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SUPPLEMENT

Circular RNA: New Regulatory Molecules

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Circular RNA are a family of covalently closed circular RNA molecules, formed from pre-mRNA of coding genes by means of splicing (canonical and alternative noncanonical splicing). Maturation of circular RNA is regulated by *cis*- and *trans*-elements. Complete list of biological functions of these RNA is not yet compiled; however, their capacity to interact with specific microRNA and play a role of a depot attracts the greatest interest. This property makes circular RNA active regulatory transcription factors. Circular RNA have many advantages over their linear analogs: synthesis of these molecules is conservative, they are universal, characterized by clearly determined specificity, and are resistant to exonucleases. In addition, the level of their expression is often higher than that of their linear forms. It should be noted that expression of circular RNA is tissue-specific. Moreover, some correlations between changes in the repertoire and intensity of expression of circular RNA and the development of some pathologies have been detected. Circular RNA have certain advantages and can serve as new biomarkers for the diagnosis, prognosis, and evaluation of response to therapy.

Key Words: *circular RNA; noncanonical alternative splicing; deposition of minor interfering RNA depot; gene expression; diagnosis*

Circular RNA (circRNA) are a family of circular covalently closed single-stranded RNA molecules. Up to recent time they were assumed to be by-products of canonical splicing [35,58]. CircRNA have received little attention because of their low content and unknown functions. However, the development of modern technologies of RNA sequencing, quantitative PCR analysis, and bioinformation methods has made it possible to distinguish circRNA as a special class of RNA molecules. Total analysis of non-polyadenylated transcripts by high-throughput deep sequencing has detected rather high incidence of circRNA: more than 10% of all genes expressed in various cells and tissues are presented by circRNA [25,30,37,39,41,54,75,81]. The level of circRNA is tissue-specific [2,28,36,60,63,72]. In eukaryotic cells, circRNA are mainly a result of alternative noncanonical splicing of pre-mRNA. Cyclization is realized with participation of spliceosome and regulated by *cis*- and *trans*-elements [9,20,37,42,48,66,81]. Due to a rather long lifespan, circRNA are important element of post-transcriptional regulation of gene expression: they bind miRNA and create a sort of depot for these molecules preventing their interactions with mRNA targets [32,54]. Other mechanisms of gene expression regulation with circRNA participation are now also proposed [16,32,41,47,53,54,84]. The interest to these molecules is explained primarily by the fact that circRNA are involved, in this or that way, in the key cell processes, such as self-regeneration, proliferation, and apoptosis. Hence, disorders in the system of expression of circRNA proper or their target molecules can lead to the development of many diseases. In addition,

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the presence of circRNA in virtually all biological fluids suggests that these molecules can be potential biomarkers of certain diseases.

The first evidence of circRNA existence was documented in 1976: electron microscopy showed a single-stranded circular covalently closed RNA molecule in some plant viroids [64]. Later, it was shown that hepatitis D virus genome is presented by circRNA [26]. It was then hypothesized that circular single-stranded RNA form was a mode of viroid genome existence. However, several years later, studies of *DCC* oncosuppressor gene in human cells showed that the exon sequence in some transcripts differed from their sequence in genomic DNA [58], and a mechanism of circRNA formation was suggested. However, it was then concluded that this splicing process was more likely an exclusion.

The existence of "exotic" circRNA was almost completely ignored over two subsequent decades, as these molecules could not be directly sequenced and their position in genomic DNA could not be mapped. In addition, the standard molecular biological methods for RNA studies failed to catch circRNA, as these molecules had no free 3' and 5' ends or a polyadenylated tail [38]. New sequencing technologies together with bioinformation approaches brought circRNA back into molecular biology and showed them as important contributors to vital cell activity [73,84]. High-throughput deep sequencing of ribosomal RNA-deficient circRNA libraries with high coverage made it possible to identify new circRNA in organisms of all classes, starting from protists, plants, fungi, and up to animals [51,73]. CircRNA were found in many *Metazoa*, including humans, mice, *Danio rerio* fish, nematodes, and *Drosophila*, and also in plants, which indicates evolutional conservative nature of this class of RNA molecules [73]. Interestingly, circRNA isoforms were found in various groups of organisms, for example, in the fungi, plants, and protists, whose latest common ancestor had existed more than a billion years before [73]. The conservative nature of coding sequences from 223 human circRNA and their orthologs from mouse cells was studied [54]. The codon nucleotide sequence of the respective linear RNA forms served as control. It was found that the third position in circRNA codons (often invariant for coding an amino acid), was more conservative in comparison with the control, which could also indicate the important functional role of this class of biomolecules.

BIOGENESIS OF circRNA

Our knowledge of the entire variety and functions of circRNA is so scanty, that these molecules can be classified only by their structure. Like mRNA, circRNA are transcripts of protein-coding genes, and their precursors are pre-mRNA proper synthesized by RNA polymerase II [9,48,66,81]. However, the structure of circRNA differs from the structure of its linear analogs because of differences in the splicing mechanisms leading to formation of these RNA forms. The majority of circRNA in eukaryotic cells are synthesized by the canonical spliceosome by the head-to-tail mechanism with excision of exons from thousands of genes normally leading to the formation of linear mRNA forms [9,25,30,37,48,41,54,66,81]. Generally, circRNA can consist of exons (sense parts of the gene) — circular exon RNA (ceRNA), introns (missense parts of the gene) — circular intron RNA (cinRNA), or have a mixed exon-intron structure — circular exon-intron RNA (ceiRNA). The existence of circRNA with one or several exons indicates probable contribution of alternative splicing of pre-mRNA to their formation and the existence of various circRNA isoforms [63,75,81,84]. Despite the fact that canonical splicing forms are involved in both processes, the rules of their regulation differ [74].

Let us discuss the probable mechanisms of formation of various circRNA forms.

