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**RNA-RNA ligation: Methods, Prospects and Applications**

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**Abstract**

Ligation is a widely used molecular biology tool, used for the purpose of joining the ends of linear DNA or RNA molecules, either to the same (intramolecular ligation) or different molecule (intermolecular ligation). The 5’-3’ end to end ligation of a linear, single-stranded mRNA molecule results in a circular mRNA, which has many advantages in comparison to its linear counterpart. The methods for circularization of mRNA have been discussed with special emphasis on enzyme mediated ligation.

**Keywords:** Ligation, T4 DNA ligase, T4 RNA ligase, Circular mRNA.

**Introduction**

In the field of molecular biology, researchers are curious to know the detailed mechanism of central dogma, with regard to the structure and functions of the key components including DNA, RNA and the proteins. In the majority of living cells, DNA is the genetic material, whereas in several viruses and bacteriophages, RNA is the genetic material. The three types of RNA found in the cells are mRNA (messenger RNA), rRNA (ribosomal RNA) and tRNA (transfer RNA) (Carpi, 2003).

Generally, mRNA is a linear, single-stranded molecule. But, circular RNAs can also be found in nature. The non-linear form of RNA is found, although not frequently, in case of viroids, where the RNA molecules exist as single stranded covalently closed circles (Sanger et al., 1976). Such naturally occurring RNA circles are also found in plant pathogens, viruses and the satellite viruses (Puttaraju and Been, 1996). Circular mRNA offers many unique advantages over linear ones; viz., increased stability, allowance of topology investigations, creation of long repeating polypeptides and usage as a model system (Chan et al., 1988; Umekage and Kikuchi, 2009; Ford and Ares, 1994). Hence, circular mRNA molecule can be constructed for effective use as a molecular biology tool.

**Circular mRNA**

The main features and advantages of circular mRNA are discussed below. The circular form of mRNA possesses higher stability than their linear counterparts making them more resistant to nuclease degradations (Puttaraju and Been, 1996). The half-life of excised group I intron circularized RNA is much higher, in comparison to the half-life of a typical E. coli mRNA at 37°C (Chan et al., 1988). Moreover, the topological information of RNA can be obtained in its circular form, which is not possible in its linear form as the information may be lost upon analysis of linear products. As the circular mRNA does not possess free 5’ or 3’ ends, this property can be effectively utilized to study the functions of the free ends of a linear mRNA (Ford and Ares, 1994). Furthermore, the potential changes in the functions of certain regulatory proteins, involved with the mRNA could be analyzed by the circularization of mRNA.

Circular mRNA can also have strong implication in protein research. In one study, circular mRNA was used to create a very long, repeating polypeptide on a stop codon deficient open reading frame (Perriman and Ares, 1998). In eukaryotes, translating mRNA is shown to be circular by molecular interaction between the proteins binding to 5’ cap and 3’ poly A tail (Wells et al., 1998). It has been proposed that circularization ensures that the mRNA has been correctly processed and thus aids in proof-reading. The recent knowledge of different initiation modes of translation in bacteria suggests that the circularization of mRNA may facilitate direct recycling of ribosomes or the ribosomal subunits after the first-round of translation termination. This has not been experimentally proved and hence still is a theoretical model (Preiss, 2003). Thus, putting these evidences and predictions together, the circular mRNA serves as a suitable model to analyze the effect of variations.
The circularization of RNA molecule provides an advantage to the study of RNA structure and function, especially in case of the molecules that are prone to folding in an inactive conformation (Wang and Ruffner, 1998). Circular mRNA can also be particularly interesting and useful for in vivo applications, especially in the research area of RNA-based control of gene expression and therapeutics (Puttaraju and Been, 1996). MicroRNAs, which serve as the regulators of gene expression, constitute potential therapeutic target. One novel method of detection of such miRNAs involves their circularization, followed by reverse transcription of circularized miRNA to produce tandem repeats and subsequent amplification of segments by qPCR (Kumar et al., 2011).

RNA ligation

RNA ligation is important for creating long repetitive or chimeric RNA molecules, wherein, one or several small RNA oligonucleotides are ligated together by chemical or enzymatic methods discussed below. In addition, base modifications can be easily introduced in the ligated RNA by using precursor RNA fragments containing desired modifications (Hartmann et al., 2009).

The RNA-RNA ligation technique can be extrapolated to create a circular mRNA molecule. The RNA circularization can be achieved by ligating the 5'-phosphate end to the 3'-hydroxyl end of the same RNA molecule. Several methods for mRNA circularization have been attempted. Below we briefly discuss the method of chemical ligation and enzymatic ligation.

Methods of RNA ligation

Chemical ligation

The chemical ligation of RNA molecules is a flexible way to create circular RNA, as it can be carried out on a large scale under a variety of conditions. Due to simplicity of this method large RNA molecules could be circularized rather easily (Wang and Ruffner, 1998) and different reactions could be set up in parallel, with optimum control over each reaction (El-Sagheer et al., 2011). Moreover, the chemical reagents used for ligation usually do not hinder the desired biological activity of the circularized molecule (El-Sagheer et al., 2011).

