Circular RNA Translation

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A new RNA family has emerged, circular RNAs (circRNAs), generated by a process of backsplicing. CircRNAs have a strong impact on gene expression via their sponge function, and form a new mRNA family revealing the pivotal role of 5′ end-independent translation. CircRNAs are translated into proteins impacting various pathologies including cancer and neurodegenerative diseases, and are key players in aging. RNA circle translation also provides many perspectives for biotechnological and therapeutic applications.

Keywords: Circular RNA ; sponge function ; translation ; ribosome ; IRES ; m6A

1. Introduction

The potential of circular RNA to be translated has been studied since the 1970s. In 1979, an experiment was designed to determine the ability of circular mRNA to attach ribosomes [1]. A synthetic RNA was circularized with T4 RNA ligase and the binding of bacterial 70S ribosomes versus wheat or rabbit 80S ribosomes was assessed, showing that only the prokaryotic ribosomes were able to attach to RNA circles while the eukaryotic ribosomes were not. This demonstration supported the hypothesis of a ribosome scanning mechanism depending on the RNA 5′ end to explain initiation of translation in eukaryotes. According to this model, the 40S ribosome small subunit was expected to be recruited only at the mRNA capped 5′ end [2]. Consequently it was thought that eukaryotic ribosomes were unable to initiate translation by internal entry, rendering impossible the translation of circular RNA.

Ten years later, the discovery of translation initiation mediated by internal ribosome entry sites (IRESs) broke the rule [3][4][5]. Furthermore, the presumed inability of eukaryotic ribosome to bind circular RNA was contradicted in 1995: artificial circular RNA containing an IRES was generated [6]. The authors observed a significant translation of circular RNAs containing the IRES of encephalomyocarditis virus (EMCV). This work definitely demonstrated two main points in contrast with earlier suggestions (i) the 40S ribosomal subunit is not necessarily recruited at the mRNA 5′ end but can be recruited internally onto an IRES, and (ii) a circular RNA can be translated.

Despite these demonstrations, IRES function in cellular mRNAs remained questioned for a long time, although obvious in the case of picornaviruses whose genomic mRNAs are uncapped [7][8]. From here on many studies have demonstrated the role of IRESs to permit translation of specific classes of capped mRNAs when the cap-dependent initiation mechanism is blocked, which occurs during stress [7][9]. The IRES-dependent mechanism has now revealed its crucial role in the translational response to stress and is regulated by specific proteins called IRES trans-acting factors (ITAF) [3]. IRESs are also responsible for an increased translation of these mRNAs in cancer cells, a process related to abnormal rRNA modifications [10]. Covalently closed RNA circles resulting from splicing were identified at the beginning of the 1990s and were first considered as aberrant splicing products [11][12]. More than 20 years later it appears that hundreds of human and animal genes express circular RNA isoforms called circRNAs [13]. They are post-transcriptional regulators and in several cases they are translated, mostly via IRESs [14]. Translation of cellular circRNAs thus provides full physiological relevance to IRES-dependent translation.

CircRNAs may also be translated by another cap-independent mechanism mediated by the methylation of the nitrogen at position 6 in the adenosine base within mRNA, N^6-methyladenosine (m^6A) [14][15]. M^6A is a reversible epitranscriptomic modification found in many eukaryotic mRNAs [9]. When present in the 5′ untranslated region (5′UTR), a single m^6A promotes cap-independent translation at sites called “m^6A-induced ribosome engagement sites” (MIRESs) [16]. As IRESs, MIRESs stimulate selective mRNA translation in stress conditions by a mechanism involving direct binding of the initiation factor eIF3. Translation of circRNAs definitively put an end to the debate about 5′ end requirement and IRES existence in cellular mRNAs [8][17]. CircRNAs form a new class of mRNAs whose stability is far more important than that of their linear counterpart.

