. Assignment of the inexact start codon leads to a series of subsequent relevant errors in the experimental study of the relative cDNA. We therefore introduced the term '5' end mRNA artifact' to refer to the incorrect assignment of the first translation codon (AUG sequence) in an mRNA, due to the incomplete determination of its 5' end sequence (29).

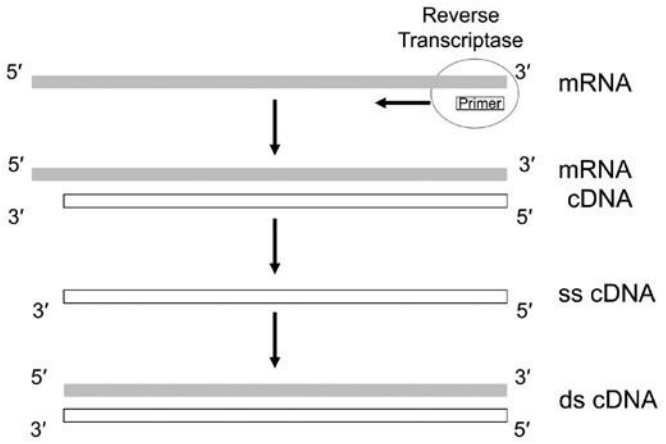


Figure 1. The 5' end mRNA artifact. cDNA is typically obtained through a primer starting polymerization from the 3' region of the mRNA by reverse transcriptase. The natural processivity of the enzyme, as well as its quality, the integrity of the RNA and the secondary structures assumed by the 5' region of the mRNA may hamper the reverse transcriptase progression, causing a failure in the polymerization of the first-strand cDNA along the full length of the mRNA template toward its 5' end, affecting all further experiments, including the assignment of the first AUG codon. ss, single-stranded; ds, double-stranded.

From the experimental point of view, the recognition of this technical issue, although often without systematic investigation of its possible consequences for genome annotation, has led to the development of several methods to determine the full length mRNA sequence on a large scale. Some were based on the presence of the 'cap' at the true 5' end of the mRNA [reviewed in (30)], such as 5' cap trapping (31) and cap analysis of gene expression (CAGE) (32). Systematic empirical annotation of a set of transcript products by 5' RACE (33) has also been employed, as well as after the introduction of microarray-based platforms, hybridization of RNA on high-density resolution tiling arrays (34). However, these techniques were found to be experimentally labor-intensive and they have not been routinely applied.

Concurrently, the growing incorporation of information derived from individual cDNA and large-scale sequencing projects, including those specifically designed to characterize mRNA 5' end (31,35,36), led to a continuous refinement and improvement of completeness at the 5' region of deposited and verified mRNA reference sequences (e.g., RefSeq, https://www. ncbi.nlm.nih.gov/refseq/), as also regarding the corresponding protein-coding sequences. Therefore, it became possible to exploit the data from EST or other large-scale RNA sequencing projects to verify if sequence analysis could be optimized to reveal the extension of the 5' region of known mRNAs and possibly the consequential redefinition of the amino acid sequence of the encoded products.

The recent availability of massive RNA-sequencing (RNA-Seq) methods for the generation of transcriptome sequence databases (37) offers a new potential tool to deal with the issue, although to date it appears not to have been systematically used to this aim. Moreover, information about sequences possibly extending the knowledge of the 5' end of mRNA is not easily derivable from RNA-Seq data in comparison with the EST-based approach, due to short sequence reads typically obtained by this technique, as well as difficulty in building full-length transcript structures.

Furthermore, a ribosome footprinting profiling strategy based upon high-throughput sequencing of ribosome-protected mRNA fragments has been developed, enabling the genome-wide investigation of translation (38). This technique, used in combination with initiation-specific translation inhibitors, allows the identification of translation initiation with subcodon or even single-nucleotide resolution and was successfully exploited in order to predict also additional upstream AUG codons (39-41).

Finally, we should note the existence of ORFs and out-of-frame AUGs located in the 5'-UTR, upstream of the main coding region (42). These situations are different from the artifact reported herein as they do not extend the known coding region, but are implicated in the regulation of gene expression by modulating mRNA stability and translation (42,43).

3. Systematic identification of incomplete 5' end region in human known mRNAs

The theoretical possibility that the presence of a more precise knowledge of the mRNA 5' end sequence may lead to consequential correction of the previously accepted predicted product appeared in several reports in the form of anecdotal evidence randomly found for single genes that were under detailed investigation. For example, mRNA CDS was extended in this way for *RANBP9*/*RanBPM* gene (RAN binding protein 9, on 6p23), where the study performed by Nishitani *et al* (44) allowed the addition of 230 new amino acids. In the case of nuclear factor, erythroid 2-like 3 (*NFE2L3*) gene (on 7p15.2), the corresponding #AB010812.1 mRNA sequence of 2,174 bp in length derived from Kobayashi *et al* (45) was replaced by the sequence #AF134891.1 of 2,618 bp, leading to the addition of 294 new amino acids to the predicted protein. The study performed by Nomura *et al* (46) for *SP2* gene (Sp2 transcription factor, on 17q21.32) allowed the release of the #D28588.1 mRNA sequence entry recording a CDS of 3,288 bp leading to the addition of 111 new amino acids compared to the previous #M97190 entry of 2,063 bp provided by Kingsley and Winoto (47). The coding nature of these extensions was also supported by very high similarity with the respective murine hortologs (29). These and other similar reports suggested that a high-throughput approach was desirable to discover all the incompletenesses in the CDSs (Table I).

