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The Discovery of Rolling Circle Amplification and Rolling Circle Transcription

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CONSPECTUS

Nucleic acid amplification is a hugely important technology for biology and medicine. While the polymerase chain reaction (PCR) has been highly useful and effective, its reliance on heating and cooling cycles places some constraints on its utility. For example, the heating step of PCR can destroy biological molecules under investigation and heat/cool cycles are not applicable in living systems. Thus, isothermal approaches to DNA and RNA amplification are under widespread study. Perhaps the simplest of these are the rolling circle approaches, including rolling circle amplification (RCA) and rolling circle transcription (RCT). In this strategy, a very small circular oligonucleotide (e.g., 25–100 nucleotides in length) acts as a template for a DNA or an RNA polymerase, producing long repeating product strands that serve as amplified copies of the circle sequence. Here we describe the early developments and studies involving circular oligonucleotides that ultimately led to the burgeoning rolling circle technologies currently under development.

This Account starts with our studies on the design of circular oligonucleotides as novel DNA- and RNA-binding motifs. We describe how we developed chemical and biochemical strategies for synthesis of well-defined circular oligonucleotides having defined sequence and open (unpaired) structure, and we outline the unusual ways in which circular DNAs can interact with other nucleic acids. We proceed next to the discovery of DNA and RNA polymerase activity on these very small cyclic DNAs. DNA polymerase “rolling circle” activities were discovered concurrently in our laboratory and that of Andrew Fire. We describe the surprising efficiency of this process even on shockingly small circular DNAs, producing repeating DNAs thousands of nucleotides in length. RNA polymerase activity on circular oligonucleotides was first documented in our group in 1995; especially surprising in this case was the finding that the process occurs efficiently even without promoter sequences in the circle. We describe how one can encode cleavable sites into the product DNAs and RNAs from RCA/RCT, which can then be resolved into large quantities of almost pure oligonucleotides.

Our Account then proceeds with a summary describing a broad variety of tools and methods built in many laboratories around the rolling circle concept. Among the important developments are the discovery of highly efficient DNA polymerases for RCA; the invention of exponential (“hyperbranched”) RCA amplification made possible by use of a second primer; the development of the “padlock” process for detection of nucleic acids and proteins coupled with RCA; the use of circular oligonucleotides as vectors in cells to encode biologically active RNAs via RCT; and the

Notes

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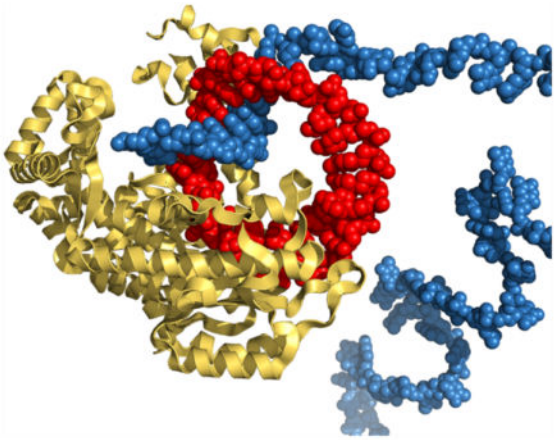
use of small DNA circles to encode and extend human telomeres. Finally, we finish with some ideas about where the field may go in the future.

Graphical Abstract



1. INTRODUCTION

1.1. The Importance of Nucleic Acid Amplification

Nucleic acid amplification is one of the most enabling technologies in all of science during the last 30 years. Much of modern biology and medicine depends on the ability to amplify DNA and RNA; indeed, one would be hard-pressed to find published studies in experimental biology or biomedicine that do not make use of amplification. Nucleic acid amplification methods not only are applied as tools for basic science but are highly practical in nature. They are central to a large fraction of molecular diagnostics methods and thus play a major role in medical practice.

1.2. The Utility of Simple Isothermal Amplification Methods

The majority of DNA amplification is carried out with the polymerase chain reaction (PCR).[1] It is reasonably simple, requiring only the DNA sample, two primer oligonucleotides, nucleotides, and polymerase enzyme. A small complicating factor is that the amplification process itself requires heating and cooling cycles, for the simple reason that primers cannot bind to fully double-stranded DNA, and thus the DNA polymerase cannot initiate a new DNA copy unless the product DNA duplex is thermally denatured. Thus, PCR requires thermocyclers to control heating and cooling cycles, and the method also requires specialized heat-stable DNA polymerases.[2]

As a result of these constraints of PCR, there is growing interest in simple nucleic acid amplification methods that can be performed isothermally.[3,4] Single-temperature methods require neither specialized equipment nor heat-stable enzymes. The isothermal approach can often be carried out simultaneously with other biochemical reactions or in the presence of native biomolecules, many of which would be destroyed by the high heat of PCR cycles, or even directly in living cells.

Isothermal DNA/RNA amplification methods have been under rapid development over the last 20 years.[3–5] Of these, rolling circle amplification (RCA) is among the earliest and most widely used;[5] other prominent methods include nucleic acid sequence based amplification (NASBA),[6] strand displacement amplification (SDA),[7] and loop-mediated isothermal amplification (LAMP).[8] Each of these has its own applications, advantages, and limitations. Among them, only RCA uses a circular template, presenting unique opportunities and applications.

1.3. An Account of Early Work Developing RCA and RCT

Reviews have been published before on the growing field of rolling circle amplification.[5] Our purpose here is not to present a comprehensive review of this large field but rather to give an account of how some of the early work and discoveries in the field were made, with particular emphasis on contributions from our laboratory.

2. SYNTHESIS AND UNUSUAL PROPERTIES OF CIRCULAR OLIGONUCLEOTIDES

2.1. Early Literature Examples of Synthetic Circular DNAs

The observation that small circular oligonucleotides could act as templates for DNA and RNA polymerases first required the synthesis and development of circular oligonucleotides as a structural class. As of 1990, when we entered the field, circular oligonucleotides were quite rare in the literature; indeed, the only examples of unpaired circles were very small[9] (2–10 nucleotides (nt), see Figure 1) and so could not be active with polymerases. The only larger circular synthetic DNAs known at the time were a few literature examples of “dumbbell”-type cyclic molecules,[10] which consisted of duplex DNAs capped at the ends with short 3–6 nt loops. Because such duplexes were highly stable and had very small loops, they were not ideal (or even feasible) as templates for polymerases, since there was little unpaired sequence to which primers could bind.

In 1990, we introduced the first literature examples of circular oligonucleotides having open, unpaired structure.[11,12] This was part of a long-term research plan to study the unusual properties of circular DNAs, including both their DNA/RNA binding capabilities and their ability to act as substrates for polymerases. Both topics were presented in proposals written in 1988 by one of the authors (E.T.K.) and submitted to universities in applications for assistant professorship positions. Happily, one of these applications was successful, allowing the author to begin to work on these ideas in a new laboratory of his own.