Spliceosome-dependent biogenesis. Several mechanisms of circRNA formation are known [6]. The simplest of them is circRNA formation by the canonical splicing process. Generally, the information about the primary structure of proteins in eukaryotic cells is translated from DNA via mRNA (Fig. 1, *a*). First, RNA copy of genomic DNA is synthesized during transcription process. This immature mRNA (or pre-mRNA) consists of coding sequences carrying information about protein structure (exons) and noncoding sequences (introns; Fig. 1, *a*, stage I). Spliceosome is assembled on the intron directly before the intron is excised from pre-mRNA. Short sequences at the intron terminals (GU located at the 5'-terminal and AG at the 3'-terminal) are essential for their correct interactions. As a result, the intron forms a loop-like structure, lariat (Fig. 1, *a*, stage II) and is excised with the formation of exon-exon junction (Fig. 1, *a*, stages III, IV). This leads to the formation of linear form of mature RNA with consecutively arranged exons (Fig. 1, *a*, stage V). Normally, the excised introns are degraded by exonucleases. However, intron loop rather often is not excised in a regular manner; instead, the 3'-terminal fragment located below the branching site is "clipped", which leads to the formation of a stable circRNA (Fig. 1, *a*, stage V). CircRNA found in human and *Xenopus tropicalis* cells originate from the lasso element formed during splicing of various RNA types [84]. In *Archaea* and *Metazoa* animals circRNA with 3',5'-phosphodiester bond have been found — a cyclic form of sequence from introns excised from

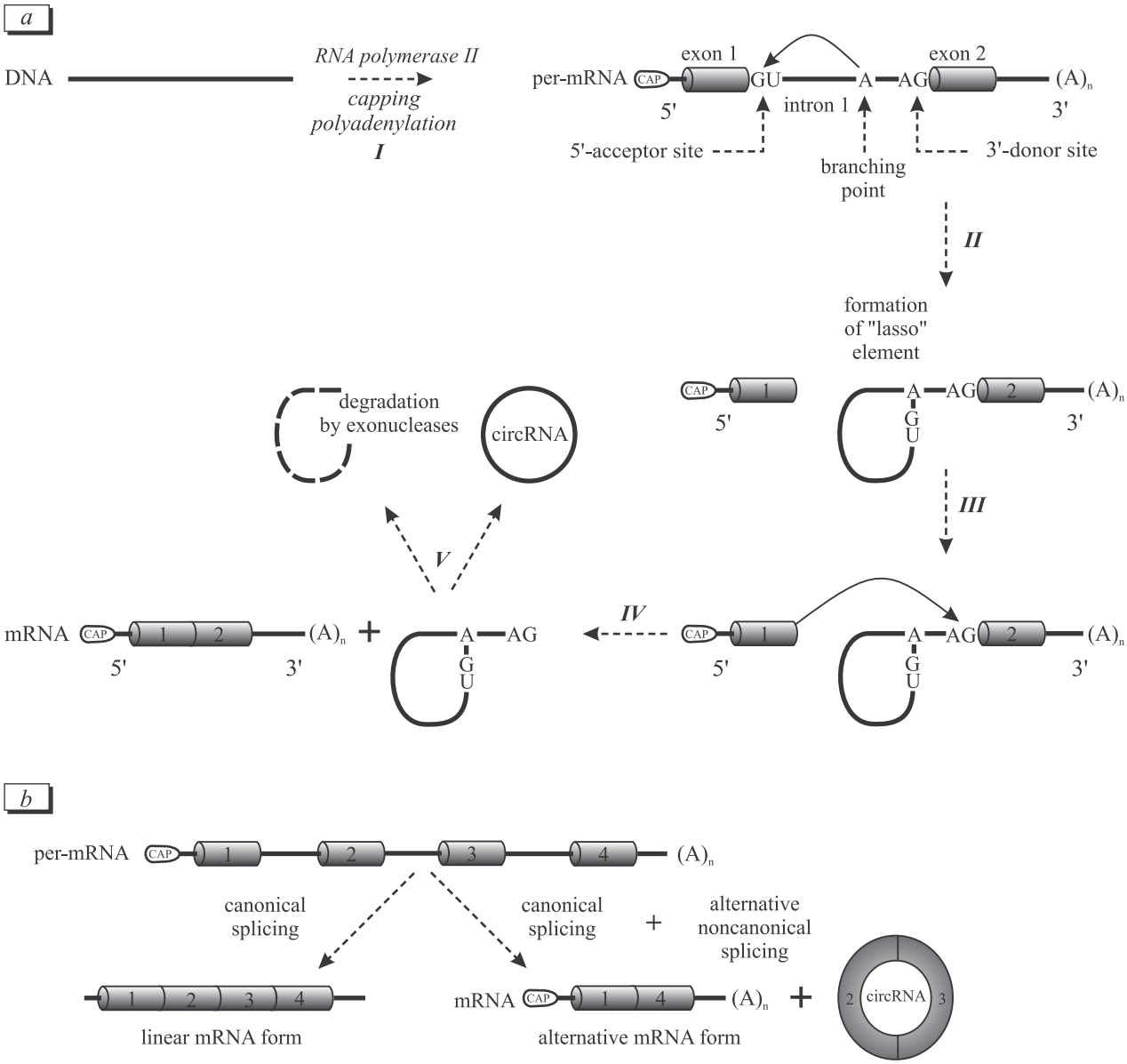

Fig. 1. Spliceosome-dependent mechanism of circRNA formation by canonical splicing (*a*) and formation of alternative mRNA and circRNA forms by noncanonical splicing (*b*).

tRNA during the splicing process [3,52,61]. Another method for circRNA production is ligation of viral RNA genomes by host cell enzymes with the formation of 3',5'- or 2',5'-phosphodiesrter bond [21,26,64]. In addition, circRNA can emerge during rRNA processing or under conditions of disordered tRNA biogenesis in *Archaea* and algae [22].