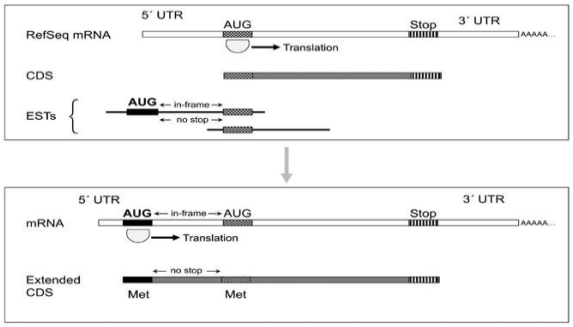
Regarding our group, as a first approach to the issue, due to our interest in an integrated route to identifying new pathogenesis-based therapeutic approaches for trisomy 21 (Down syndrome) (48,49), we focused on the known, well-characterized genes present in the original map of human chromosome 21 (Hsa21), manually analyzing 109 RefSeq mRNA sequences catalogued as 'category: known' by Hattori *et al* (50), and linked to at least one published report, for the

Table I. Main published results of systematic search for completeness of

Ref.	Year	Organism	Method
(35)	2000	*H. sapiens*	Oligo-capping
(29)	2003	*H. sapiens*	Manual and automated sequence
(53)	2007	*D. rerio*	Automated sequence analysis
(39)	2011	Mouse embrionic stem cells	Ribosome footprinting profiling and support vector machine (SV machine learning strategy
(54)	2012	*H. sapiens*	Fully automated sequence analys
(40)	2012	*H. sapiens*	Ribosome footprinting profiling neural network prediction
(55)	2014	*M. musculus*	Fully automated sequence analys
(41)	2014	*H. sapiens*	Ribosome footprinting profiling manual analysis
		M. musculus	Ribosome footprinting profiling manual analysis

[a]Estimation. CDS, coding sequence; *H. sapiens*, *Homo sapiens*; *D. rerio*, *Danio r*

Figure 2. Identification and correction of incomplete 5' end regions. Possible EST sequen selected for the presence of an upstream in-frame AUG codon and absence of any stop co codons. The upstream in-frame AUG codon becomes the actual translation start codon, thus sequence of the mRNA product. EST, expressed sequence tag; Met, methionine.



presence of an in-frame stop codon upstream of the described ATG. In 49 cases, the finding of such a stop codon allowed the exclusion of the possibility that the recorded 5'-UTR sequence may actually be part of a longer CDS (51). The sequence of the remaining 60 mRNAs in which bases in the 5'-UTR could on the contrary be consistent with the presence of translated codons was systematically aligned with sequences available in databanks using Basic Local Alignment Search Tool (BLAST software, http://www.ncbi.nih.gov/BLAST/), leading to the discovery of a total of 20 genes for which EST (or also non-EST RNA sequences) homology suggested the existence of mRNAs more complete at 5' terminus. They putatively encode for protein products longer at their amino terminus, due to the presence of a previously unknown start codon in frame with and upstream of the described one (Fig. 2). Experimental evidence for the existence of these transcripts was finally obtained, following RT-PCR and sequencing, for five loci: down syndrome critical region 1 (*DSCR1*) [now regulator of calcineurin 1 (*RCAN1*)], disco interacting protein 2 homolog A (*DIP2A*; *KIAA0184*), URB1 ribosome biogenesis 1 homolog (*URB1; KIAA0539I*), SON DNA binding protein (*SON*) and trefoil factor 3 (*TFF3*) (29). In these cases, both of the following conditions occurred: an extension of described exon 1 predicted new coding codons upstream of the known AUG; and a novel AUG was present upstream of these codons, in frame with the previously described AUG and without any intervening stop codon. This thus suggests that, following the rules of translation initiation [reviewed by Kozak (21)], the actual CDS should be considered as the one included between the novel 'first-AUG' and the known stop (Fig. 2). It was observed that no known mechanism hampers the possibility that the newly identified start codon is not the point of actual translation as the use of 'internal' AUGs, enabling additional initiation events at downstream AUG codons in some mRNAs may occur only in three well-defined circumstances (21): re-initiation, which does not apply to the mRNAs investigated by this approach, as the newly determined AUG is not part of a small upstream ORF separated from the main ORF by a stop codon; context-dependent leaky scanning, which may be excluded as we considered the concordance with the Kozak sequence (21,52) for the novel AUGs, observing full (sometimes better) compatibility with the use of the novel AUG (29); a third mechanism, that is the use of internal ribosome entry site (IRES) sequence modules, adopted only by some known viral mRNAs.

These positive results suggested to extend the approach to the whole set of human RefSeq mRNAs known at the time (n=13,124), following automation by a simple program to detect the presence or the absence of an in-frame stop in the described 5'-UTR of an mRNA. The percentage of the latter type of mRNA in the set (51%) was very similar to the one found for the Hsa21 gene set (55%), thus estimating that, in proportion, the CDS of 556 known human mRNAs might be incomplete at the 5' end (29).

