

Expert Opinion

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A novel single-stranded DNA expression vector

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A novel expression vector system has been developed that can intracellularly generate any single-stranded DNA (ssDNA) molecule, such as a triplex-forming oligonucleotide (TFO), antisense oligodeoxynucleotide (ODN) and DNA enzyme, by our laboratory at CytoGenix, Inc. 'Proof of concept' studies from our laboratory as well as our collaborators' indicate that this ssDNA expression vector system is capable of producing, intracellularly, antisense ODNs, DNA enzymes or TFOs for the purpose of downregulating gene expression or inducing targeted genome modification. This technology provides new research tools and has potential applications in gene target validation and drug development.

Keywords: DNA enzyme, expression vector, gene target validation, single-stranded DNA

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1. Introduction

There has been increasing interest in oligodeoxynucleotides (ODNs) as tools for understanding gene function by knocking-out or knocking-down target genes, for validating new genomic drug targets and ultimately as potential therapeutic agents. Development of conventional therapeutics often requires non-rational screening of thousands of compounds to find an active molecule. Unfortunately, non-rational screening does not usually provide information about the mechanism of action of the molecule and the compound identified by such screening often lacks specificity. In contrast, ODN-based therapeutic screening allows for the rational design of sequence-specific drugs. Oligonucleotides consist of i) DNA-based oligodeoxynucleotides such as triplex-forming oligonucleotides (TFO), antisense ODNs, DNA enzymes and ii) RNA-based oligoribonucleotides such as ribozymes. These molecules modulate gene expression by interacting with DNA or mRNA in sequence-specific manners. Recently, the use of double-stranded RNA (dsRNA) has proven to be a power tool to suppress gene expression through a process known as RNA interference (RNAi). However, the reduction of gene expression is transient which limits its applications [1].

The sequencing of the human genome has created a tremendous opportunity for development of ODN-based drugs. A number of ODN-based drugs have already entered advanced clinical trials [2]. Vitravene™ (Novartis Ophthalmics), the first ODN-based drug, is now marketed for the treatment of cytomegalovirus (CMV) retinitis infections in AIDS patients.

The cellular uptake of naked ODNs is generally inefficient since only a small number of ODN molecules actually gain entry to the cells. Many different strategies have been attempted for the effective delivery of ODNs and resulted in varying degrees of success [3]. Ribozyme can be synthesised endogenously by a viral vector, for example, or delivered exogenously like DNA-based ODNs. Although an RNA expression vector has been successfully used to produce ribozyme molecules in cells, there is presently no vector delivery system available for DNA-based ODNs. Recently a novel single-stranded DNA (ssDNA) expression vector that can