2.2. Development of Synthetic Strategies

The study of circular synthetic oligonucleotides required the development of synthetic strategies for making these molecules. Ultimately we studied two different strategies for forming this last and crucial bond: enzymatic ligation and chemical ligation (Figure 2). Both of these approaches made use of a separate DNA “splint”, which served to bring the reactive ends together in close proximity, greatly increasing their effective molarity. These methods are described elsewhere in detail.[13]

The ligase enzyme approach is broadly applied now. However, there are some limitations that come with the enzymatic approach, including the cost of enzyme and difficulties in splint binding if the precircle folds in a nonoptimal way. As an alternative, we developed a nonenzymatic approach to synthesis of circular oligonucleotides.[11] In our strategy, a splint was used to bring the reactive ends together, but activation of the phosphate group was carried out with chemical reagents. We tested both water-soluble carbodiimides and cyanogen bromide/imidazole-based coupling chemistries.[14] Interestingly, we found that we could perform chemical ligation either with a 5′ phosphate or 3′ phosphate group (unlike ligase enzymes). Ultimately, combining a range of methods,[15] we were able to demonstrate synthesis of DNA circles as small as 13 nt and as large as 105 nt.

2.3. Unusual Biomolecular Recognition Properties of Circular DNAs

The first subject studied under our research plan was the design of circular DNAs that could bind to single-stranded nucleic acids via triple helix formation.[11,12] Pioneering work by Dervan and Hélène had shown that linear C,T-containing oligonucleotides could bind to double-stranded DNA in sequence-specific fashion, forming Hoogsteen hydrogen bonds with the duplex.[16,17] Our binding target was quite different, being single-stranded DNAs and RNAs, and our concept was to design circular ligands that could bind a target by sandwiching it between two domains (Figure 3). This nucleic acid binding strategy is reviewed elsewhere;[18] suffice it to say that this unusual mode of recognition results in very tight binding and exceedingly high sequence selectivity. Also important for the ensuing work with polymerases is that the DNA circles in the project were largely unstructured, thus allowing them to bind primers without interference from internal structure. Moreover, they were usually rich in pyrimidines (C,T), which later became important for RNA polymerase activity.

3. DISCOVERY OF DNA POLYMERASE ACTIVITY ON SMALL CIRCULAR DNAs

3.1. Earliest Rolling Circle Reports

Rolling circle replication has been long known as the mechanism by which naturally occurring circular plasmids and viral genomes are replicated. In that sense, the concept of copying a circular DNA template with a DNA polymerase was not new in the early 1990s. However, plasmid DNAs are several thousands of nucleotides in length and are biological in origin. In contrast, the circular DNAs we were preparing were more than a hundred times smaller, and they were completely synthetic. Both of these differences are exceedingly important to the technological applications of RCA and RCT. When we began our studies, it was not at all clear that such a small, bent template could even fit in the active site of a polymerase enzyme, much less act as an efficient template.

In 1992, we began testing the ability of DNA polymerases such as the Klenow fragment (Kf) of DNA polymerase I to extend primers using circular DNAs 34 nt in size as the template. Primers were 5′-end-radiolabeled with ^{32}P, and their extension measured by gel electrophoresis. It was immediately obvious that long DNA polymers were formed with these small circles. We began characterizing these products, work that proceeded somewhat

slowly at first because of our inexperience with polymerases and sequencing. We also simultaneously proceeded with studies of RNA polymerases on small circular templates (see below), which we published first because of the simplicity (requiring no primers) and the novelty of promoterless transcription.

We were quite excited by these early findings, recognizing that multiple technological uses could arise from this “rolling circle” amplification process. We applied for a patent in April 1993,[19–21] which was ultimately issued as three patents covering the circles themselves as “vectors” encoding amplified DNA and RNA, the long repeating nucleic acid probes made by the rolling process, and the use of circles to synthesize quantities of unit-length DNAs and RNAs. Of course, numerous subsequent “rolling circle” patents and technological developments have arisen since that early work, from many laboratories including our own. Some important examples are described below.

In 1995, the group of Andrew Fire (then at Carnegie Institute, now a colleague of ours at Stanford) published a study entitled “Rolling Replication of Short DNA Circles.”[22] This was the first peer-reviewed literature example of “rolling circle” DNA polymerization on a very small circular DNA template and preceded by a few months our first paper on the topic. The authors observed that *Escherichia coli* DNA polymerase I could extend a primer on partially randomized DNA circles 42 and 52 nt in size, with lengths well in excess of 180 nt observed. Notably, they found no such long products for T4, Klenow, or Sequenase polymerases. They converted these DNA products to double stranded form and confirmed their repetitive nature by digestion with a restriction endonuclease. The authors suggested this rolling reaction to be potentially useful for synthesis of repetitive double-stranded DNA libraries for *in vitro* selection.[22] Interestingly, the Fire lab did not publish any subsequent papers on rolling circle topics, turning their focus instead on embryonic development in *Caenorhabditis elegans* and the role of double-stranded RNAs, for which they are widely recognized.[23]

We noted that the above paper did not suggest biotechnological uses of the rolling circle replication beyond the construction of double-stranded concatemer libraries. We had envisioned, in contrast, that the extended repetitive single-stranded DNA itself could be potentially directly useful in a number of amplification strategies.[20,21] Thus, we proceeded with our studies to characterize the effects of varied templates, enzymes, and properties of the repeating products. In our first paper on rolling circle DNA synthesis (appearing in early 1996),[24] we surveyed the effects of varied circle size and varied enzymes on the isothermal polymerization reaction (Figure 4). As for enzymes, we found that T4, T7, Sequenase, Taq, Klenow, and Pol I DNA polymerases all produced products longer than 2000 nt as measured by gel electrophoresis. A time course of extension with the Klenow enzyme showed lengths reaching >12 000 nt after 3 h on a 42mer template, which corresponds to >460 times around the circular template.[24]

3.2. Effects of Circle Size and Topology on Rolling

Both we and Fire noted that very small DNA circles present unusual topological issues with polymerase-mediated DNA synthesis.[22,24] As templates, they are bent on the length scales on which common DNA synthesis occurs. Thus, it seemed to us quite unlikely that a

polymerase could simply synthesize around a small circle and back to the starting point. Indeed, this is a common misconception about rolling circle amplification: that a DNA-unwinding polymerase enzyme is required for efficient amplification, in order to unwind this product in front of the polymerizing enzyme. This is not the case, because a duplex cannot be made in unstrained form in a circle smaller than roughly 120 nt.[25]

To gather data on the effects of circle size, we synthesized circular oligonucleotides ranging from 26 to 74 nucleotides. The results showed efficient rolling circle synthesis for all these circle sizes, suggesting that there were no problematic topological issues limiting synthesis.[24] We noted that the smallest of these, the 26mer (Figure 5), had a diameter smaller than that of the polymerase itself!

This brings up a second interesting topological issue of very small circular templates: we noted that the circle itself must rotate about its single bonds (the nucleotides spinning like beads on a necklace) to alleviate the twisting tendency that would otherwise require the long DNA tail to pass through. We proceeded in a second study to test even smaller circular DNAs, to see what is the lower size limit of rolling circles, essentially “tightening the belt” on the enzyme.[26] In a collaboration with the Pedroso lab in Barcelona, we prepared and tested very small DNA circles, 13–28 nt in size. We found that the Kf enzyme succeeded in synthesizing DNA on all four templates. The efficiency on the smallest (13mer) was clearly less than that on the 28mer, and some sequence errors were observed, showing clear signs of strain at that smallest size. However, both the 23mer and 28mer gave relatively high efficiency.