This approach required manual curation to analyze in detail, by sequence comparison, any mRNA candidate to have an incomplete CDS at 5' region. An improvement of the algorithm was then published and applied with success to zebrafish [see below (53)], showing that the automated detection of putative additional bases at the known 5' end of a set of mRNAs following elaboration of multiple results of sequence comparison analysis (by BLAST tool) was possible. Some technical limitations of the used environment made the implementation of this pipeline difficult for the much more numerous human sequences which hampered progress in this direction for a while. Further improvement of the automated EST-based approach (5'_ORF_Extender 2.0, freely available at http://apollo11.isto.unibo.it/software/) finally made the systematic identification (Fig. 2) of CDSs at the 5' end of all human known mRNAs possible, parsing >7 million BLAT alignments and thus finding 477 human loci out of 18,665 analyzed (Table I), with an extension of their RNA 5' CDS identified in detail (54). In addition, in this study, a proof-of-concept confirmation was obtained by *in vitro* cloning and sequencing for some example genes: *GNB2L1* [now receptor for activated C kinase 1 (*RACK1*)], glutaminyl-tRNA synthetase (*QARS*) and tyrosyl-DNA phosphodiesterase 2 (*TDP2*) cDNAs. On the other hand, a list of 20,775 human mRNAs where the presence of an in-frame stop codon upstream of the known start codon indicates completeness of the CDS at 5' end in the current form was generated (54). This approach could also be aimed at the different 5'-UTR sequence identification, but the length of the bases aligned upstream of the novel AUG is usually too short to allow this type of investigation. In addition, should the length be long enough, the analysis would require an ad hoc algorithm able to discriminate mRNA isoforms of this type, including mapping of the newly determined 5'-UTR to the genome to derive the alternative transcription/splicing events responsible for the different 5'-UTR sequences.

While this review is more focused on human mRNAs for the possible repercussion in medicine, it should be noted that similar results are to be expected for the genomes of other organisms due to the sharing of common molecular techniques, whose limitations are at the basis of the artifact. Actually, studies on two of the most commonly used model organisms for the investigation of the human genome, *Danio rerio* (zebrafish) and *Mus musculus* (domestic mouse) have confirmed this expectation. A novel proposed automated approach (5'_ORF_Extender 1.0) was able to systematically compare available ESTs with all the zebrafish experimentally determined mRNA sequences, identify additional sequence stretches at 5' region and scan for the presence of all conditions needed to define a new, extended putative ORF. The tool identified 285 (3.3%) mRNAs with putatively incomplete ORFs at the 5' region and, in three example selected cases (*selt1a*, *unc119.2* and *nppa* or selenoprotein T 1a, unc-119 lipid binding chaperone B homolog 2 and natriuretic peptide A, respectively), the extended coding region at 5' end was experimentally demonstrated (53). As regards the mouse mRNAs, the application of the improved method used for human transcripts (54) showed that in 351 mouse loci, out of 20,221 analyzed, an extension of the mRNA 5'-coding region could be identified. Experimental confirmation was obtained by *in vitro* cloning and sequencing for adenomatosis polyposis coli 2 (*Apc2*) and MAP kinase-interacting serine/threonine

kinase 2 (*Mknk2*) cDNAs and a list of 16,330 mouse mRNAs with estimated complete CDS at 5' end was provided (55). Remarkably, 82% of the results were original and have not been identified by the annotation pipelines used in the main mouse genome databases and genome browser (55). The diffusion of the 5' end mRNA artifact may thus be considered approximately constant from lower vertebrates to humans because the methods used to characterize the relative mRNAs are the same or very similar (Table I).

The identification of the most upstream definable start codon does not exclude that a downstream AUG codon may also be used by the ribosome, a phenomenon known as alternative translation (56). It has been shown that alternative translation start sites tend to be conserved in eukaryotic genomes, providing a functional mechanism under selection for increased efficiency of translation and/or for obtainment of different N-terminal protein variants (57). It has also already been noted that this type of analysis cannot formally exclude that the extended ORF may derive from alternative transcription starting site (due to alternative promoter usage) and/or splicing of the investigated locus (53). However, it reveals in any case that additional coding sequences not previously identified exist, as may be confirmed by phylogenetic comparison at the amino acid level (53). As in the case of any other computer prediction, further investigation is required, *in silico* but especially *in vitro*, for a fine characterization of the putative model.

While the published approaches have considered algorithms assuming that the start codon has an AUG sequence, it should be noted that in a minor percentage of mRNA CDSs the start codon may have alternative sequences, particularly CUG, UUG, GUG, ACG, AUA and AUU (58). Actually, recent experiments have confirmed this phenomenon and suggested that it may be more frequent than was previously assumed. Therefore, when the use of a non-AUG codon is known or suspected, further analysis not included in standard pipelines should be performed in individual cases to identify in frame upstream non-AUG start codons which may also be responsible of encoding proteins longer that the ones previously described.