The length of circRNA found in eukaryotic cells greatly varies: from 100 to 4000 nt, which indicates different intron-exon structure of these molecules [63,81]. As a rule, circRNA have exons, no more than 5. These structures form also during the work of the canonical splice machine, but only at the stage of exon transposition in various combinations. This kind of splicing is called "head-to-tail", noncanonical alternative splicing, or "backsplicing" (Fig. 1, *b*) [32,39,54]. In this case it seems most likely that the lariat type structure is not removed correctly [18,47,67]. The existence of mixed exon-intron forms of circRNA molecules can be also explained by, for example, ligation of groups I and II introns with the formation of circRNA with 3',5'- or 2',5'-phosphodiester bond by self-splicing, that is, by non-spliceosome-dependent processing of pre-mRNA [17,29,48,50].

Most circRNA consist of several exons, usually from two or three [81]. It seems that no specific sequence of any kind is required for the formation of circRNA, except the splicing site [66]. In addition,

several circRNA types, with or without introns, can be produced from the same transcript by alternative splicing (Fig. 2) [12,30,47,63,81]. In this case, spliceosome brings two sites closer to each other: splicing 5'-site (acceptor site) of the exon more distant from the start of pre-mRNA transcript and splicing 3'-site (donor site) of the previous exon with the formation of circRNA with abnormal order of exons; for example, exons 4 and 2 can be connected in this circRNA.

Interestingly that exon length should be minimum for noncanonical alternative splicing and product cyclization. It is shown for circRNA with high levels of expression that the exons are longer in single-exon circRNA than those in the multiexon forms [68,81].

Artificial introduction of mutations in circRNA-expressing plasmid constructions has shown that the efficiency of cyclization of the excised exon depends on the presence of a canonical splicing site at the terminals of these exons [9,30,66,74]. There are no data on the cyclization kinetics in RNA terminals up to the present time; however, generally we can speak about lesser efficiency of this process in comparison with the efficiency of canonical splicing, as the constant level of circRNA expression is often significantly lower than the expression of the respective linear form [9,48,66,81]. In addition, poor efficiency of cyclization can be attributed to the fact that the process of association of 3'-acceptor site with and its ligation to 5'-donor site is not preferable for spliceosome. However, despite poor efficiency of noncanonical alternative splicing, synthesis of circRNA correlates with high level of new mRNA transcription [9,82].

The details of cyclization process with spliceosome involvement are not yet clear. It is shown that the production of circRNA ceases with alteration of the polyadenylation signal sequence in pre-mRNA [48]. This fact gives grounds to suggest that cyclization may be posttranscriptional. It is shown however that the polyadenylation signal is not an obligatory condition for circRNA production in plasmid-transfected cells and, presumably, the production of circRNA is paralleled by transcription process [42]. For example, circRNA can be found among chromatin-associated RNA isolated from the *Drosophila* heads, which is one more evidence supporting this theory [9]. However, it is worthy of note that chromatin-associated RNA is strictly speaking not an equivalent of the initial RNA forming in transcription, as RNA-transcripts with mature polyadenylated 3'-terminals have also been found among the chromatin-associated fractions [14]. Hence, it remains unclear, how, precisely, the circRNA biogenesis is related to the transcription process. Studies of circRNA precursors may help to detect the kinetics of circRNA biogenesis and the potential relationship between this process and other stages of mRNA maturing at the level of the entire genome.

Contribution of intron complementary sites to formation of circRNA. Formation of the circular product often implies the presence of complementary sites inside introns flanking the circularized exons. The majority of mammalian and *C. elegans* circRNA and some of *D. melanogaster* circRNA form from internal exons adjacent to the long flanking introns often containing sequences with reverse complementarity (Fig. 2, *a*) [37,39,42,81]. These sequences easily "anneal" each other with the formation of a duplex RNA structure, this eventuating in the formation of a circRNA molecule and facilitating the formation of its alternative forms [48,72,81]. This mechanism of circRNA formation is hypothesized in studies of cyclization process with the use of expressing vectors and methods for complete genome analysis [39,42,48,81]. Duplex RNA can be formed with the use of repeating sites, for example, Alu-repeats in mammals, or non-repeating RNA fragments [42,48,81]. The minimum length of a fragment capable of initiating circRNA biogenesis can be 30-40 nt, but formation of longer duplex structures increases significantly the output of circRNA [81]. It is noteworthy that the efficiency of circRNA production also depends on the efficiency of formation of a duplex between RNA sites flanking circularized exons and the RNA sites inside one intron (Fig. 2, *b*). The possibility of formation of an RNA duplex between two sites of the same intron shifts the balance towards canonical splicing, while formation of a duplex between intron-flanking RNA sequences leads to an increase of circRNA output [81]. Moreover, the presence of several Alu-repeat sequences with opposite orientation in the intron theoretically can lead to emergence of several circRNA from the same gene transcript during alternative splicing (Fig. 2, *b*) [81].

Hence, complementarity sites in flanking introns can be regarded as regulatory elements. Presumably, their retention in the initial genes is essential for maintenance of interspecies conservative nature of the RNA formation process. However, it is noteworthy that not all circularized exons are flanked by complementary sequences. In *D. melanogaster* the sites that can be circularized with the formation of circRNA usually have no obvious mutually complementary sequences in the intron edges, while in *O. sativa* only some circularized exons have sites complementary to intron repeats [51,75]. *Schizosaccharomyces pombe* circRNA-forming gene *mrps16* (mitochondrial ribosomal protein of the small subunit) also lacks flanking complementary sequence, and hence, its circRNA forms during canonical splicing by the lariat formation mechanism [12]. As for human cells, formation of circRNA is detected in them even by using expressing vectors with intron