3.3. Unusual Stoichiometry of the Rolling Circle Reaction

In our first study, we pointed out the surprising efficiency and stoichiometry of this rolling circle DNA synthesis.[24] The overall stoichiometry of the reaction is conversion of a pot of nucleotides directly to a pot of single-stranded DNA (Figure 6). The enzyme and circle are both catalysts, used in relatively tiny quantities, and the primer is also substoichiometric, used only once to yield hundreds of product DNA repeats. This stoichiometry is quite different from that of the PCR reaction, which consumes two primers for every amplicon.

We pointed out that the RCA stoichiometry could be harnessed in the amplified synthesis of oligonucleotides. We demonstrated this by encoding a restriction enzyme site into circular DNAs, showing that after a rolling circle reaction, the product could be cut by the enzyme into predominantly unit lengths.[24] The net result of this appealingly efficient process is isothermal conversion of a pot of nucleotides into a pot of oligonucleotides of precise sequence and length.

A final note on the stoichiometry: with a given polymerase enzyme, a small circle is expected to yield more copies than a large circle. Polymerases have inherent processivity, defined as the average length of DNA (RNA) they synthesize before dissociating. An enzyme such as T7 DNA polymerase, with processivity of up to a few thousand nucleotides, therefore might yield 100 concatenated copies of a 50mer circle, whereas on a 7000 nt plasmid, the enzyme would in most cases dissociate even before reaching one complete

copy. This is an important reason why rolling circle processes on very small DNA circles are especially useful and are distinct from the biological process.

4. APPLICATIONS AND METHODS INVOLVING RCA

Although the early papers mentioned above documented the use of circular oligonucleotides as efficient polymerase templates and characterized the long repeating DNA products, much subsequent work was necessary to develop RCA into useful technological tools for a wide range of applications. A few important examples are mentioned below.

4.1. Repeating Probes

The direct product of the rolling circle process is a long repeating DNA, and if the repeats can be detected, this can concentrate many signals in a localized area (Figure 7a). The remote 5′ end of the primer can also carry useful functional or binding groups (such as biotin), allowing the repeating probe to be anchored in wells and on solid supports. Examples of applications include RCA performed on glass, beads, microwell plates, paper, or microfluidic devices. For instance, immuno-RCA on a solid surface is utilized for the detection of antigens.[27] Smolina et al. have employed this strategy for detecting cell-surface markers on cancer cells.[28]

4.2. Branched RCA for Exponential Amplification

One notable development for RCA technology was converting the original linear rolling circle amplification process into an exponential one. This can be done by using at least one additional primer complementary to the original repeat DNA. The most common type of exponential RCA is hyperbranched RCA[29] (HRCA, also termed ramification amplification[30] (RAM)), using a reverse primer complementary to the RCA product (Figure 7b). During extension, strand displacement also occurs, creating a “ramified” or “hyperbranched” DNA complex. The resulting exponential amplification is highly efficient and can be applied multiple ways.[5]

4.3. Padlock Probes Coupled with RCA

The novel concept of using circularizable oligonucleotides as “padlock” probes was reported by Nilsson et al. in 1994,[31] and using these probes with polymerases in RCA amplification was demonstrated in 1998.[32] In the padlock approach, which is used to detect specific DNAs and RNAs, the target DNA serves as a ligation template by hybridizing to the ends of the padlock probe, which then can be joined by DNA ligase, resulting in the circle being topologically linked to the target (Figure 7c).[31] With the appropriate design, even a single mismatch between the probe and target strands can prevent circularization of the padlock probe. If RCA is subsequently performed to detect the cyclization, one can discriminate the presence of specific DNA sequences.[32] The versatile padlock probe approach can also be used for mRNA and miRNA detection, using the end of the RNAs as primers.[33,34]

4.4. Highly Efficient Polymerases

The discovery and characterization of DNA polymerases with very high processivity is critically important in maximizing amplification in rolling circle processes. A spectacularly

successful example of such an enzyme is Phi29 polymerase, a bacteriophage replicating enzyme.[35] It is among the most processive of all single-subunit polymerases, with a processivity of tens of thousands of nucleotides. This enzyme is among the most widely used of all polymerases in RCA applications.

4.5. Protein Detection Coupled with RCA

In addition to the detection of nucleic acids, RCA can also be used for the sensitive detection of proteins and enzymes. This is usually achieved by the generation of primers or circular templates after a target-binding event occurs. Yin et al. developed a strategy whereby DNA aptamers compete between a protein target and a circular DNA.[36] Chu et al. devised a nonaptamer method to detect DNA repair activity; the 5′-phosphorylated DNA product resulting from 8-oxoguanine excision served as the padlock probe for an RCA reaction that generated autocatalytic DNAzymes used to produce a fluorescent signal.[37]

4.6. Telomere Elongation

A telomere is a repeating hexamer DNA sequence $(TTAGGG)_n$ at the end of human chromosomes that protects them from degradation or fusion with other chromosomes.[38] Telomeres shorten with age, and very short telomeres are associated with diseases of aging. Human telomeres are synthesized *in vivo* by the telomerase enzyme.

Our lab has shown that this process can be mimicked using RCA (Figure 8). DNA circles of various sizes comprising the reverse complement of the human telomere repeat $(CCCTAA)_n$ were synthesized[39] and used as templates for DNA polymerase, producing long RCA products that were essentially artificial human telomeres.[40] Excitingly, it was shown that these artificial telomeres could be “grown” directly on human chromosomes via the rolling circle process in fixed human cells by providing a circle and a polymerase.

4.7. Detection of miRNAs

miRNAs are biomedically important short RNAs (20–22 nucleotides in length) that can be difficult to detect by standard PCR, so RCA is an attractive potential method for detecting them. miRNA sequences can differ by only one or two nucleotides, but the specificity of ligases and the specificity of DNA priming can be used in RCA to discriminate these small differences. Padlock probes have been shown by Liu et al. to be effective in discriminating between members of the miRNA family with single-nucleotide selectivity.[34] Our lab used preformed circular probes and miRNAs as primers to generate long repeating products by RCA, which were subsequently detected by a second isothermal amplification,[41] using templated fluorogenic probes, or by luciferase signaling with specialized nucleotides.[42]

5. DISCOVERY OF RNA POLYMERASE ACTIVITY ON SMALL CIRCULAR DNAs

5.1. Transcription of Circles without Promoters

RNA polymerases (RNAPs) are different from DNA polymerases in three important respects.[43] First, they recognize and initiate RNA synthesis at specific sequences called

promoters, whereas DNA polymerases are nonselective in sequence and initiate with a primer. Second, RNAPs are typically active on double-stranded DNA, and they have unwinding activities to separate template strand from the complement. In contrast, DNA polymerases must initiate at a single-stranded starting point just downstream of the primer. Third, RNAPs initiate polymer synthesis *de novo*, without a primer.

In considering whether circular oligonucleotides might act as templates for RNA polymerases, we suspected in 1990 that it might be necessary to include a promoter in the circle in order for the polymerase to bind and initiate RNA synthesis. Nevertheless, during 1992–1993, we tested pyrimidine-rich circular oligonucleotides as templates for RNA polymerases, even though they had no promoters. The first circle tested was one off the shelf from earlier studies, a 34mer rich in T's and C's, with no internal secondary structure. This is reported in our initial paper on the subject (1995),[44] the first description of rolling circle transcription in the literature.