4. Consequences of 5' end mRNA artifact in biology and medicine

The 5' end mRNA artifact is expected, and demonstrated, to cause a chain of consequences in biomedical research, that will be now listed and discussed (Table II). The first obvious issue associated with the artifact is the negative consequence on the study of product structure and function (59). The possibility that vast amounts of studies are based on incorrect starting data is real. For instance, it occurred in the functional characterization of a polypeptide expressed from its predicted incomplete DNA (60) and in a functional study of the cytokine interleukin 16 (IL16) (61), where the product appears to be expressed from an incomplete cDNA (Table II).

The recording of protein sequences incomplete at their amino terminus in the genomic databases may also cause the failure to identify functionally remarkable protein domain sequences (Table II); in particular, sequences located at the amino terminus of proteins may be represented by signal

Table II. Possible consequences of incomplete determination of mRNA 5' CDS region for example human genes.

[a]AAs or nts added to the previously recorded protein or nucleic acid sequence, respectively, following the analysis cited as Ref. 2. CDS, coding sequence; AAs, amino acids; nts, nucleotides; ALDOC, Aldolase, Fructose-Bisphosphate C; QARS, glutaminyl-tRNA synthetase; IL16, interleukin 16; SON, SON DNA binding protein; RANBP9, RAN Binding protein 9; UMOD, uromodulin; DSCR1, down syndrome critical region 1; *ADAR*, adenosine deaminase, RNA specific; *DIP2A*, disco interacting protein 2 homolog A; *TFF3*, trefoil factor 3.

peptide sequences directing delivery of the protein to its final destination (62,63) and may also affect its half-life (64).

In addition, there is the possibility to underestimate alternative splicing at the 5' terminus of genes and to not predict the corresponding alternative protein gene products (Table II). The statement in the classic article by Okayama and Berg still holds true: 'indeed, it was comparison between cloned cDNAs and their genomic counterparts that uncovered the existence of intervening sequences and splicing' (23). Moreover, the design of a mutation screening aimed at identifying pathological variations in the coding sequences could be affected by the incomplete knowledge of the CDS, a circumstance that could occasionally explain the failure to find expected mutations in candidate or established disease genes, and could possibly lead to inaccurate genotype/phenotype correlations (Table II). From a functional point of view, the new amino acid sequence could be responsible for new interactions. The possibility of designing molecules with pharmacological activity based on binding to proteins expressed as bait in a two-hybrid test from incomplete cDNAs (65) emphasizes the importance of knowing the actual primary structure of the protein. Finally, the presence of a truncated protein sequence in the genomic databases may also be at the origin of a chain of errors in the prediction of orthologs in other species. In particular, the genome annotation pipelines will tend to propagate the truncated sequence in the predicted model proteins. For instance, the error in determination of the highly similar corresponding murine DSCR1 ortholog (66) underlines that a bias deriving from the original human incomplete data negatively affected the modeling of the murine DSCR1 product sequence.

Due to the complex structure of the human loci (67-70), errors in establishing an accurate cDNA sequence may also finally cause drawbacks in the study of genomic organization of a gene due to the tight connections between DNA and RNA (Table II). If a cDNA incomplete at its 5' terminus is used to establish the genomic structure of a locus, this could cause failure to recognize genomic sequences as part of the locus (71). As a secondary consequence, classification of a genic region as intergenic may keep the 'search space' for novel genes artificially expanded (71). Due to the physical proximity of the gene promoter region and the corresponding mRNA 5' region, a sequence supposed to be proximal to the transcription start site and annotated as promoter could be actually part of a longer mRNA, as was shown for *TFF3* (72,29). This issue may further increase the difficulty in identifying promoter sequences that do not have regular start and stop signals or characteristic cross-species conservations as the CDSs, and can even present with diverged sequences among distant species, while being functionally conserved (73). On the other hand, a non-exact delimitation between 5'-UTR and CDS could lead to errors in the knowledge of the 5'-UTR sequence itself and in the interpretation of its role in the control of translation (74). Although this last class of consequences does not directly affect the prediction of the CDS, they should be considered as a further incentive to not underestimate the relevance of this artifact due to the central role of the 5' terminus in gene expression regulation pathways. The knowledge of the true mRNA end is also useful in designing probes specific for this region that may be more variable between similar loci or isoforms from the same locus rather than the central, coding region. This is relevant regarding the possibility to extract from publicly available microarray datasets quantitative reference measures for the expression values of the whole complement of the genes of both normal (75) or pathologic (76) transcriptomes. Exact knowledge of mRNA 5' region also affects the choice of morpholino oligonucleotides, in particular in zebrafish (77), used in knockdown experiments (Table II).

The artifact may also be a source for errors in other types of genomic analysis, although in these cases the consequences are expected not to be relevant, as the alteration of calculations is likely to represent a small deviation, and not for immediate medical application of these analyses [e.g., estimation of codon usage at a genomic scale (78), although the knowledge of the whole set of codons in a cDNA could affect the technology of the production of the translated product in a host (79)].

5. Possible solutions for improving the knowledge of the 5'-coding regions in mRNAs

Several methods have been described with the aim of knowing with more precision the 5' mRNA end, thus excluding that its CDS may be incompletely predicted. The first were devised in the 1990s and were based on experimental protocols exploiting the capability of dedicated techniques to identify the first bases transcribed from DNA or the first bases following the cap in mature mRNAs. These methods have been cited in the Introduction section and remain valid, although they were often labor-intensive and not routinely used.