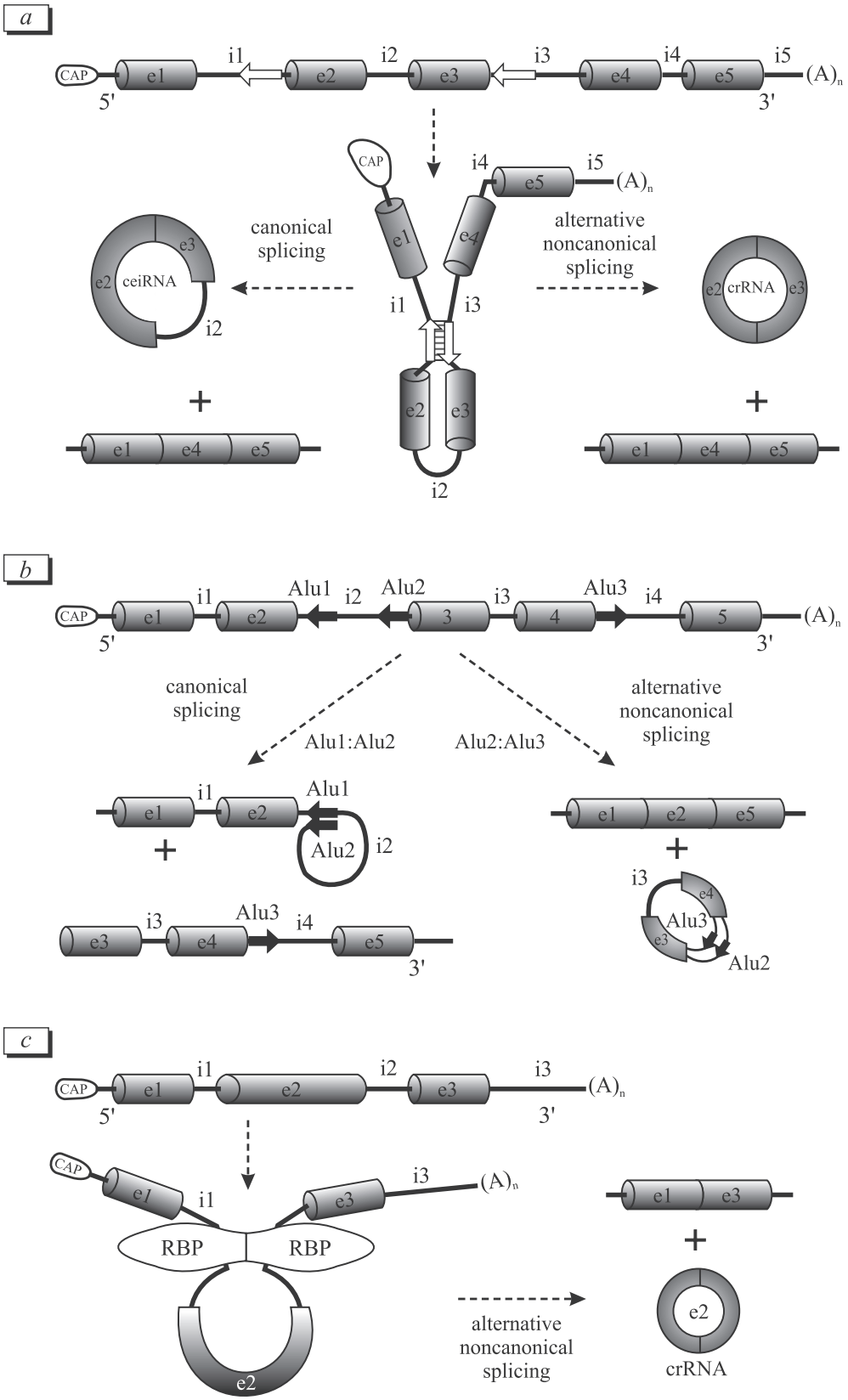

Fig. 2. Formation of circRNA product with participation of self-complementary sites (*a*), inverted Alu-repeats, flanking the sequence excised from pre-mRNA (*b*), and RNA-binding proteins (*c*). RBP: RNA-binding protein; ceRNA: circular exon RNA; ceiRNA: circular exon-intron RNA; e: exon; i: intron.

flanking regions no longer than 20 nt, but without inverted repeats [66]. It remains unclear up to the present time, whether these intron sequences serve as the motive force proper during the formation of circRNA or a cell can make use (as an alternative) of their interactions with RNA-binding proteins during exon cyclization [9,20]. In addition, canonical regulatory complementation sites (splicing *cis*-elements), known as enhancers or silencers, can differently modify the cyclization efficiency when alone or in combination with RNA-binding proteins [74].

Contribution of RNA-binding proteins to formation of circRNA. It is shown not once that RNA-binding proteins regulate circRNA biogenesis (Fig. 2, *c*) [9,20,37,60,62]. In *D. melanogaster* Miscleblind (MBL) splicing factor regulates the formation of circRNA from its own pre-mRNA. Multiple sites of MBL binding have been identified in introns flanking *Mbl* gene pre-mRNA circularizing exon [9]. High expression of *Mbl* or an increase in the number of MBL binding sites in the target intron sequences stimulate the exon cyclization process and can lead to an increase in the respective circRNA output. One more RNA-binding protein, stimulating the formation of circRNA from the respective pre-mRNA, is quaking, QKI [20]. The synthesis of many circRNA increases in human cells under the effect of QKI in epitheliomesenchymal transition. This fact gives grounds to suggest that the mechanism of circRNA formation is regulated in a tissue-specific manner. Similarly as MBL, QK15 protein regulates the cyclization process via specific binding sites; these sites are located in intron sequences flanking the circularizing exon [20]. Artificial insertion of QKI-binding sites into introns stimulates significantly the production of circRNA. It has been hypothesized [20] that due to QKI capacity to form dimers, the RNA-binding protein can bind two flanking introns and promote spatial connection between the terminals of circularizing exons at the expense of protein-protein interactions, this leading to stimulation of circRNA synthesis.