In parallel to the studies on covalently closed circular (CCC) RNA, a series of reports have shown that translation involves the functional circularization of mRNA. Already in the 1980s circular polysomes were observed by electron microscopy [18]. It was demonstrated a few years later that the mRNA 3′ untranslated region (UTR) is functionally interacting with the 5′UTR via the interaction of PABP with eIF4G [19][20]. The model of functional circularization involving eIF4G as a ribosome adapter was quickly adopted [21][22]. This mechanism involves both cap-dependent and cap-independent translation, as eIF4G can bind to the mRNA via IRESs independently of the cap-binding factor eIF4E [23]. The closed-loop phenomenon promotes ribosome recycling and thus enhances translation. Functional circularization of mRNAs occurs through several mechanisms in addition to interaction of PABP with eIF4G and appears as a pivotal parameter.

Can we still consider an mRNA as linear? That is the question.

2. Circular RNAs, from the Artefact to a New Gene Family

The first RNA circles were observed in 1976 by electron microscopy in viroids (plant pathogens), then in 1979 in human HeLa cell cytoplasm [24][25]. More than 10 years later in the 1990s the existence of such circles was confirmed and attributed to a scrambled splicing process, using the acceptor site of an exon located upstream of the donor splice sites [11][12]. The authors described the first cases of circular RNA generated from pre-mRNA processing, but the biological significance of such RNA molecules remained questioned. Today we know that these studies described what is presently called backsplicing [14].

Shortly after, a circular transcript was identified after RNase H digestion of RNAs extracted from adult mouse testis as the most abundant transcript expressed from the Sry sex determination gene [26]. This transcript specific to adult testis shows a cytoplasmic localization and a strong stability despite the absence of cap and poly(A). The stability of RNA circles was not a surprise as they do not give access to exoribonucleases. Such a stability had been observed previously for the circular RNA genome of the hepatitis delta virus, as well as for plant viroids and virusoids [27][28]. A long open reading frame (ORF) was detected in the Sry circular RNA. The authors made the assumption that it could have either a positive role by being translated by internal ribosome entry or a negative role as a noncoding RNA by preventing efficient translation [26]. When suggesting a link with translation, the authors were in the right direction: twenty years later Sry circRNA has been shown to function as a sponge for the microRNA miR-138 with 16 putative sites for that miRNA [29]. Sry circRNA thus indirectly acts on translation by preventing translational inhibition of miR-138 targets involved in activation of tumor cell growth and invasion [30].

In the last decade, the emergence of RNA deep sequencing technologies and of sharp bioinformatics analyses has generated a major leap forward in the field of circRNAs. The abundance of the circular transcript observed for Sry in 1993 turned out to be a general feature for thousands of genes in human and mouse tissue and in various cell types [13][31]. RNA-seq analyses revealed that many scrambled splicing isoforms are expressed at levels comparable to that of their linear counterparts. The circular status of these scrambled isoforms was demonstrated using RNase R, a 3′–5′ exoribonuclease that degrades all linear RNA molecules. Most circRNAs are located in the cytoplasm. The expanded landscapes of circRNAs have been determined by RNA-Seq in 44 tissues of human, macaque and mouse, revealing 104,388, 96,675 and 82,321 circRNAs from the three species respectively [32]. Initially considered as splicing background noise, circRNAs constitute according to the current studies 20% of the top 1000 most abundant transcripts in human and macaque tissues while only 8% in mouse tissue. In human tissue, 61% of the coding genes express at least one circular transcript. All these reports demonstrate that expression of circRNAs is far from being an epiphenomenon.

CircRNAs exhibit different modes of action, depending on their composition which itself affects their localization. CircRNAs that contain intronic (called ciRNAs) or intronic plus exonic sequences (EIciRNAs) are nuclear and mainly regulate the expression of their parental gene. EIciRNAs have been shown to interact with RNA polymerase II subunits, with U1 snRNP and with the parental gene promoter where they behave as transcriptional enhancers [33]. Another study has shown that circRNA expression can influence the splicing of the parental gene by competing with canonical splicing [34]. The third class of circRNAs, composed of exonic sequences exclusively (ecRNAs), are cytoplasmic and act via two types of mechanisms: on the one hand they act by sponging miRNAs or RNA binding proteins (RBPs), on the other hand they can be translated [14][35].

Translation of circular RNA has demonstrated its relevance in many diseases [84,85]. Several circRNA products are involved in cancer [49,50], while the deregulated expression of circRNAs acting as sponges or being translated is involved in neurodegenerative diseases. CircRNAs appear as key players in aging [88,89].