Gel electrophoretic analysis of the reaction with T7 RNAP revealed that long RNA strands were rapidly produced with the circle, whereas linear controls yielded little RNA (Figure 9). Excitingly, experiments showed that the rolling circle process yielded 7-fold more RNA than did canonical promoter-initiated RNA synthesis.[44] Thus, the RCT process occurs with high efficiency. Gel analysis showed lengths of up to 9000 nucleotides in these RNAs, which were confirmed by banding patterns to be repeating complements of the circle sequence. The data indicated that the T7 enzyme was initiating with GTP (as it does with canonical promoters), but at many of the C's in the circle.[44]

Varying the sizes of circular DNAs showed that the T7 enzyme could efficiently transcribe circles as small as 23 nt.[26] Overall, we concluded that an RNA polymerase can indeed bind a very small "structureless" single-stranded DNA circle and initiate the synthesis of long repeating strands of RNA. The efficiency can be exceptionally high, with at least 450-fold amplification observed in some cases.

5.2. Evaluating Varied RNA Polymerases

We explored the scope of this rolling circle transcription by comparing RNA polymerases from bacteriophage T7 and *E. coli*.[44,45] The data showed that both enzymes could in fact use a small circular template; in the case of the *E. coli* enzyme, initiation occurred with ATP, the canonical starting nucleotide for this enzyme at *E. coli* promoters. Later experiments by K. Ryan showed that human RNA polymerases can also be active in rolling circle transcription.[46]

We collaborated with the Hansma laboratories at UCSB to visualize rolling circle transcription at the single molecule level.[47] Atomic force microscopy images showed very long RNA strands being produced, some of them with RNA polymerase still at the end. This imaging revealed a fascinating picture of rolling circles as biochemical motors: the engine (RNA polymerase) drives a single wheel (the circle), driven by fuel (the nucleotides), laying down a complementary track of RNA behind as it proceeds.

5.3. Encoding Self-Cleaving RNAs

Having determined the basic process and properties of RCT, we next began a series of studies to encode functional RNAs on circular templates.[48–50] Especially intriguing was the notion of encoding RNA-cleaving ribozymes. We realized that if we encoded both a ribozyme and its cleavage sequence (Figure 10), the long repeating transcript might cleave itself into progressively shorter segments, ultimately resulting in monomer oligoribonucleotides. Also important was the notion that these self-cleaved ribozymes might then be able to go on and cleave a separate target RNA (for example, a disease-related RNA).

We found that both monomer RNAs and repeating ribozymes produced by RCT could in fact cleave HIV RNAs *in vitro*.[48] To us, this suggested the exciting possibility of introducing a circular DNA into a cell as a synthetic vector, where it could encode a disease-fighting ribozyme in highly amplified quantities.

We ultimately studied this ribozyme-synthesis strategy with three different ribozyme motifs: the hammerhead, the hairpin, and the hepatitis delta motifs, using DNA circles 63–105 nucleotides in size.[48–50] We observed that the self-cleavage reaction begins to take place during the transcription reaction itself and culminates in primarily monomer-length RNAs. This is especially appealing in efficiency: the overall reaction stoichiometry is the conversion of mononucleotides into almost pure single ribozyme RNAs.

5.4. In Vitro Selection for Efficient RCT

Very interestingly, we found strong differences in the ability of T7 versus *E. coli* RNA polymerase to transcribe these ribozyme-encoding circles.[49] We surmised that different secondary structures were preferred by one enzyme over another. This led us to the idea that it should be possible to use *in vitro* selection techniques to find optimal “pseudo-promoters” for RCT driven by a particular enzyme.

To pursue this idea, we constructed a hairpin ribozyme-encoding RNA library that contained a 40-nt randomized domain.[51] Transcription of this library results in transcripts that are enriched in sequences that folded for enhanced transcription. Proceeding through several rounds of transcription and selecting “winners” yielded optimal pseudopromoters for *E. coli* polymerase enzyme. Importantly, these sequences and structures could be transplanted from one circle to another.

5.5. RCT *in Vivo*: A New Class of Vector

Having developed a DNA circle folded structure that is strongly transcribed by *E. coli* RNAP *in vitro*, we then proceeded to test whether a circular DNA containing it could be transcribed directly in live bacterial cells.[51] We encoded in the circle a ribozyme that could cleave a drug resistance gene (marA) *in vitro*. On treating live bacteria with this circle, we were able to show a drop in marA gene expression of 80%, whereas a single nucleotide mutation resulted in a complete loss of ribozyme activity. Thus, our experiments confirmed the use of circular oligonucleotides as “nanocircle vectors” encoding biologically active

RNAs in living cells. This is likely to be general; it has been recently shown that circles can be active as vectors for expressing siRNAs with biological activities in tumor cells.[52]

5.6. RNA Polymers with Biological Activity

The P. Hammond laboratory has constructed circular DNAs encoding repeating hairpin RNAs. After RCT in vitro, these partially self-complementary RNAs assemble into large "micro-sponge" complexes. Upon uptake into cells, the RNA micro-sponge was processed by the cell's machinery to yield many short interfering RNAs, thus providing a novel approach for siRNA delivery.[53]

6. SUMMARY AND FUTURE DIRECTIONS

It is by now clear that the rolling circle strategy is a highly efficient, yet appealingly simple, approach to amplification of nucleic acids and of biological macromolecules and signals. Creative work in many laboratories has led to the development of dozens of methods using this amplification as a platform. Although the work on rolling circles has proceeded now over two decades, it is still growing in popularity (Figure 11). An increasing number of laboratories are either adopting the methodology for its favorable capabilities or developing new methods and applications around the process.

It is interesting to speculate on the directions of future research on rolling circles. Given its current rapid growth, there will clearly be a large number of new applications of RCA appearing soon. In particular, the notion of the use of nanocircle vectors in living systems for encoding biologically active RNAs and DNAs in cells is exciting and only in its beginning stages. Rolling circle transcription could be used increasingly to encode repetitive long RNAs or self-processed short RNAs having biological activity.[46,51] Future work will also be interesting in the field of telomere extension, where nanocircles encoding telomeric DNA repeat might extend the protective ends of chromosomes,[40] extending the replicative lifespan of the cells.

To date, the large majority of rolling circle research has been for applications in biotechnology and in biology. However, it seems likely that the methodology will also find increasing use in nanotechnology, since many DNA and RNA nanostructures require relatively large or long repetitive structures.

Acknowledgments

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Biographies

Michael Mohsen received his B.S. from NYU in 2015 and is currently pursuing his Ph.D. at Stanford with Eric Kool.

Eric Kool was born near Chicago, raised in Wisconsin, and attended college at Miami of Ohio. He received his Ph.D. at Columbia with Ronald Breslow and did his postdoctoral training with Peter Dervan at Caltech. His first academic position was at the University of Rochester, and in 1999, he joined the faculty at Stanford, where he is the George and Hilda Daubert Professor of Chemistry. His interests lie in designing modified forms of DNA and RNA for applications in biology and medicine.