An alternative example of the relationship between RNA-binding protein and circRNA output is adenosine deaminase 1 (ADAR1), the main function of which is RNA correction. This enzyme can suppress the level of circRNA expression by various modes [37,60]. It catalyzes adenosine transformation into inosine during interaction with RNA duplex, formed by two exon-flanking sequences. Emergence of inosine leads to attenuation of the complementary interactions in the duplex up to its complete dissociation, and hence, the circRNA product output drops. The expression of circRNA is regulated in this way for just several genes, only those in which ADAR1 catalyzes transformation of one base into another [60]. In addition, ADAR1 can nonenzymatically inhibit circRNA biogenesis, as it can melt the hairpin double-stranded RNA in case the inverted complementary sequence is located in the terminal intron of circularized exon [19,37,60].

As there are hundreds of RNA-binding proteins, detection of the molecules involved in circRNA biogenesis is an important problem. It is also interesting, in this connection, to study the combined effects of RNA-binding proteins on the levels of expression of certain circRNA. The formation of the product of noncanonical alternative splicing of *Lac-case2* gene in *D. melanogaster* is regulated by interactions between intron repeats and by a combination of interactions between hnRNP (heterogeneous nuclear ribonucleoprotein) and SR proteins (Ser/Arg) [42]. Hence, regulation of circRNA production in the cells is a significantly more intricate process than it could seem at first.

PREVALENCE OF circRNA

circRNA formed from pre-mRNA are rarely detected in profiling of polyadenylated RNA, as they have no polyA tail. CircRNA are usually detected in these cDNA libraries or in cDNA libraries, created by circRNA fraction enrichment by processing with RNAse P (enzyme hydrolyzing only linear RNA forms), by sequencing and subsequent computer analysis, aimed at search for various orientations of RNA sequences in readings. This method has promoted detection of more than 10,000 circRNA in various *Metazoa* — from Drosophila and worms to mice [37,75]. By the present time about 100,000 various circRNA are identified in human cells; according to tentative estimations, they may constitute 1 to 10% of all transcription forms. In addition, circRNA are detected in plant cells (*Oryza sativa* and *Arabidopsis thaliana*) and in fungal and protist cells [12,51,73]. Fibroblast circRNA are transcripts of ~14% of all transcribed genes [39]. An appreciable part of transcripts subjected to splicing are transformed into circRNA [18,62]. This fact may indicate that this type of molecules is rather traditional for cellular vital activity.

The level of circRNA expression is often extremely low, which fact once more indicates that the greater part of circRNA molecules are products of splicing [25,30,37,39,54,63,75,81]. However, the quantity of circRNA of certain human genes (about 50) can be by an order of magnitude higher than the quantity of their linear isoforms in each of the studied cell lines [30,39,63]. In addition, thousands of circRNA molecules accumulate in brain cells [54]. There are also several tens of genes for which the expression in the form of circRNA is a tissue-specific sign or a sign specific for a certain cell type [48,54,66].

circRNA, formed as a result of exon excision in the process of noncanonical alternative splicing, are

formed mainly in neuron gene splicing; these circRNA can be potentially significant for the brain cell functioning processes [9]. A detailed analysis of sequences of RNA isolated from mammalian brain tissues, neuron-differentiated cell lines, and brain cells of mice at different stages of development, has shown high expression of thousands of circRNA [60,79]. Correlations of this kind are detected for *D. melanogaster*: the expression of circRNA in neuronal tissue cells increases with age [75]. Moreover, the expression of rather many circRNA in neurogenesis is significantly higher in comparison with the expression of linear RNA isoforms; in addition, in synaptogenesis the circRNA pool is enriched significantly in comparison with the parameters for linear isoforms [60,79]. Comparison of various mouse, human, and even *D. melanogaster* cells shows that this parameter is rather conservative for circRNA, which indicates a probable relationship between circRNA and the neuron work [60]. As circRNA are stable and can accumulate in nonproliferating cells, low rate of neuron division can be responsible for passive accumulation of circRNA [10]. Hundreds of circRNA molecules are detected, whose expression is also changing in epitheliomesenchymal transformation in humans, which indicates the probable impact of circRNA for cell migration, invasion, or metastatic growth [20]. However, the specific functions of circRNA in brain cells or in carcinogenesis remain unclear.

Some circRNA are produced from transcripts of genes associated with the organism development or disease, this supporting once more the theory of the functional activity of circRNA in health and disease in some cases. For example, curcSRY (circRNA of Y chromosome site responsible for gender identification) plays the key role in the development of the testes in mice [32]. CircRNA suppressing *ANRIL* long noncoding RNA *INK4/ARF*-locus is involved in transcription repression; the expression of circRNA from *ANRIL* increases the expression of *INK4/ARF* and the risk of atherosclerosis associated with it [16]. One of the best studied circRNA molecules, ciRS-7, works as a sort of miR-7 accumulator in nervous tissues [32,53,54]. The level of ciRS-7 expression is extremely low or zero in other tissues, including tissues with very high expression of miR-7 [54]. Moreover, the expression of ciRS-7 decreases significantly during the development of sporadic Alzheimer's disease [32,53,54]. In addition, the profile of circRNA expression is changing in cell proliferation associated with oncogenesis; their quantity may drastically drop during the development of colorectal cancer [10].

Despite a vast scope of data, correlation between the expression of mRNA and the corresponding circRNA remains unclear [33,30,48,60,63,79]. The above examples indicate that high expression of circRNA in some cells and tissues does not reflect the common increase of pre-mRNA expression but presumably plays some role. It seems essential to detect the basic mechanisms determining the levels of circRNA under various conditions in various types of tissues. For example, RNA-Seq analysis of more than 7000 human circRNA has shown that the tissue specificity of their expression levels does not appreciably differ from tissue specificity of their linear analogs expression in various cell types [30]. Moreover, as circRNA molecules are more stable than their linear isoforms and can accumulate in the cells with different division rate, it remains unclear whether the detected level of circRNA really reflects the kinetics of these molecules processing [39,54,84].