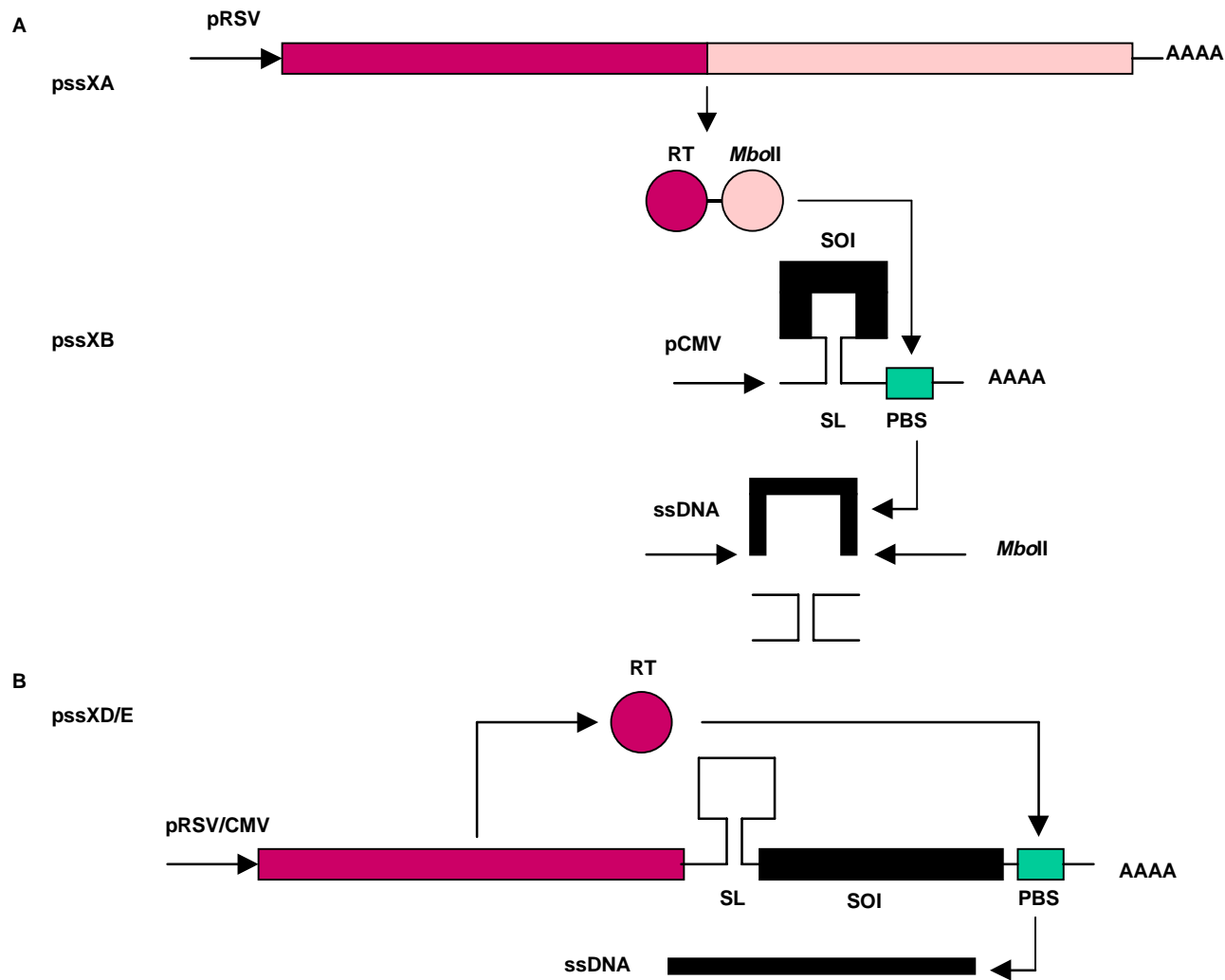


Figure 1. Design of the ssDNA expression vector systems.

A. First generation ssDNA expression vector system (two-component system). A reverse transcriptase RT-*MboII* fusion protein is expressed by pssXA vector. By binding to the PBS, RT initiates the cDNA or ssDNA synthesis from an engineered mRNA transcript generated by pssXB vector. ssDNA is released from the flanking sequences by the cleavage of *MboII*. **B. Second generation ssDNA expression vector system (single-component system).** A stop codon is inserted at the end of RT gene coding sequence so that the translation of RT gene can be terminated. Reverse transcriptase initiates ssDNA synthesis from the mRNA transcript and the process is terminated by the stem-loop structure. CMV: Cytomegalovirus; PBS: Primer binding site; RT: Reverse transcriptase; SL: Stem-loop structure; SOI: Sequence of interest; ssDNA: Single-stranded DNA.

intracellularly generate any ssDNA molecule, including a TFO, antisense ODN or DNA enzyme, has been developed [4]. Studies from our laboratory as well as our collaborators' indicate that this ssDNA expression system is capable of producing a DNA enzyme for downregulating *c-raf* kinase gene expression [4] and a TFO for inducing genomic recombination [5]. This review will discuss the development of the ssDNA expression vector system and a number of applications of this technology.

2. First generation ssDNA expression vector

2.1 Two-component ssDNA expression vector set

The first generation ssDNA mammalian expression vector is a two-component system that includes pssXA and pssXB plasmids. The key feature of the expression vector set is shown in

Figure 1A. pssXA expresses an active fragment of the Moloney mouse leukaemia viral (MoMuLV) reverse transcriptase (RT) and the *Moraxella bovis MboII* restriction enzyme as a single chain fusion protein. pssXB expresses an engineered RNA transcript from which the desired cDNA or ssDNA can be generated. The resulting RNA transcript contains a primer binding site (PBS) along with some flanking regions of the promoter that are essential for the reverse transcription initiation by MoMuLV RT [6]. The RT, expressed by pssXA, generates a copy of ssDNA from the RNA transcript using an endogenous tRNA^{pro} molecule that binds specifically to the PBS [7]. Digestion of the RNA strand by either endogenous RNase H activity or the RNase activity of the RT [8] releases the newly synthesised ssDNA. The sequence of interest (SOI) is nested within the flanking sequences that contain the com-