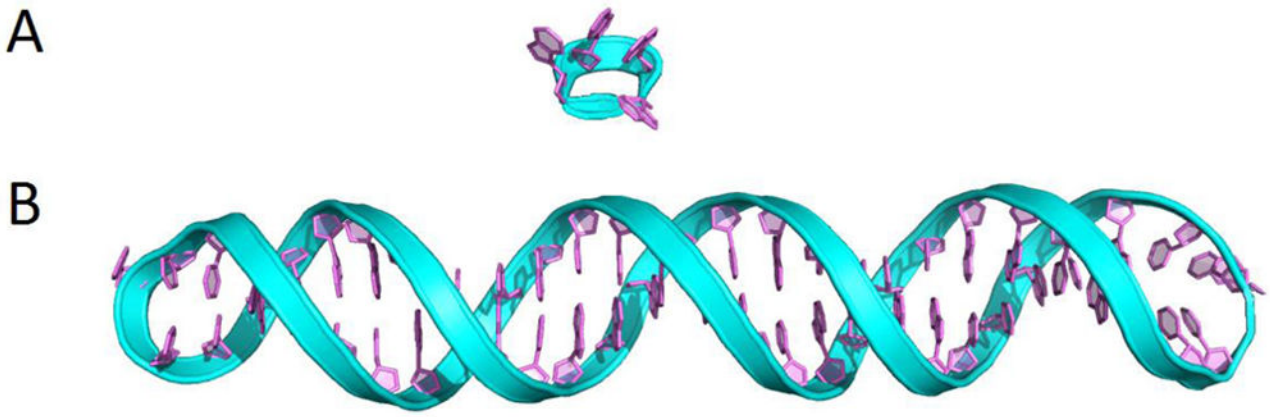

Figure 1.
Types of circular oligonucleotides ca. 1990: (a) very small unstructured cyclic oligomers ~2–10 nt in size; (b) “dumbbell” double-stranded structures. Neither of these were suitable for rolling circle methods.

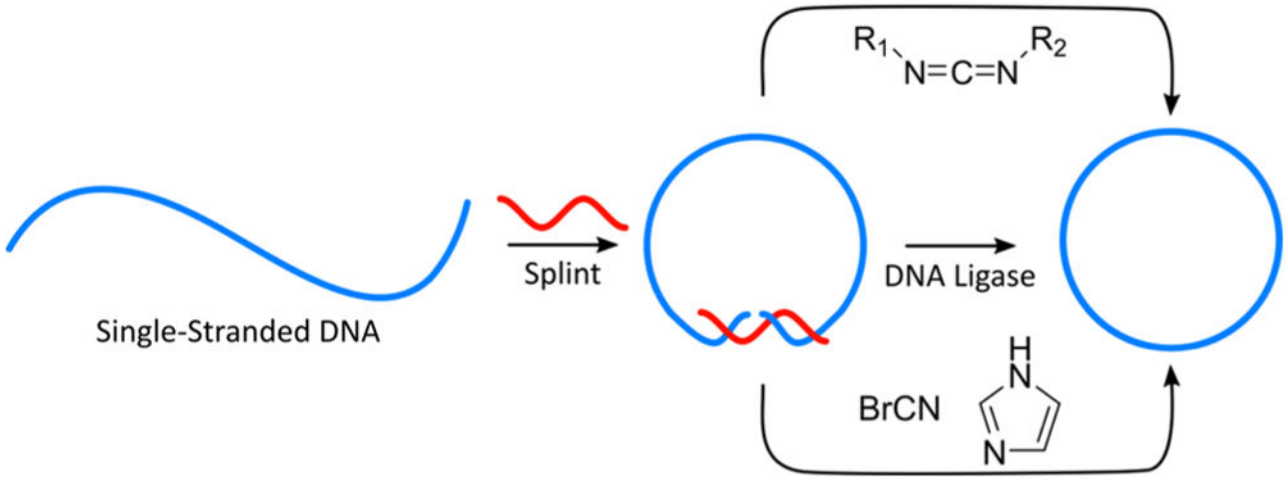

Figure 2.
Ligation methods for synthesis of circular oligonucleotides.

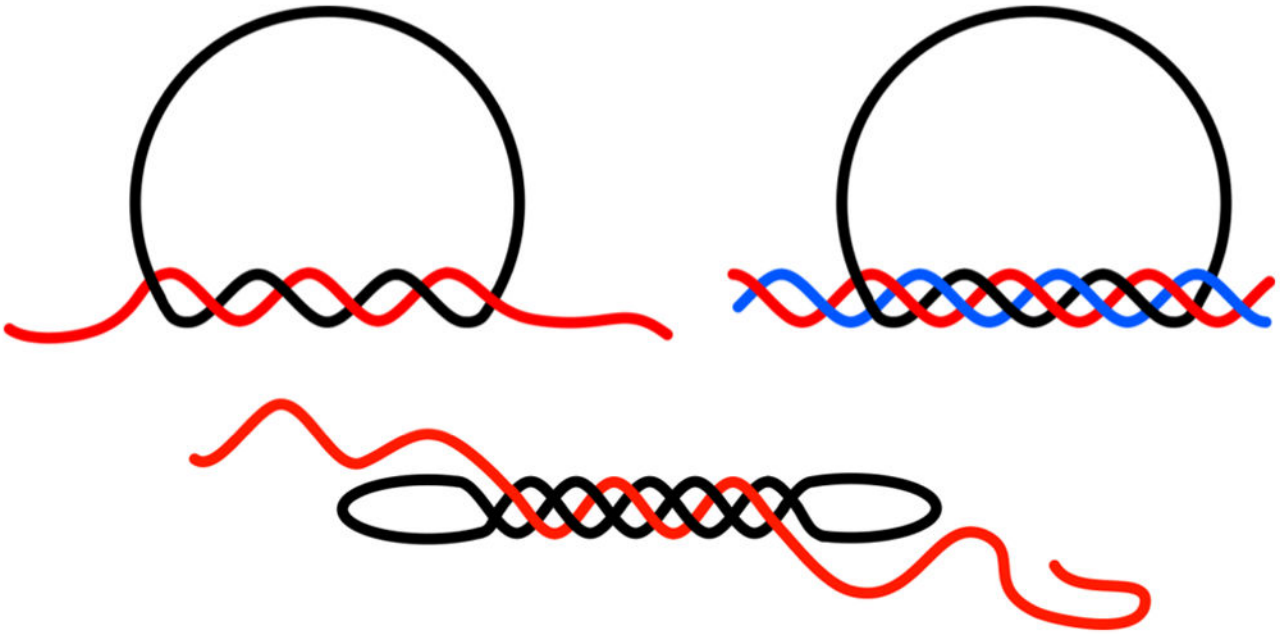

Figure 3.
Three DNA/RNA binding modes of circular oligonucleotides, involving duplex formation by a circle, triplex formation with duplex DNA, and triplex formation (sandwiching) with single-stranded DNA/ RNA.[18]

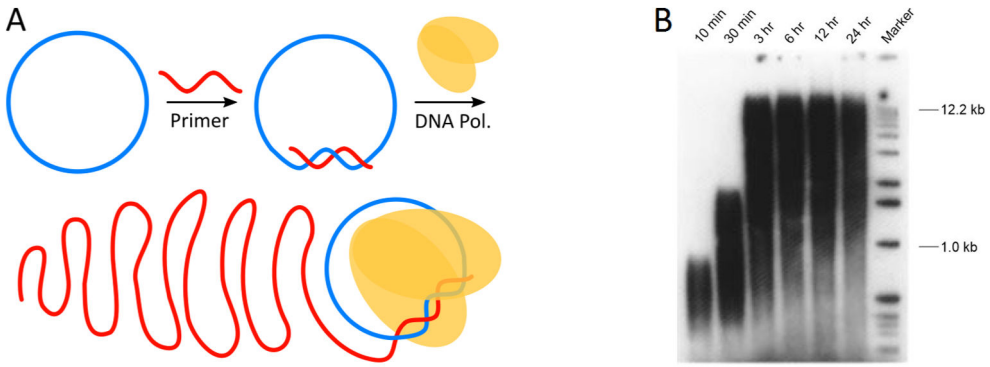

Figure 4.