It is known by the present time that the function of circRNA is largely determined by the molecule location. Circular exon RNA (ceRNA) are located mainly in the cytoplasm, but it is not clear whether cinRNA and ceiRNA are alternatively nuclear forms or they can be present in the cytoplasm as well [30]. These data are essential for more ample understanding of circRNA functions.

THE KNOWN FUNCTIONS OF circRNA

Regulation of gene expression by circRNA: Buffer for minor interfering RNA. It may seem at first that a low level of circRNA expression generally indicates that this RNA form is just a by-product of the eukaryotic transcriptoma work. However, it is shown that some circRNA are physiologically important and contribute to regulation of gene expression at various levels.

A great part of circRNA formed as a result of noncanonical alternative splicing are located mainly in the cytoplasm [39]. Despite the fact that artificially created circRNA, containing a ribosome binding site (RBS), can be subjected to translation, there are no data on the direct interactions of endogenous circRNA with ribosome [20,39,74,84]. This can mean in fact that in the cytoplasm more stable circRNA can compete with mRNA for binding to miRNA and thus modify the miRNA function as postreplicative suppressors or indirectly modulate the stability of the target mRNA [39,70]. All circRNA capable of miRNA deposition are really located in the cytoplasm and contain no introns [30]. Moreover, circRNA of this type can contain several polymorphic sites in the target miRNA binding site [69]. By the present time miRNA are much better characterized than circRNA; it is well known that many miRNA are in this or that way associated with human diseases. Presumably, the details of miRNA-circRNA interactions will help to develop new biomarkers for the diagnosis of human diseases.

The first evidence of miRNA accumulation by endogenous circRNA or at least of its competition with endogenous RNA is presented in two independent reports [33,54]. By the present time numerous data indicate that more stable circRNA may be involved in the RNA interference process and compete with mRNA for binding miRNA in the cytoplasm and hence, take part in regulation of gene transcription. The best characterized circRNA in this aspect is ciRS-7, formed from *CDR1* gene antisense transcript preferably in the brain of mice and humans; this gene is associated with neurological diseases [23,32]. CircRNA ciRS-7 has about 70 miR-7 binding sites and plays a peculiar role of this molecule antagonist in regulation of the target mRNA transcription [32,54]. The expression of ciRS-7, located in the cytoplasm, is rather high; this molecule is characterized by a high capacity to bind miR-7 — up to 20,000 miR-7 molecules per cell [54]. Multiple interactions between ciRS-7 and miR-7 isoform can modify the system of expression of *PARP* and *SP1* mRNA (miR-7a targets in myocardial cells), which can eventually lead to myocardial infarction [27]. The deposition function is realized under conditions of simultaneous expression. For example, coexpression of ciRS-7 and miR-7, particularly in the neocortical and hippocampal neurons, leads to a significant suppression of miRNA activity and to an increase of the target genes expression [33]. Importantly that the function of ciRS-7 is conservative. The expression of human ciRS-7 in *Danio rerio* cells leads to deterioration of the midbrain development; suppression of miR-7 expression causes a similar effect [54].

In addition to ciRS-7, there are several circRNA potentially capable of working as buffers for miRNA. CircRNA of SRY mice has 16 binding sites for miR-138, involved in some physiological and pathological processes [31,32,83]. However, human homologous circRNA has one miR-138 binding site [30]. According to some data, there are several binding sites for respective miRNA in circRNA sequences formed from transcripts of genes coding for transcription factors with the "zinc fingers" domains [30]. CircRNA cirITCH has multiple sites for interactions with miR-7, miR-17, and miR-124; this buffering of miRNA leads to an increase of E3-ubiquitinligase gene (*INCH*) expression and inhibition of the Wnt-dependent signaling pathway in tumor-transformed cells in esophageal squamous cell carcinoma [44]. CircRNA HIPK3, isolated from exon 2 of homeodomain interacting protein kinase 3 gene mRNA, accumulates miR-124 and other miRNA; hence, suppression of HIPK3 circRNA leads to an inhibition of cell growth, which indicates a probable role of this molecule in cell proliferation, mediated by changes in miR-124 interactions with its target mRNA [85]. Some circRNA, synthesized from cattle casein gene (*CSN*) transcripts with high expression in mammary glands, accumulate miR-2284 family miRNA with targets *CSN1S1* and *CSN2* mRNA [80].

In order to work as an accumulator, circRNA should contain conservative miRNA binding sites in their structure. However the greater part of human and mouse circRNA in fact have no specific miRNA binding sites at all, which makes doubt the high prevalence of the sponging (accumulator) function of these molecules [30,79].

In contrast to mammals, *D. melanogaster* circRNA have more than a thousand of conservative binding sites for miRNA, but it remains unclear whether these molecules work as sponges in the moth cells [75]. Quantitative studies of miRNA functions have shown that generally the changes in the concentration of miRNA binding sites are inessential for the level of the target gene expression [3].

These data indicate that high capacity of circRNA to accumulate various miRNA serves as a very fine intracellular mechanism for regulation of various processes.

Role of circRNA in transcription. Circular exon-intron RNA often have transcription sites, and hence, the hypothesis on their involvement in the transcription complex assembly and regulation of transcription seems quite probable [47]. However, the majority of circRNA are located in human cell cytoplasm [39,47,84]. It remains unclear in which way the circRNA are transported to the nucleus and remain there. Potentially circRNA may use the same mechanisms for penetration into the nucleus as the linear RNA forms, often found in the nucleus and containing introns [15]. Stable lariat intronic RNA, produced by the lasso mechanism, have been detected in *Xenopus tropicalis* oocytes. In human cells these cinRNA more intensely accumulate in the nucleus and regulate the gene transcription similarly as the *cis*-elements [84]. Some cinRNA with high expression, for example, ci-ankrd52 and ci-sirt-7, interact with RNA polymerase II elongation complex [84]. Suppression of expression of these cinRNA leads to a decrease of the transcription levels of the respective genes *ANKRD52* and *SIRT7*, which fact gives grounds to suggest that cinRNA stimulate the transcription of other related genes, though the mechanism of this effect remains unclear [84].