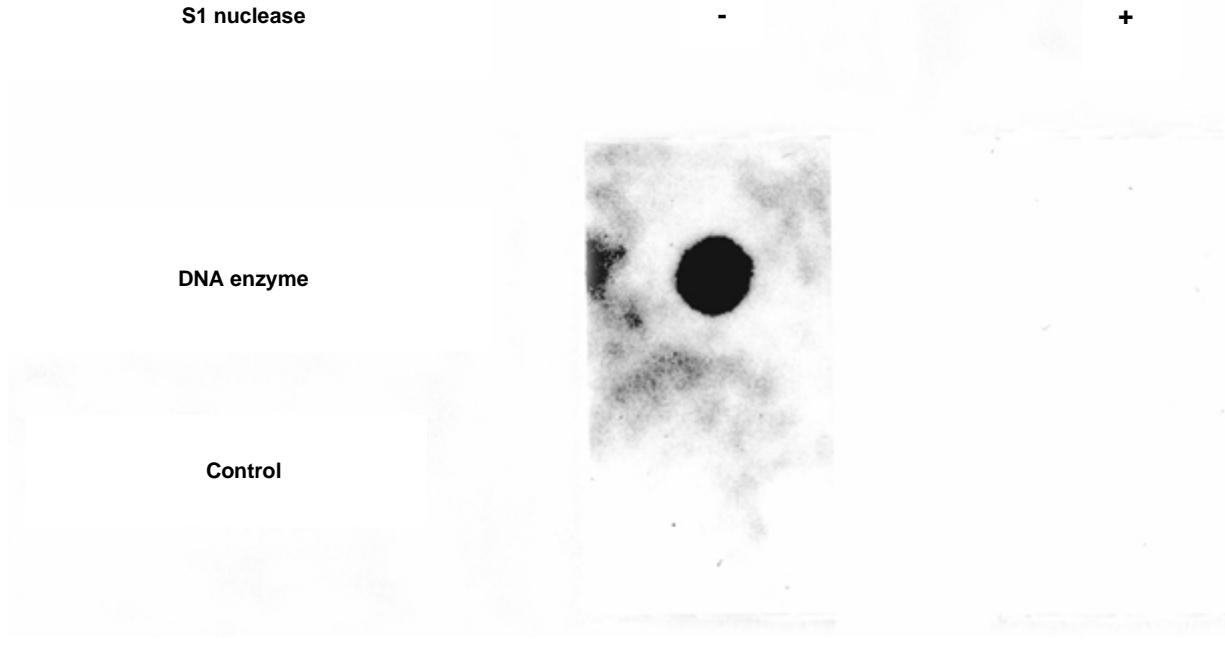


Figure 2. Detection of c-raf DNA enzyme by dot-blot analysis.

A549 cells were transiently transfected with the pssXD vector expressing the DNA enzyme or a control vector. Forty-eight hours after transfection, ssDNA was isolated with total RNA using Trizol[®] solution [4]. Samples were pretreated with RNase A in the presence or absence of S1 nuclease for 30 min at 37°C. The treated samples were then used for dot-blot analysis. North2South Chemiluminescent Nucleic Acid Hybridisation and Detection Kit (Pierce) was used following the manufacturer's instruction. The probe for dot-blot analysis is a c-raf specific and biotin-labelled ODN [4].
ODN: Oligodeoxynucleotide; ssDNA: Single-stranded DNA.

plementary inverted repeats. The ssDNA produced can form an internal stem-loop structure due to the inverted repeats and the 'stem' includes a recognition site for *Mbo*II so that the enzyme cleaves and releases the ssDNA containing the SOI from the flanking sequences.

2.2 Production of c-raf kinase mRNA-cleaving DNA enzyme

A recently identified ODN, referred to as '10 – 23' DNA enzyme, has been demonstrated to cleave any RNA targets containing a purine-pyrimidine junction [9,10]. This '10 – 23' DNA enzyme consists of a 15 nucleotide catalytic domain flanked by two RNA target binding domains of 7 – 8 nucleotides each. The catalytic efficiency of the '10 – 23' DNA enzyme meets or exceeds that of a comparable ribozyme [11]. Another advantage of the '10 – 23' DNA enzyme compared with the use of a ribozyme is that the biologically unstable RNA structure of the ribozyme is replaced with more stable DNA chemistry.