The current method of chemical ligation is mostly employed for chemical synthesis of RNA from ribonucleotides, which allows production of RNA molecules of length upto only 50-100 nucleotides. Thus, one approach to create a long RNA molecule, would be to synthesize small RNA fragments and then, to ligate each individual fragment (Hartmann et al., 2009). The phosphodiester bond synthesis via chemical ligation can be carried out either by activating the phosphomonoester group using reactive imidazolide, involved in internucleotide bond formation or by the use of a condensing reagent such as cyanogen bromide (Fedorova et al., 1996). But, the disadvantage of chemical ligation is that it often results in the creation of 2'-5' phosphodiester linkage, along with the desired 3'-5' phosphodiester linkages. This can result in improper functioning and branching of the circular mRNA (Wang and Ruffner, 1998). The efficiency of creating small circular RNA molecules by chemical ligation has remained unexplored.

Enzymatic ligation:

The drawbacks of chemical ligation can be avoided by means of enzymatic ligation. Large RNA molecules can be circularized by the enzymatic approach of ligation using enzymes T4 DNA Ligase (E.C 6.5.1.1) and T4 RNA Ligase (E.C 6.5.1.3). It is unknown whether this approach is equally effective on small molecules. T4 DNA ligase is a polypeptide of around 68 kDa, which catalyzes the ligation of DNA molecules with 3'-hydroxy and 5'-phosphate termini, in the presence of ATP and Mg++. It requires an uninterrupted, unbulged double helical structure to perform its action. It does not display any sequence specificity and hence, can be used for a variety of sequences (Hartmann et al., 2009). This enzyme can be used to ligate RNA molecules as well, although not with high efficiency. One of the approaches of RNA circularization via template-mediated ligation involves the use of a bridging deoxyoligonucleotide, which binds to the 3' and 5'-end of a linear RNA molecule, holding them in close vicinity to each other. This is followed by T4 DNA ligase treatment, which considers this construct as a double-stranded (ds) nick and hence, ligates it (Chen and Sarnow, 1995). However, T4 DNA ligase can also be used for circularization of RNA by direct ligation, although not preferred due to its low efficiency. This enzyme is extensively used for other purposes in the field of molecular biology such as the 5'-adenylation of RNA using complementary DNA oligonucleotide. For this purpose, T4 DNA ligase is preferred over T4 RNA ligase as it provides a good yield avoiding side-product formations and does not require extensive optimizations (Wang and Silverman, 2006).

RNA ligases are a vital class of enzymes, used widely in the field of molecular biology. They catalyze the phosphodiester bond formation between the 5'-phosphate and 3'-hydroxyl termini of RNA in a sequential nucleotidyl transfer reactions. Based upon polynucleotide substrate specificity, RNA ligases are classified into 2 classes, RnI
and Rnl2 (Torchia et al., 2008). Rnl1 ligase catalyzes the joining of broken ends of single stranded RNA generated by an endonuclease, while Rnl2 repairs the breaks in double stranded RNA.

T4 RNA ligase 1, also called as T4 Rnl1, is a polypeptide of 347 amino acids, encoded by gene 63 of T4 bacteriophage. It catalyzes the ligation of the RNA molecules via phosphodiester linkages and is involved in RNA splicing, repair and editing pathways. The RNA ligation ability of T4 RNA ligase with regard to its biological role was first observed in T4-bacteriophage infected E. coli (Hartmann et al., 2009). Upon induction of T4-infection, a nuclease is produced, which cleaves the host tRNA\textsuperscript{Lysine} at the wobble position of the anticodon loop. Lysine is one of the common amino acids required for synthesis of most of the proteins. The loss of lysine tRNAs, can be fatal for the phage replication, and hence, needs to be repaired. The repair of this damage is catalyzed by T4 RNA ligase and polynucleotide kinase (PNK), which is seen as a phage rebuttal against the bacterial host (Amitsur et al., 1987).

The T4 Rnl1 is favored for creation of circular mRNA molecules via intramolecular ligation of single stranded linear mRNA (Nichols et al., 2008) as seen in Fig. 1. They tend to ligate the unprotected 3’ to 5’ ends of the same or different RNA molecules at random and hence result in a combination of linear, multimeric and circular products. T4 Rnl1 cannot perform blunt-end DNA ligations by itself, but, stimulates T4 DNA ligase activity in this regard.

This enzyme also ligates specific nicks on double-stranded molecules, but, is less efficient than T4 RNA ligase 2. The T4 Rnl-1 is also used in construction of cDNA libraries, creation of single-stranded DNA/RNA hybrids and in determination of the length of 3’ poly A tail (Nichols et al., 2008). T4 RNA ligase 2, also called as T4 Rnl2, is an enzyme, that catalyzes the covalent joining of 3’ hydroxyl terminus of RNA to 5’ phosphorylated DNA/RNA, preferably in double-stranded conformation. The mode of action of T4 RNA ligase 2 is similar to that of T4 RNA ligase 1, but, the former prefers double-stranded structures. Thus, this enzyme is efficient in creating hybrid or chimeric double stranded molecules. The truncated T4 Rnl-2 can be used to ligate adenylated oligo in a DNA:RNA hybrid, to join a single-stranded adenylated primer to small RNAs for cloning and in miRNA cloning, wherein, an adenylated DNA linker is ligated to single stranded RNA (Nichols et al., 2008; Viollet et al., 2011).

Recently, a 381- amino acid homodimeric protein Methanobacterium RNA ligase (MthRnl) is shown to catalyze intramolecular ligation of single stranded RNA to form a covalently closed circular RNA molecule (Torchia et al., 2008).

Conclusion

Circularization of single stranded mRNA has many advantages and applications as discussed above. Although researchers have been successful in circularizing mRNA, there is still a need to optimize the conditions to obtain a higher yield of functional circular mRNA. Thus, deriving an efficient way of construction of circular mRNA can create a benchmark in the field of molecular biology for RNA and protein synthesis related studies.

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