Regulation of circRNA transcription, associated with RNA polymerase, has been also demonstrated by RNA immunoprecipitation in studies of the noncoding RNA function [78]. It has been shown that in the nucleus ceiRNA interact with RNA polymerase II and thus modify its transcription activity. Specifically, suppression of *ElciEIF3J* and *ElciPAIP2* gene expression attenuates transcription of other related genes.

In addition, it is shown that ciRS-7, also known as circCdr1as, regulates the level of insulin transcription by accumulating miR-7, though the precise mechanism of this effect is still unknown [78].

These data demonstrate that nuclear circRNA act as *cis*-regulators of the gene transcription; the mechanism of their action is still not quite clear. It remains unknown whether these molecules can regulate the transcription process as *trans*-elements. Anyway, circRNA can be detected far from the site of their synthesis, and hence, can have some alternative functions, for example, serve as a sort of a "bait" for RNA binding proteins. Aggregations of TAR-DNA binding protein 43 (TDP43) accumulate in degenerative neurons of the majority of patients with lateral amyotrophic sclerosis. As excessive accumulation of TDP43 is toxic and can lead to disorders in the normal work of neurons, the presence of the appropriate cinRNA can lead to suppression of TDP43 toxicity at the expense of alternative interactions. Presumably, in the absence of DBR1 enzymatic activity responsible for excision of lariat structures with an intron inside and their conversion into the linear form, cinRNA, produced by splicing, work in the cytoplasm as the binding element for TDP43 [8].

Role of circRNA in the splicing process. The process of circRNA formation can determine the type of the initial pre-mRNA splicing, as circRNA are mainly produced from the central exons of pre-mRNA of the genes, coding for proteins [81]. Despite the fact that circRNA are formed more rarely than mature mRNA, the use of the same 5'- or 3'-sites for splicing in circRNA biogenesis can be paralleled by splicing of pre-mRNA. In this case the quantity of the resultant linear RNA forms, containing circularized intron, is to decrease in inverse proportion to the quantity of intron-containing circRNA [9,41,81]. Competitive interactions between canonical splicing of pre-mRNA and biogenesis of circRNA have been really detected. Exon 2 of *Mbl* gene pre-mRNA can form circMbl annular structure, including flanking introns, which specifically binds to MBL. This complex regulates the activity of *Mbl* pre-mRNA splicing and competes with the canonical spliceosome; this can lead to emergence of alternative mRNA transcripts [9].

Exon missing is the most incident form of alternative splicing of pre-mRNA in human cells. Theoretically the quantity of newly formed circRNA is to increase with an increase in the quantity of mature mRNA subjected to alternative splicing. This theory is validated in experiments with circRNA-expressing vectors and in full genome studies of RNA sequences of human endothelial cells processed by TNFα or TNFβ [41]. It is noteworthy that circRNA can form not from all exons missed in alternative splicing [41]. This fact indicates the existence of an additional regulation level in biogenesis of circRNA molecules of this kind.

Translation of circRNA. Generally, at the beginning of mRNA translation process the translation machine recognizes 5'-UTR-cap, scans mRNA for the start codon, and synthesizes the protein till it meets the stop codon. Non-cap-dependent translation is also probable in some cases, but it implies the presence of a specific IRES sequence, which can be characteristic of circRNA [59]. Interestingly that because of its origin, circRNA has no stop codon. This means that *in vitro* translation of this molecule can run nonstop. Screening of circRNA has shown that these molecules are not associated with polyribosomes and hence, cannot be translated *in vivo* [39]. However, it is possible that no polyribosomes are needed for their translation. Moreover, the synthesis of small proteins or micropeptides in the cell is possible only in the presence of several ribosomes. Micropeptides translated from linear noncoding RNA forms and regulating the muscle performance and 16 kDa protein, translated from viral circRNA, were really detected in infected rice cells [4,5]. Bioinformatic analysis of several circRNA, containing IRES elements, has shown that these molecules can translate small proteins or micropeptides [24]. Moreover, circRNA with IRES sequences, synthesized *in vitro*, effectively bind ribosomal 40S subparticle and initiate the translation process. An artificial chimerical circRNA with a green fluorescent protein open reading frame is created for the expression system in *E. coli* cells [57] and the non-cap-dependent translation of mRNA, containing N6-methyladenosin in the 5'-terminal UTR sequence, with involvement of the eukaryotic translation factor 3 (eIF3), is demonstrated. Hence, it remains unclear which form — IRES sequence or m6A — supports the translation of circRNA and whether this process is non-cap-dependent.

Dynamic interactions of circRNA. Association of circRNA with miRNA can modify the level of mRNA translation or stability. In addition, circRNA can directly interact with other RNA molecules, such as mRNA or long noncoding RNA. Formation of a loop in mRNA improves the efficiency of translation, and hence, it is obvious that circRNA interactions with the target mRNA 5'- and 3'-terminals, making them closer to each other and promoting the formation of the loop structure, is essential for the process of this mRNA molecule translation [40]. In addition, circRNA can interact with RNA-binding proteins and even accumulate them: interactions of HuR, KSRBP, TTP, and AUF-1 with their target mRNA can be modified by binding to respective circRNA, this eventuating in modification of the pre-mRNA splicing type and translation of the target gene or the target gene transport and storage [1,76]. Consequently, binding of

RNA-binding protein to mRNA, in turn, can modify the processing, work, concentration, and subcellular location of circRNA. Importantly that, despite its certain stability, it remains unclear how, specifically, the circRNA concentration changes in response to changes in the level of the respective RNA-binding protein. In addition, circRNA can interact with transcription factors, modifying their transcription activity or the mechanism of protein transposition to the nucleus. These interactions have been demonstrated for long noncoding RNA, for example, for GAS5, which works as a "bait" for the glucocorticoid receptor transcription factor, preventing its binding to DNA and thus stimulating the transcription activation [77].