In the initial experiment with our ssDNA expression vector, we chose to express a DNA enzyme that cleaves c-raf kinase mRNA in A549 lung carcinoma cells [4]. DNA enzyme was designed to specifically bind to the 3'-UTR (untranslated region) of c-raf mRNA, a region similar to that targeted by antisense ODN, ISIS 5132 [12]. By transfecting pssXA into A549 cells, a stable cell line, designated as E10, was established. The expression of the RT enzyme activity was confirmed by a RT-PCR (polymerase chain reaction following

reverse transcription) assay [13]. E10 cells were then transiently transfected with pssXB coding for the c-raf DNA enzyme or a random control sequence. The newly synthesised c-raf DNA enzyme molecules were detected by either dot-blot or RT-PCR analysis 24 or 48 h after transfection [4]. To determine whether DNA enzyme molecules, generated by the ssDNA expression vector set, can modulate c-raf gene expression, a northern blot analysis was performed. The result showed that the expressed DNA enzyme molecules could reduce c-raf mRNA level in cells by up to 36% compared to cells transfected with the control vector [4].

2.3 Production of ssDNA for triplex formation

In their early study, Glazer's laboratory at Yale University showed that a G-rich 30mer TFO (AG30) can mediate targeted genome modification in mammalian cells via directed mutagenesis or induced recombination [14,15]. They established a mouse fibroblast cell line (FL-10) that carries a pair of mutant thymidine kinase (TK) genes in a single locus as direct repeats. A 30 bp G-rich polypurine sequence amenable to high-affinity, third-strand binding in the antiparallel triplex motif by AG30 TFO molecules was inserted into the region between the two mutant TK genes. Since parental mouse LTK⁻ cells lack cellular TK genes, recombination between the two mutant TK genes, induced by the AG30 TFO molecules, has the potential to generate wild type TK and can be selected by growth in the presence of HAT medium.

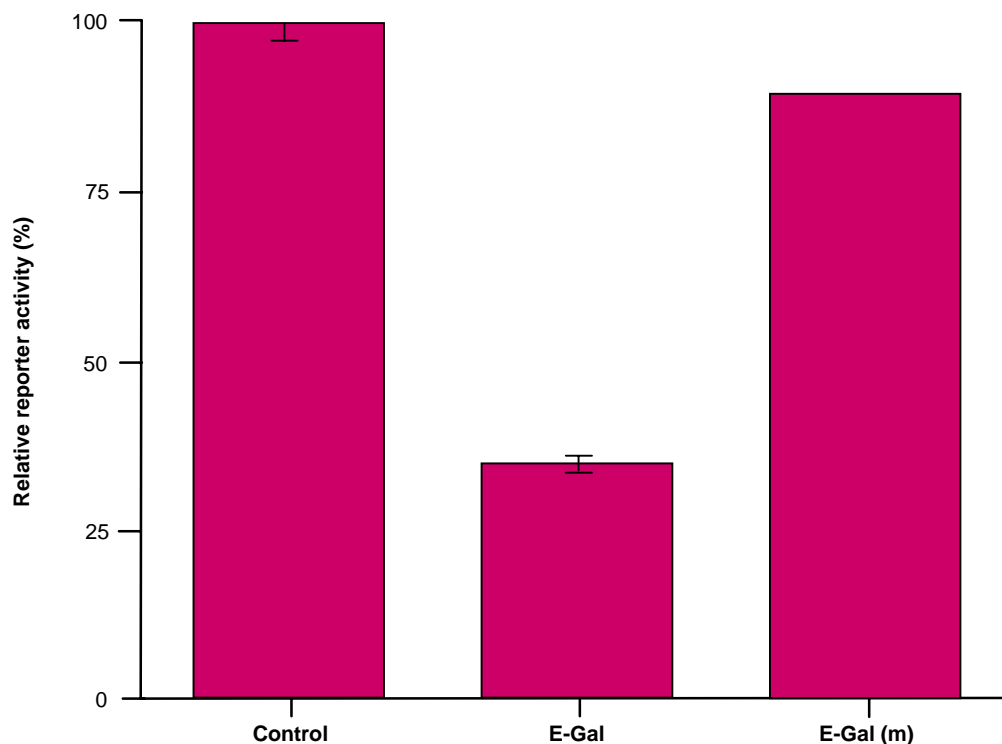


Figure 3. Inhibition of β -gal expression by DNA enzyme produced *in vivo*.