(a) Cartoon showing rolling circle DNA synthesis process. (b) Observation by electrophoresis of polymerase synthesis of long DNAs on a circular 34-nt DNA template.

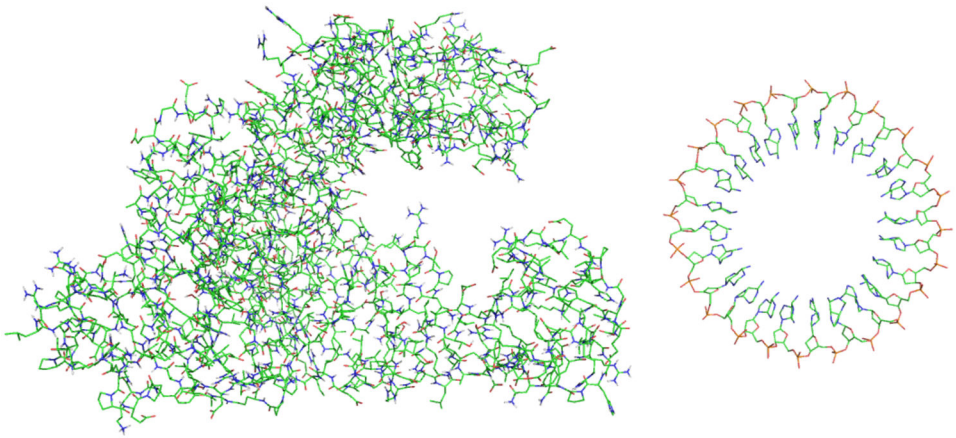

Figure 5.
Comparison of sizes of Kf DNA polymerase and a 24 nt circular oligonucleotide.

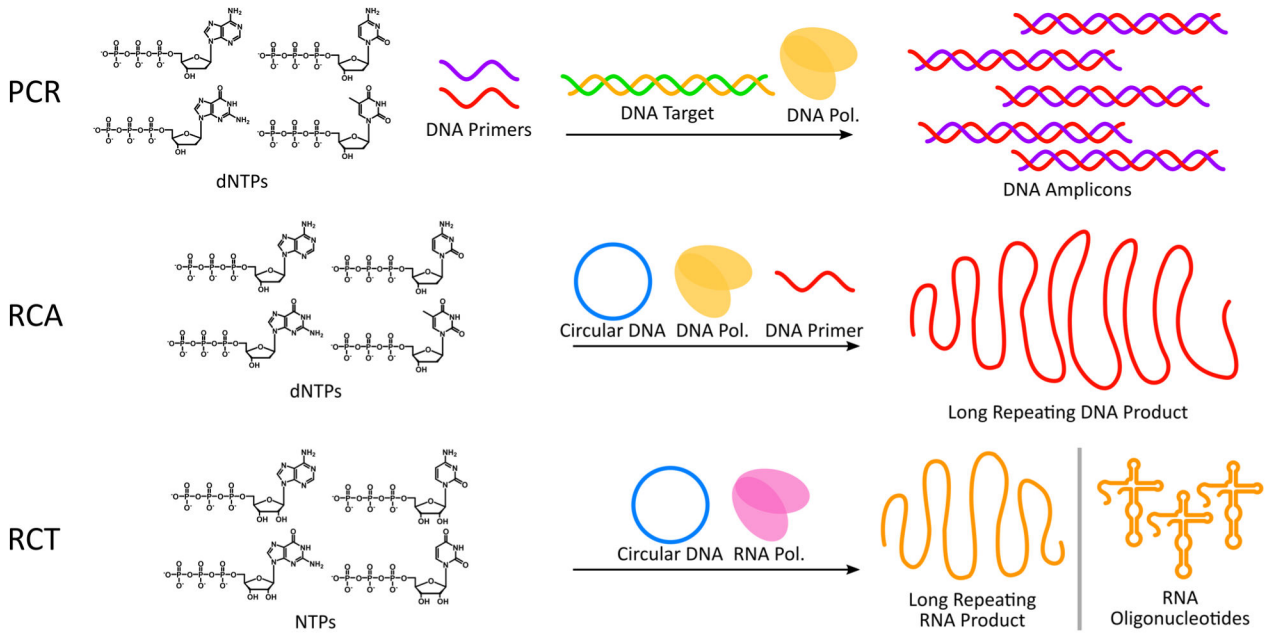

Figure 6.

Stoichiometry of RCA and RCT reactions as compared with PCR. The rolling circle reactions convert a pot of nucleotides into long repeating DNAs or RNAs; all other components are catalytic or substoichiometric. With catalytic cleaving functions, they convert a pot of nucleotides into pure oligonucleotides. PCR, by contrast, converts primers and nucleotides into elongated primers.

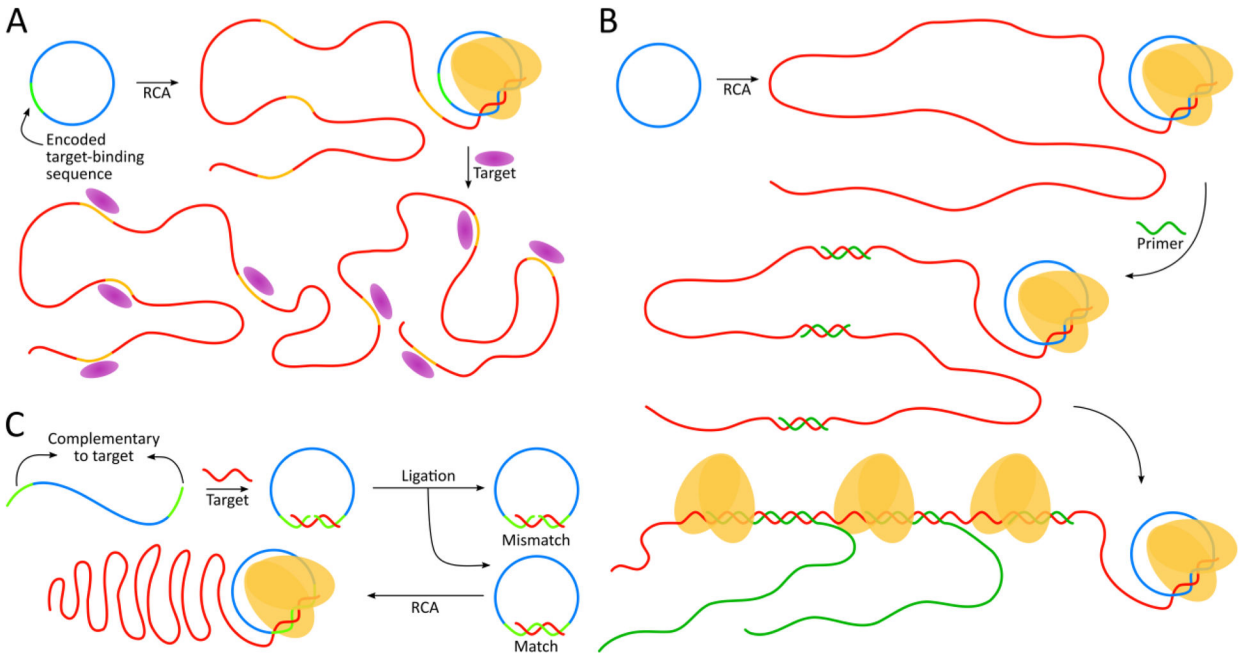

Figure 7.
Examples of important developments in RCA technology: (a) repeating probes; (b) branched RCA; (c) padlock probes.