A second group of methods is based on computational biology approaches, with the advantage of providing a first systematic screening leading to exclusion of a relevant number of genes as candidates for the 5' end mRNA artifact. Due to the availability of throughput results of an EST-based approach of this type (54), it is advisable to perform a simple first check against these results for a gene of interest before assuming that the predicted product is the one recorded in the current version of databases. Continuous refinement over time of the human mRNA sequences has led to the current estimation of 259 nucleotides as the mean 5'-UTR size (80), so there is the concrete possibility that extended protein-coding sequences could actually be hidden in longer 5'-UTRs. Further developments of the computational analysis of high-throughput cDNA sequencing methods (RNA-Seq) should also provide a means to increase the characterization of whole sequences of human transcripts. Several studies have been performed to implement RNA-Seq methods of profiling mRNA 5' ends in *Drosophila melanogaster* (81,82).

Finally, recent developments of proteomics research open the way for a different, specular approach to the problem. Knowledge of protein sequences obtained by massive analysis of polypeptide nuclear magnetic resonance (NMR) or mass spectrometry (MS) spectra, in particular oriented to N-terminal sequencing (83,84), might be used for a reverse search for genomic sequences predicted to be translated in the corresponding identified protein sequences. This thus

resembles the first protein-toward-DNA experimental flow but at on a genomic scale and largely based on computational methods.

In conclusion, we have presented evidence that current methods of genomics research are subject to a possible artifact regarding the exact determination of the mRNA 5' region sequence and the consequences that this may have on the annotation, as well as on the experimental study of both genes and gene products. While there are several strategies to deal with this issue, the most important issue appears to bring this possibility to the attention of the scientific community so that it is taken into account when planning experiments