3. Future Perspectives for Biotechnological and Therapeutic Applications of RNA Translation in Circles

The emergence of circular RNAs, much more stable than their linear counterparts, opens a new avenue for protein production in biological systems and development of therapeutic vectors. In view of the stability of circular RNA one can envisage cell transfection by circular RNA produced in vitro. The challenge of optimizing such a vector resides in its translation efficiency. The study by Wesselhoeft et al. has pioneered the use of exogenous circRNA for robust and stable protein expression in eukaryotic cells [36]. These authors have engineered a technology of circRNA production for potent and stable translation in eukaryotic cells, based on self-splicing by using a group I autocatalytic intron. They found that the most efficient intron is that of Anabaena pre-tRNA while the optimal IRES is the Coxsackievirus B3 (CVB3) IRES. The efficiency of the IRES is however cell-type-dependent. Such circRNAs containing the luciferase reporter ORF were produced by in vitro transcription and purified using high-performance liquid chromatography (HPLC). They were then used for transfection of human cell lines, revealing that the circRNA produces 811% more protein than the corresponding capped and polyadenylated linear RNA. CircRNA exhibited a protein production half-life of 80–116 h, compared to 43–49 h for the linear counterpart. These authors reported that circRNAs are less immunogenic than linear RNAs [37]. Synthetic circRNAs were also produced by simple ligation of in-vitro-transcribed linear RNA molecules containing microRNA binding sites [38]. Such sponge circRNAs, containing miR-21 binding sites, were shown to suppress proliferation of three gastric cell lines. It should be noted that synthetic circRNAs containing m^6A modifications instead of the IRES failed to produce any translation product, suggesting that m^6A-mediated translation would require the binding of nuclear RBPs [37].

In another contribution, Meganck et al. designed a recombinant adeno-associated virus (rAAV) vector expressing a circRNA coding GFP under the control of the CMV promoter, with the EMCV IRES to drive initiation of translation [39]. Vector intravenous delivery into mice demonstrated a robust transgene expression in cardiac tissue as well as in brain and eye tissue while expression was less efficient in liver tissue. This was attributed to the EMCV IRES but might also result from the weak activity of the CMV promoter in liver. The above studies demonstrate that IRESs drive the efficient production of proteins from circular RNA-producing vectors but also underline the sensitivity of IRESs to the cellular context. In future applications, the choice of IRES and promoter may be adapted according to the targeted tissue and it will be of particular interest to test cellular IRESs rather than only viral IRESs in such vectors, as these IRESs are often tissue-specific in vivo [40][41].

A recent study has developed a cell factory for recombinant protein production in Chinese hamster ovary (CHO) cells, based on rolling-circle translation [42]. Cells were transfected with a plasmid containing the sequence of human erythropoietin (EPO) ORF flanked by adequate splicing sites to obtain a circRNA. The EPO ORF was made infinite by removal of the stop codon which was replaced by a 2A element to obtain a ribosome “stop-go” process (and not a cleavage as mentioned in many publications) [43]. The 2A-mediated stop-go drives immediate reinitiation through ribosome skipping and prevents the formation of multimers. Costello et al. showed that the EPO coding “infinite” circular mRNA improves the production of secreted EPO compared to linear mRNA or circRNA with a stop codon. Another original approach is to produce ribozyme-assisted circRNAs (racRNAs), in the so-called “Tornado” (Twister-optimized RNA for durable overexpression) expression system [44]. The “Tornado” transcript is flanked by two Twister ribozymes that undergo autocatalytic cleavage and generate termini that are ligated by the endogenous RNA ligase RtcB. racRNAs containing protein-binding aptamers were successfully expressed in different mammalian cell types. In particular, the NF-kB pathway was efficiently inhibited by this way. RacRNAs might also be useful to express proteins of interest if containing an ORF. Altogether, these different studies provide numerous perspectives for a new generation of gene therapies [45]. Synthetic circRNAs, plasmids or viral vectors expressing circRNAs offer an exciting perspective to expression of genes of interest and also combinations of therapeutic genes that could be translated either with IRESs or by rolling-circle translation with 2A elements.

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