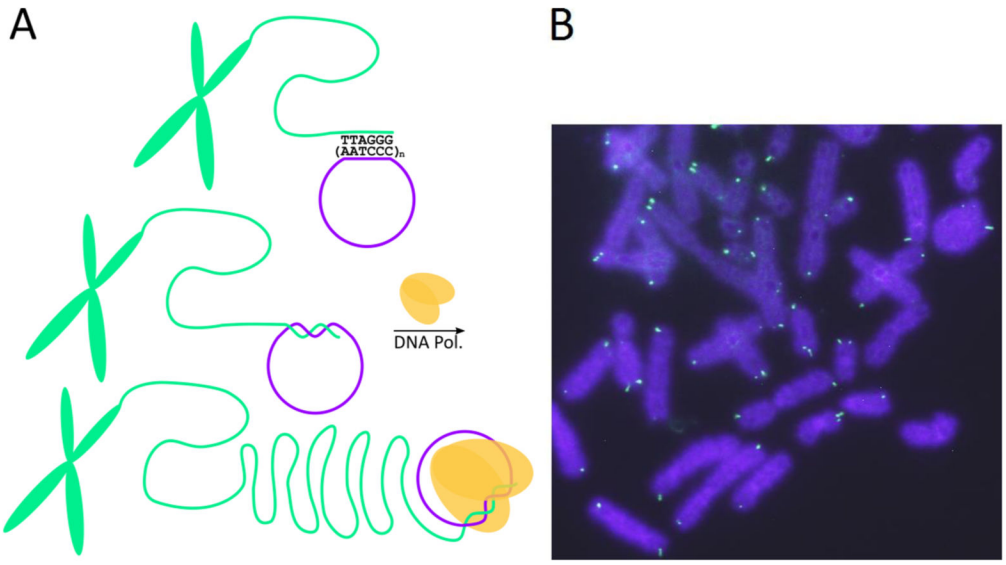

Figure 8.
Telomeric DNA extension by rolling synthesis with nanocircle vectors: (a) cartoon of the process on a chromosome end; (b) image of human chromosomes in fixed cells with telomeres extended (green) via rolling circle vector.

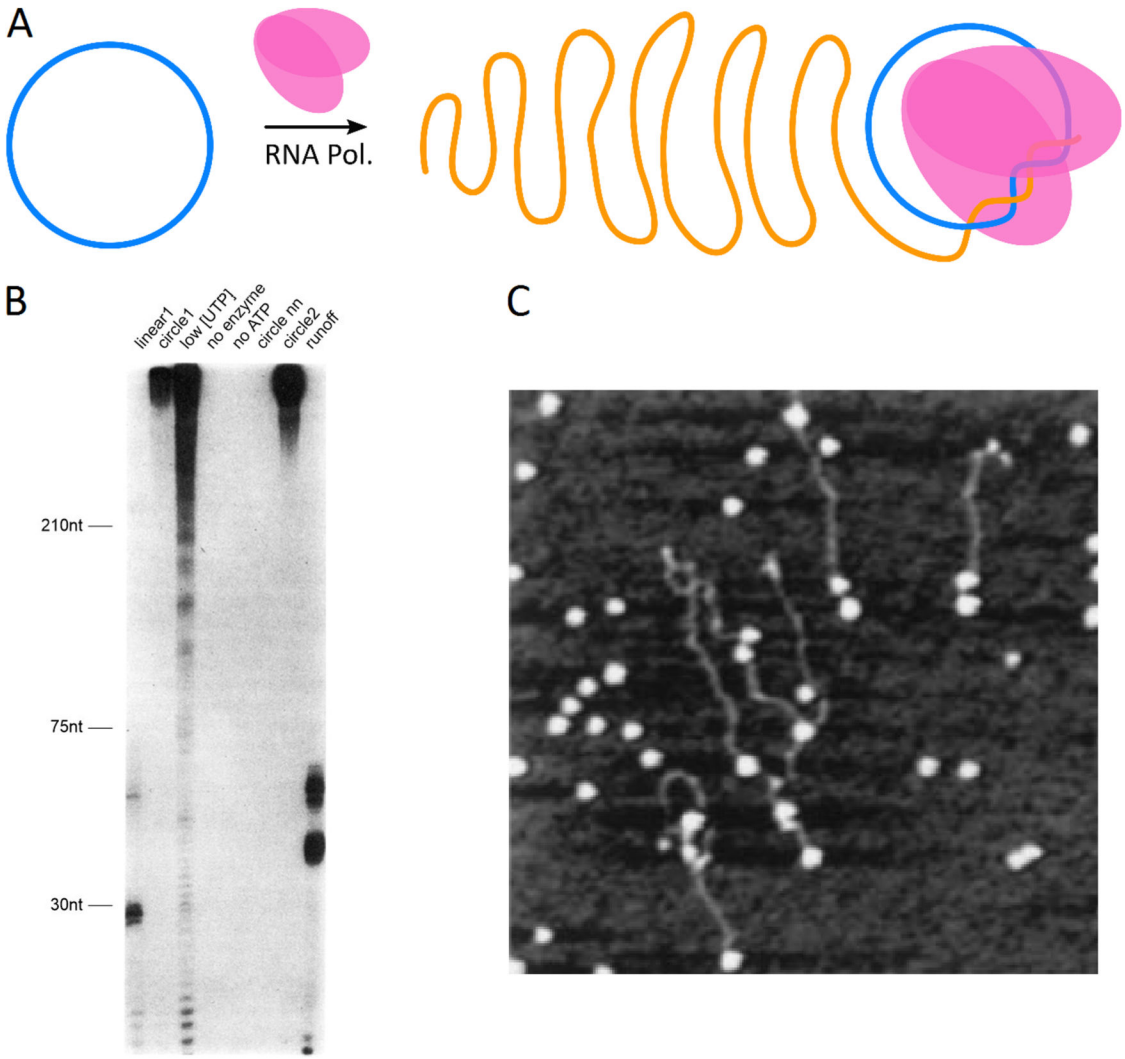

Figure 9.

RNA polymerase activity on promoterless circular oligonucleotides: (a) cartoon of RCT process; (b) gel electrophoretic observation of long extended RNAs from RCT; (c) atomic force microscopy image of long RNAs produced by RCT; RNA polymerase is visible at ends of some RNAs.