References

1. Borsani G, Ballabio A and Banfi S: A practical guide to orient yourself in the labyrinth of genome databases. Hum Mol Genet 7: 1641-1648, 1998.
2. Pandey A and Lewitter F: Nucleotide sequence databases: A gold mine for biologists. Trends Biochem Sci 24: 276-280, 1999.
3. Baxevanis AD and Bateman A: The importance of biological databases in biological discovery. Curr Protoc Bioinformatics 50: 1.1.1-1.1.8, 2015.
4. Tropp BE (ed): Molecular Biology: Genes to Proteins. 3rd edition. Jones & Bartlett Publishers, Sudbury, MA, 2008.
5. Sambrook J and Russel DW (eds): Molecular Cloning: A Laboratory Manual. Vol 2. 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001.
6. Vitale L, Casadei R, Canaider S, Lenzi L, Strippoli P, D'Addabbo P,
Giannone S, Carinci P and Zannotti M: Cysteine and tyrosine-rich 1 (CYYR1), a novel unpredicted gene on human chromosome 21 (21q21.2), encodes a cysteine and tyrosine-rich protein and defines a new family of highly conserved vertebrate-specific genes. Gene 290: 141-151, 2002.
7. Zhang J, Lou X, Shen H, Zellmer L, Sun Y, Liu S, Xu N and Liao DJ: Isoforms of wild type proteins often appear as low molecular weight bands on SDS-PAGE. Biotechnol J 9: 1044-1054, 2014.
8. Adams MD, Kelley JM, Gocayne JD, Dubnick M, Polymeropoulos MH, Xiao H, Merril CR, Wu A, Olde B, Moreno RF, *et al*: Complementary DNA sequencing: Expressed sequence tags and human genome project. Science 252: 1651-1656, 1991.
9. Boguski MS, Lowe TM and Tolstoshev CM: dbEST - database for 'expressed sequence tags'. Nat Genet 4: 332-333, 1993.
10. Nagaraj SH, Gasser RB and Ranganathan S: A hitchhiker's guide to expressed sequence tag (EST) analysis. Brief Bioinform 8: 6-21, 2007.
11. Parkinson J and Blaxter M: Expressed sequence tags: An overview. Methods Mol Biol 533: 1-12, 2009.
12. Gill RW and Sanseau P: Rapid in silico cloning of genes using expressed sequence tags (ESTs). Biotechnol Annu Rev 5: 25-44, 2000.
13. Carulli JP, Artinger M, Swain PM, Root CD, Chee L, Tulig C, Guerin J, Osborne M, Stein G, Lian J, *et al*: High throughput analysis of differential gene expression. J Cell Biochem Suppl 30-31: 286-296, 1998.
14. Sorek R, Shamir R and Ast G: How prevalent is functional alternative splicing in the human genome? Trends Genet 20: 68-71, 2004.
15. Bonizzoni P, Rizzi R and Pesole G: Computational methods for alternative splicing prediction. Brief Funct Genomics Proteomics 5: 46-51, 2006.
16. Brent MR: Genome annotation past, present, and future: How to define an ORF at each locus. Genome Res 15: 1777-1786, 2005.
17. Sanger F: La structure de l'insuline. Bull Soc Chim Biol (Paris) 37: 23-35, 1955 (In French).
18. Yanofsky C, Carlton BC, Guest JR, Helinski DR and Henning U: On the colinearity of gene structure and protein structure. Proc Natl Acad Sci USA 51: 266-272, 1964.
19. Sanger F, Nicklen S and Coulson AR: DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463-5467, 1977.
20. Ruddle FH: The William Allan Memorial Award address: Reverse genetics and beyond. Am J Hum Genet 36: 944-953, 1984.
21. Kozak M: Pushing the limits of the scanning mechanism for initiation of translation. Gene 299: 1-34, 2002.
22. Sambrook J and Russel DW (eds): Rapid amplification of 5' cDNA ends. In: Molecular Cloning: A Laboratory Manual. Vol 3. 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp8.54-8.60, 2001.
23. Okayama H and Berg P: High-efficiency cloning of full-length cDNA. Mol Cell Biol 2: 161-170, 1982.
24. Baralle F: Complete nucleotide sequence of the 5' noncoding region of human alpha-and beta-globin mRNA. Cell 12: 1085-1095, 1977.
25. Proudfoot NJ: Complete 3' noncoding region sequences of rabbit and human beta-globin messenger RNAs. Cell 10: 559-570, 1977.
26. Marotta CA, Wilson JT, Forget BG and Weissman SM: Human beta-globin messenger RNA. III. Nucleotide sequences derived from complementary DNA. J Biol Chem 252: 5040-5053, 1977.
27. Efstratiadis A, Kafatos FC and Maniatis T: The primary structure of rabbit beta-globin mRNA as determined from cloned DNA. Cell 10: 571-585, 1977.
28. Ullrich A, Shine J, Chirgwin J, Pictet R, Tischer E, Rutter WJ and Goodman HM: Rat insulin genes: Construction of plasmids containing the coding sequences. Science 196: 1313-1319, 1977.
29. Casadei R, Strippoli P, D'Addabbo P, Canaider S, Lenzi L, Vitale L, Giannone S, Frabetti F, Facchin F, Carinci P, *et al*: mRNA 5' region sequence incompleteness: A potential source of systematic errors in translation initiation codon assignment in human mRNAs. Gene 321: 185-193, 2003.
30. Harbers M: The current status of cDNA cloning. Genomics 91: 232-242, 2008.
31. Carninci P, Kvam C, Kitamura A, Ohsumi T, Okazaki Y, Itoh M, Kamiya M, Shibata K, Sasaki N, Izawa M, *et al*: High-efficiency full-length cDNA cloning by biotinylated CAP trapper. Genomics 37: 327-336, 1996.
32. Kodzius R, Kojima M, Nishiyori H, Nakamura M, Fukuda S, Tagami M, Sasaki D, Imamura K, Kai C, Harbers M, *et al*: CAGE: Cap analysis of gene expression. Nat Methods 3: 211-222, 2006.
33. Frohman MA, Dush MK and Martin GR: Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA 85: 8998-9002, 1988.
34. Denoeud F, Kapranov P, Ucla C, Frankish A, Castelo R, Drenkow J, Lagarde J, Alioto T, Manzano C, Chrast J, *et al*: Prominent use of distal 5' transcription start sites and discovery of a large number of additional exons in ENCODE regions. Genome Res 17: 746-759, 2007.
35. Suzuki Y, Ishihara D, Sasaki M, Nakagawa H, Hata H, Tsunoda T, Watanabe M, Komatsu T, Ota T, Isogai T, *et al*: Statistical analysis of the 5' untranslated region of human mRNA using 'Oligo-Capped' cDNA libraries. Genomics 64: 286-297, 2000.
36. Porcel BM, Delfour O, Castelli V, De Berardinis V, Friedlander L, Cruaud C, Ureta-Vidal A, Scarpelli C, Wincker P, Schächter V, *et al*: Numerous novel annotations of the human genome sequence supported by a 5'-end-enriched cDNA collection. Genome Res 14: 463-471, 2004.
37. Metzker ML: Sequencing technologies - the next generation. Nat Rev Genet 11: 31-46, 2010.
38. Ingolia NT, Ghaemmaghami S, Newman JR and Weissman JS: Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. Science 324: 218-223, 2009.
39. Ingolia NT, Lareau LF and Weissman JS: Ribosome profiling of mouse embryonic stem cells reveals the complexity and dynamics of mammalian proteomes. Cell 147: 789-802, 2011.
40. Fritsch C, Herrmann A, Nothnagel M, Szafranski K, Huse K, Schumann F, Schreiber S, Platzer M, Krawczak M, Hampe J, *et al*: Genome-wide search for novel human uORFs and N-terminal protein extensions using ribosomal footprinting. Genome Res 22: 2208-2218, 2012.
41. Van Damme P, Gawron D, Van Criekinge W and Menschaert G: N-terminal proteomics and ribosome profiling provide a comprehensive view of the alternative translation initiation landscape in mice and men. Mol Cell Proteomics 13: 1245-1261, 2014.