During replication the genomic DNA transforms from double-stranded into single-stranded form; hence, the circRNA formed in the nucleus can potentially interact with the single-stranded genomic DNA, thus preventing the normal replication process. It is known that linear noncoding RNA forms in the protein-nucleic complexes in the nuclei can form heteroduplexes or triplexes with genomic DNA, modifying the gene expression process. For example, promoter-associated RNA forms a triplex with DNA at the site of ITTF1 transcription factor binding and attracts DNMT3b methyltransferase for suppression of rRNA expression; this process can be regarded as transcription-associated regulation with participation of the genome locus [65]. One more example is interactions of ANRASSF1 noncoding RNA with genomic DNA. It is presumed that *ANRASSF1* oncosuppressor gene RNA forms an RNA/DNA hybrid at the site of the transcription beginning, this promoting attraction of PRC2 chromatin modifying complex and leads to a specific decrease of *ANRASSF1* transcription activity [13]. One more example is HOTAIR long noncoding RNA: mapping of this molecule revealed numerous sites of interactions with DNA [56,71]. CircRNA can similarly interact with genomic DNA and modify the transcription processes.

Hence, the characteristics and type of circRNA interactions with other molecules suggest that changes in the environmental conditions involve changes in the circRNA function, type, concentration, or subcellular location. A lasting external exposure or disease development can lead to emergence of new partners of the RNA-binding protein — RNA complexes or to changes in the tertiary structure of circRNA, which will modify the capacity of this RNA molecule to interact with other nucleic acids or proteins. As circRNA is synthesized in the nucleus in the process of splicing, it can be expected that many intron-containing circRNA remain in the nucleus and modify the preRNA splicing as competitive regulators. Further analysis of dynamic interactions between circRNA, RNA, DNA, RNA-binding proteins, and transcription factors will no doubt discover the bulk of universal mechanisms making it possible for circRNA to regulate the gene expression process.

METHODS FOR DETECTION OF circRNA

Identification of circRNA in biological samples is a new rapidly developing trend of research. High-throughput deep sequencing of libraries of cDNA, produced with the use of total RNA and by various fractionation or enrichment methods, is the most reliable method for circRNA search [7,11,34]. The efficiency of this method is explained mainly by the use of new methods for enrichment of the target RNA molecules, such as CircleSeq, when RNA is removed from the samples, after which the preparations are processed by RNAse P, due to which the linear RNA molecules are selectively degraded [39]. After sequencing the resultant nucleotide sequences are more amply analyzed for identification of circRNA with emphasis on RNA forms with the exon order differing from the expected one in annotated transcript [38].

Additional databases can be used, such as CircInteractome (https://circinteractome.nia.nih.gov/), allowing identification of the potential circRNA and their protein or miRNA partners [24]. This approach makes it possible to identify circRNA molecules sponging the miRNA and specific proteins and to monitor the internal site of entry into the ribosome within the circRNA sequence, in order to evaluate the probability of this transcript translation. In addition, using CircInteractome it is possible to construct primers for detection of specific circRNA by quantitative PCR in the real time mode or miRNA sequences for further studies of circRNA location, level of expression, or functional identification in cell cultures.

Numerous web tools are used for analysis of circRNA sequences; very often more than one methodological approach is used for circRNA search in the same database for comparison of the results and evaluation of their validity.

PROSPECTS

The available circRNA sequence databases together with intensely developing bioinformation technologies allow identification of more than 10,000 circRNA molecules in various organisms. However, despite great interest to these molecules and increasing number of published reports, there are still more questions about the biogenesis and functions of circRNA than answers to these questions. It remains not quite clear how the spliceosome is involved in the noncanonical alternative splicing process. Presumably, the regulation of

this process is similar to regulation of the alternative splicing process. In addition, self-complementary sequences and *trans*-regulatory elements can be also involved in circRNA formation. It remains unclear how circRNA transport from the cytoplasm to the nucleus is regulated; presumably, we may think about its parallels with a similar process for mRNA. It is absolutely unclear how degradation of circRNA molecules is realized, as the annular structure fully protects these molecules from the activity of RNA cleaving enzymes. Presumably, in addition to the "obvious" functions (accumulation of miRNA and regulation of gene transcription), circRNA can serve as a matrix in association of macromolecular complexes with RNA-binding proteins. One more key problem is the composition and presentation of circRNA in various cells and tissues: is there a relationship of any kind between these parameters and disease emergence and development? The presence of various circRNA in human saliva, blood, and exosomes suggests that theoretically these molecules can be used as biomarkers of some diseases.

According to current concepts, the basic characteristics of circRNA are as follows: they are universal, conservative (as the cyclization principle seems to be evolutionally retained in various species), highly specific, stable, resistant to enzymes responsible for transformation of circRNA forms into linear ones and to exonucleases, and their expression is excessive (the expression of some exogenous circRNA is higher than of their linear forms) [43,55,73]. Hence, circRNA have certain advantages and can be used as new biomarkers for the diagnosis, prognosis, and evaluation of therapeutic response.

Potential sources of circRNA are the serum, plasma, and biological liquids, particularly microvesicles, such as exosomes [11,46,49,55]. The stability of circRNA seems to make possible their detection with the use of the minimum invasive methods for collection of biomaterial (blood, urine, and saliva).

A significantly changed expression of circRNA in tumor tissues in comparison with normal tissues seems to be a specific feature of circRNA, which can be useful in diagnostic studies. For example, some circRNA exhibit aberrant expression in human cancer cells, *e.g.*, in esophageal, colorectal, and gastric cancer [10,44,45]. New data on the quantitative and qualitative composition of circRNA in various cell types of humans and model organisms in health and disease are regularly published. Hence, in the nearest future we can expect an avalanche of reports on the diagnostic significance of circRNA, primarily in cancer diseases and in other spheres of medicine.

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