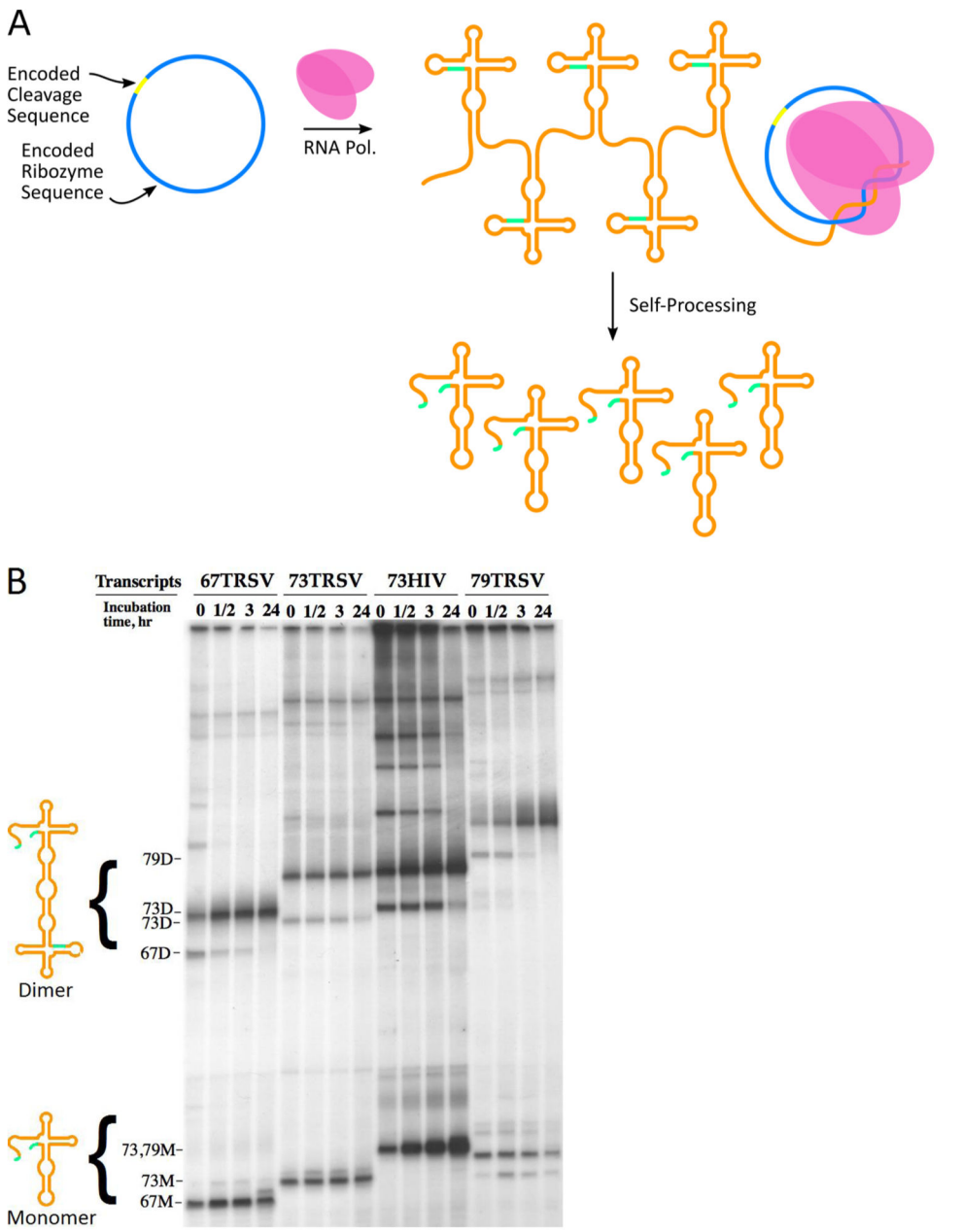

Figure 10.

Synthesis of catalytically active ribozyme RNAs by RCT: (a) cartoon of synthesis and self-processing; (b) electrophoresis gel showing long RNAs self-cleaving into unit lengths over time.

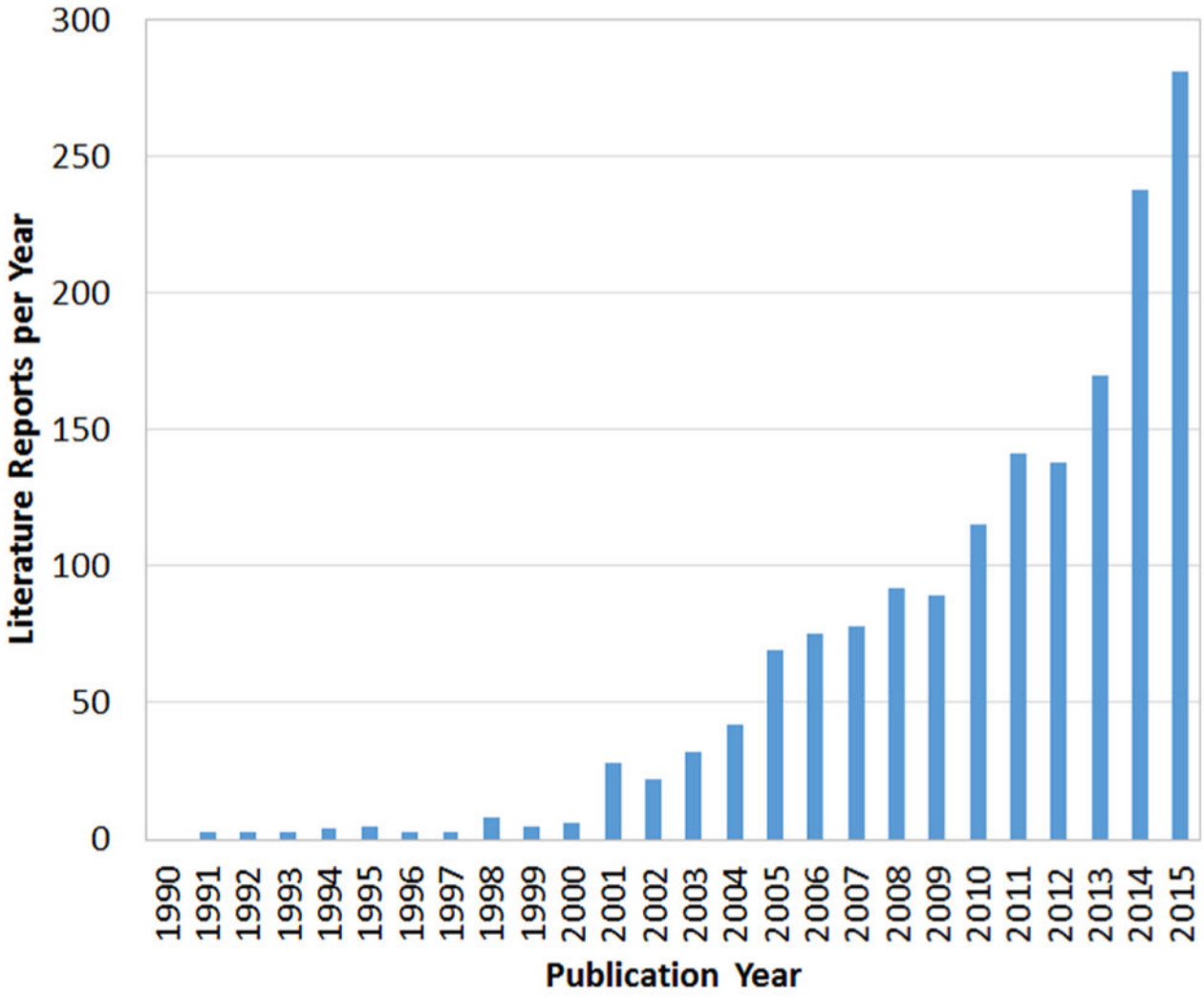

Figure 11.
Literature reports citing “rolling circle amplification” and “rolling circle transcription” are rapidly increasing (source: Web of Science).