A549 lung cells were co-transfected with DNA enzyme and β -gal expression vectors at a ratio of 3:1 (w/w). Cell lysates were prepared 24 h after transfection and β -gal enzyme activity was determined using a colorimetric assay kit from Sigma according to the manufacturer's instructions.

β -gal: β -galactosidase; Control: β -gal expression vector only; E-Gal: DNA enzyme and β -gal expression vectors; E-Gal (m): Mutated DNA enzyme and β -gal expression vectors.

Glazer's laboratory tested the ability of our ssDNA expression vector set to produce ssDNA for triplex formation and induce genome recombination using the mouse FL-10 cell line they established in their previous study [5]. By cotransfecting pssXA and pssX, coding for AG30 TFO into FL-10 cells, they were able to produce a detectable amount of AG30 TFO molecules of the size expected. An estimated 6.2×10^5 AG30 TFO molecules per cell were generated by the ssDNA expression vector set 24 h after transfection while transfection of synthetic AG30 TFO molecules yielded $\sim 1.9 \times 10^5$ molecules per cell. The relative level of AG30 TFO molecules produced by the ssDNA expression vector set was substantial (about three times more than that of TFO molecules directly introduced into cells by transfection) considering that cotransfection of the two plasmids was not optimised in these experiments [5].

Glazer *et al.* also investigated induced recombination in FL-10 cells by AG30 TFO molecules produced with the ssDNA expression vector set. AG30 TFO molecules, produced by the ssDNA expression vector set, were found to induce recombinants at a frequency sevenfold higher than stimulated by the synthetic AG30 TFO molecules.

3. Second generation ssDNA expression vector

Although the first generation ssDNA expression vector can be used *in vitro*, it would be impractical to use the two-plasmid

system in future animal and clinical studies. We constructed a second generation single-component ssDNA expression vector, designated as pssXD, based on our previous two-plasmid expression vector system (Figure 1B). In addition, the *Mbo*II gene was removed from this version of the expression vector. We observed, in our *in vitro* study, that the stem-loop structure formed by inverted repeats could terminate, at least partially, the synthesis of ssDNA (unpublished). Others have shown that a naturally-occurring stem-loop structure, a polypurine tract (PPT), could cause reverse transcription pausing during retroviral replication [16]. The expression of *Mbo*II in cells is also a concern because of its nature to digest DNA and the potential toxicity to cells, as suggested by Datta and Glazer [5]. Instead of relying on *Mbo*II to remove flanking sequence in the ssDNA generated, we have considered using the stem-loop structure to terminate the ssDNA synthesis in our second generation expression vector.

3.1 Production of c-raf kinase mRNA-cleaving DNA enzyme

Similar to our early work with the two-plasmid expression vector system, we tested the ability of the single-component expression vector to generate c-raf DNA enzyme in A549 cells. Again, we assessed the expression of c-raf DNA enzyme in cells by the dot-blot assay (Figure 2). A biotin-labelled ODN probe was used to detect the c-raf DNA enzyme mole-

cules generated in A549 cells. Positive signal could only be detected in cells transfected with pssXD expressing c-raf DNA enzyme. As expected, no detectable signal was observed if the sample was treated with S1 nuclease which specifically degrades ssDNA. In addition, there was about 67% reduction of c-raf gene expression in the cells transfected with the pssXD expressing the DNA enzyme.

3.2 Production of DNA enzyme targets a β -galactosidase reporter gene

A different version of a second generation ssDNA expression vector was constructed in which a CMV promoter replaced the Rous sarcoma virus promoter. Using this version of ssDNA expression vector (designated as pssXE), we expressed a DNA enzyme that targets a β -galactosidase (β -gal) reporter gene. The β -gal reporter gene was used as the target gene since the alteration of gene expression could be easily measured by β -gal enzyme activity. We constructed an expression vector that could generate a DNA enzyme capable of cleaving β -gal mRNA at a protein translation start site (ATG). A control vector expressing a mutant DNA enzyme has also been constructed. A single mutation from T to G in the catalytic domain of this mutant DNA enzyme abolishes targeting RNA cleavage activity [17]. A549 lung carcinoma cells were cotransfected with DNA enzyme and β -gal expression vectors. Cell lysates were prepared 24 h after transfection and β -gal enzyme activity was determined by a colorimetric assay. Compared to cells that were transfected with the control vector, significant reduction of β -gal activity (~ 61%) was observed in cells expressing the DNA enzyme (Figure 3).