42. Iacono M, Mignone F and Pesole G: uAUG and uORFs in human and rodent 5' untranslated mRNAs. Gene 349: 97-105, 2005.
43. Barbosa C, Peixeiro I and Romão L: Gene expression regulation by upstream open reading frames and human disease. PLoS Genet 9: e1003529, 2013.
44. Nishitani H, Hirose E, Uchimura Y, Nakamura M, Umeda M, Nishii K, Mori N and Nishimoto T: Full-sized RanBPM cDNA encodes a protein possessing a long stretch of proline and glutamine within the N-terminal region, comprising a large protein complex. Gene 272: 25-33, 2001.
45. Kobayashi A, Ito E, Toki T, Kogame K, Takahashi S, Igarashi K, Hayashi N and Yamamoto M: Molecular cloning and functional characterization of a new Cap'n' collar family transcription factor Nrf3. J Biol Chem 274: 6443-6452, 1999.
46. Nomura N, Nagase T, Miyajima N, Sazuka T, Tanaka A, Sato S, Seki N, Kawarabayasi Y, Ishikawa K and Tabata S: Prediction of the coding sequences of unidentified human genes. II. The coding sequences of 40 new genes (KIAA0041-KIAA0080) deduced by analysis of cDNA clones from human cell line KG-1. DNA Res 1: 223-229, 1994.
47. Kingsley C and Winoto A: Cloning of GT box-binding proteins: A novel Sp1 multigene family regulating T-cell receptor gene expression. Mol Cell Biol 12: 4251-4261, 1992.
48. Strippoli P, Pelleri MC, Caracausi M, Vitale L, Piovesan A, Locatelli C, Mimmi MC, Berardi AC, Ricotta D, Radeghieri A, *et al*: An integrated route to identifying new pathogenesis-based therapeutic approaches for trisomy 21 (Down Syndrome) following the thought of Jérôme Lejeune. Sci Postprint 1: e00010, 2013.
49. Pelleri MC, Cicchini E, Locatelli C, Vitale L, Caracausi M, Piovesan A, Rocca A, Poletti G, Seri M, Strippoli P, *et al*: Systematic reanalysis of partial trisomy 21 cases with or without Down syndrome suggests a small region on 21q22.13 as critical to the phenotype. Hum Mol Genet 25: 2525-2538, 2016.
50. Hattori M, Fujiyama A, Taylor TD, Watanabe H, Yada T, Park HS, Toyoda A, Ishii K, Totoki Y, Choi DK, *et al*; Chromosome 21 mapping and sequencing consortium: The DNA sequence of human chromosome 21. Nature 405: 311-319, 2000.
51. Reymond A, Camargo AA, Deutsch S, Stevenson BJ, Parmigiani RB, Ucla C, Bettoni F, Rossier C, Lyle R, Guipponi M, *et al*: Nineteen additional unpredicted transcripts from human chromosome 21. Genomics 79: 824-832, 2002.
52. Pesole G, Gissi C, Grillo G, Licciulli F, Liuni S and Saccone C: Analysis of oligonucleotide AUG start codon context in eukariotic mRNAs. Gene 261: 85-91, 2000.
53. Frabetti F, Casadei R, Lenzi L, Canaider S, Vitale L, Facchin F, Carinci P, Zannotti M and Strippoli P: Systematic analysis of mRNA 5' coding sequence incompleteness in *Danio rerio*: An automated EST-based approach. Biol Direct 2: 34, 2007.
54. Casadei R, Piovesan A, Vitale L, Facchin F, Pelleri MC, Canaider S, Bianconi E, Frabetti F and Strippoli P: Genome-scale analysis of human mRNA 5' coding sequences based on expressed sequence tag (EST) database. Genomics 100: 125-130, 2012.
55. Piovesan A, Caracausi M, Pelleri MC, Vitale L, Martini S, Bassani C, Gurioli A, Casadei R, Soldà G and Strippoli P: Improving mRNA 5' coding sequence determination in the mouse genome. Mamm Genome 25: 149-159, 2014.
56. Kochetov AV, Sarai A, Rogozin IB, Shumny VK and Kolchanov NA: The role of alternative translation start sites in the generation of human protein diversity. Mol Genet Genomics 273: 491-496, 2005.
57. Bazykin GA and Kochetov AV: Alternative translation start sites are conserved in eukaryotic genomes. Nucleic Acids Res 39: 567-577, 2011.
58. Ivanov IP, Firth AE, Michel AM, Atkins JF and Baranov PV: Identification of evolutionarily conserved non-AUG-initiated N-terminal extensions in human coding sequences. Nucleic Acids Res 39: 4220-4234, 2011.
59. Arakaki TL, Pezza JA, Cronin MA, Hopkins CE, Zimmer DB, Tolan DR and Allen KN: Structure of human brain fructose 1,6-(bis)phosphate aldolase: Linking isozyme structure with function. Protein Sci 13: 3077-3084, 2004.
60. Lamour V, Quevillon S, Diriong S, N'Guyen VC, Lipinski M and Mirande M: Evolution of the Glx-tRNA synthetase family: The glutaminyl enzyme as a case of horizontal gene transfer. Proc Natl Acad Sci USA 91: 8670-8674, 1994.
61. Hermann E, Darcissac E, Idziorek T, Capron A and Bahr GM: Recombinant interleukin-16 selectively modulates surface receptor expression and cytokine release in macrophages and dendritic cells. Immunology 97: 241-248, 1999.
62. Schatz G and Dobberstein B: Common principles of protein translocation across membranes. Science 271: 1519-1526, 1996.
63. Nakamura M, Masuda H, Horii J, Kuma K, Yokoyama N, Ohba T, Nishitani H, Miyata T, Tanaka M and Nishimoto T: When overexpressed, a novel centrosomal protein, RanBPM, causes ectopic microtubule nucleation similar to gamma-tubulin. J Cell Biol 143: 1041-1052, 1998.
64. Varshavsky A: The N-end rule: Functions, mysteries, uses. Proc Natl Acad Sci USA 93: 12142-12149, 1996.
65. Rothermel B, Vega RB, Yang J, Wu H, Bassel-Duby R and Williams RS: A protein encoded within the Down syndrome critical region is enriched in striated muscles and inhibits calcineurin signaling. J Biol Chem 275: 8719-8725, 2000.
66. Strippoli P, Petrini M, Lenzi L, Carinci P and Zannotti M: The murine DSCR1-like (Down syndrome candidate region 1) gene family: Conserved synteny with the human orthologous genes. Gene 257: 223-232, 2000.
67. Vitale L, Frabetti F, Huntsman SA, Canaider S, Casadei R, Lenzi L, Facchin F, Carinci P, Zannotti M, Coppola D, *et al*: Sequence, 'subtle' alternative splicing and expression of the CYYR1 (cysteine/tyrosine-rich 1) mRNA in human neuroendocrine tumors. BMC Cancer 7: 66, 2007.
68. Facchin F, Canaider S, Vitale L, Frabetti F, Griffoni C, Lenzi L, Casadei R and Strippoli P: Identification and analysis of human RCAN3 (DSCR1L2) mRNA and protein isoforms. Gene 407: 159-168, 2008.
69. Facchin F, Vitale L, Bianconi E, Piva F, Frabetti F, Strippoli P, Casadei R, Pelleri MC, Piovesan A and Canaider S: Complexity of bidirectional transcription and alternative splicing at human RCAN3 locus. PLoS One 6: e24508, 2011.
70. Casadei R, Pelleri MC, Vitale L, Facchin F, Canaider S, Strippoli P, Vian M, Piovesan A, Bianconi E, Mariani E, *et al*: Characterization of human gene locus CYYR1: A complex multi-transcript system. Mol Biol Rep 41: 6025-6038, 2014.
71. Nagase T, Seki N, Ishikawa K, Tanaka A and Nomura N: Prediction of the coding sequences of unidentified human genes. V. The coding sequences of 40 new genes (KIAA0161-KIAA0200) deduced by analysis of cDNA clones from human cell line KG-1. DNA Res 3: 17-24, 1996.
72. Ribieras S, Lefèbvre O, Tomasetto C and Rio MC: Mouse Trefoil factor genes: Genomic organization, sequences and methylation analyses. Gene 266: 67-75, 2001.
73. Doglio L, Goode DK, Pelleri MC, Pauls S, Frabetti F, Shimeld SM, Vavouri T and Elgar G: Parallel evolution of chordate cis-regulatory code for development. PLoS Genet 9: e1003904, 2013.
74. Hinnebusch AG, Ivanov IP and Sonenberg N: Translational control by 5'-untranslated regions of eukaryotic mRNAs. Science 352: 1413-1416, 2016.
75. Caracausi M, Vitale L, Pelleri MC, Piovesan A, Bruno S and Strippoli P: A quantitative transcriptome reference map of the normal human brain. Neurogenetics 15: 267-287, 2014.
76. Pelleri MC, Piovesan A, Caracausi M, Berardi AC, Vitale L and Strippoli P: Integrated differential transcriptome maps of Acute Megakaryoblastic Leukemia (AMKL) in children with or without Down Syndrome (DS). BMC Med Genomics 7: 63, 2014.
77. Manning AG, Crawford BD, Waskiewicz AJ and Pilgrim DB: unc-119 homolog required for normal development of the zebrafish nervous system. Genesis 40: 223-230, 2004.
78. Piovesan A, Vitale L, Pelleri MC and Strippoli P: Universal tight correlation of codon bias and pool of RNA codons (codonome): The genome is optimized to allow any distribution of gene expression values in the transcriptome from bacteria to humans. Genomics 101: 282-289, 2013.
79. Komar AA: The Yin and Yang of codon usage. Hum Mol Genet 25 (R2): R77-R85, 2016.
80. Piovesan A, Caracausi M, Antonaros F, Pelleri MC and Vitale L: GeneBase 1.1: A tool to summarise data from NCBI gene datasets and its application to an update of human gene statistics. Database (Oxford) 2016: pii: baw153, 2016.
81. Ahsan B, Saito TL, Hashimoto S, Muramatsu K, Tsuda M, Sasaki A, Matsushima K, Aigaki T and Morishita S: MachiBase: A

Drosophila melanogaster 5'-end mRNA transcription database. Nucleic Acids Res 37 (Database): D49-D53, 2009.
82. Machida RJ and Lin YY: Four methods of preparing mRNA 5' end libraries using the Illumina sequencing platform. PLoS One 9: e101812, 2014.
83. Helbig AO, Gauci S, Raijmakers R, van Breukelen B, Slijper M, Mohammed S and Heck AJ: Profiling of *N*-acetylated protein termini provides in-depth insights into the N-terminal nature of the proteome. Mol Cell Proteomics 9: 928-939, 2010.
84. Doucet A and Overall CM: Amino-Terminal Oriented Mass Spectrometry of Substrates (ATOMS) N-terminal sequencing of proteins and proteolytic cleavage sites by quantitative mass spectrometry. Methods Enzymol 501: 275-293, 2011.