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. ELBASHIR S, HARBORTH J, LENDECKEL W, YALCIN A, WEBER K, TUSCHL T: Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* (2000) **41**:494-498.
2. UHLMANN E: Oligonucleotide technologies: synthesis, production, regulations and application. *Expert Opin. Biol. Ther.* (2001) **1**:319-328.
3. AKHTAR S, HUGHES MD, KHAN A *et al.*: The delivery of antisense therapeutics. *Adv. Drug Delivery Rev.* (2000) **44**:3-21.
4. CHEN Y, JI Y, ROXBY R, CONRAD C: *In vivo* expression of single-stranded DNA in mammalian cells with DNA enzyme sequences targeted to c-raf. *Antisense Nucleic Acid Drug Dev.* (2000) **10**:415-422.
- This study is the first demonstration of the

4. Discussion and expert opinion

A number of ODN-based drug candidates are in various stages of preclinical and clinical development and the first antisense ODN drug, Vitravene, is on the market for the treatment of CMV infections. However, the main barrier to efficacy for most ODN-based drugs is still achievement of the delivery of ODNs in sufficient quantities to the correct target sites of action to obtain the desired level of gene inhibition in a desired time-frame.

Using a bacterial retron system, Inouye's laboratory was able to generate ssDNA in a 'multicopy DNA' form [18,19]. Multicopy DNA has a unique structure whose 5' end is linked to the 2'-OH of an internal guanosine residue of an RNA molecule by a 2', 5'-phosphodiester linkage. However, the potential side effects of this unique structure are still unknown. The ssDNA expression vector system we developed can produce any ssDNA molecules with minimal extraneous vector sequences. Furthermore, an inducible expression vector for the production of ssDNA molecules is currently under investigation. Although a number of issues still remain to be clarified, such as the stability and exact size of ssDNA molecules, this technology may offer an important research tool and has potential applications in gene target validation and drug development.

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production of single-stranded DNA by this novel expression vector.

5. DATTA HJ, GLAZER PM: Intracellular generation of single-stranded DNA for chromosomal triplex formation and induced recombination. *Nucleic Acid Res.* (2001) **29**:5140-5147.
- This study demonstrated that this novel expression vector can generate functional triplex-forming oligos for inducing genome recombination in cells.
6. SHINNICK TM, LERNER RA, SUTCLIFFE JG: Nucleotide sequence of Moloney murine leukaemia virus. *Nature* (1981) **293**:543-548.
7. MARQUET LJ, ISEL C, EHRESMANN C, EHRESMANN B: tRNAs as primer of reverse transcriptase. *Biochimie* (1995) **77**:113-124.
8. TANASE N, GOFF SP: Domain structure of the moloney murine leukemia virus reverse transcriptase: mutational analysis and separate expression of the DNA polymerase and RNase H activities. *Proc. Natl. Acad. Sci. USA* (1988) **85**:1777-1781.
9. SANTORO SW, JOYCE GF: A general purpose RNA-cleaving DNA enzyme. *Proc. Natl. Acad. Sci. USA* (1997) **94**:4262-4266.
- This study describes the development of '10-23' DNA enzyme using an *in vitro* screening method.
10. SANTORO SW, JOYCE GF: Mechanism and utility of an RNA-cleaving DNA enzyme. *Biochemistry* (1998) **37**:13330-13342.
- Kinetic study of '10-23' DNA enzyme.
11. BREAKER RR: Catalytic DNA: in training and seeking employment. *Nature Biotech.* (1999) **17**:422-423.
12. MONIA BP, JOHNSTON JF, GEIGER T, MULLER M, FABRO D: Antitumor activity of a phosphorothioate antisense oligonucleotide targeted against c-raf kinase. *Nat. Med.* (1996) **2**:668-675.

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13. SILVER J, MAUDRU T, KAZUNOBU F, REPASKE R: An RT-PCR assay for the enzyme activity of reverse transcriptase capable of detecting single-virions. *Nucleic Acid Res* (1993) **21**:3593-3594.
14. LUO Z, MACRIS MA, FARUQI AF, GLAZER PM: High-frequency intrachromosomal gene conversion induced by triplex-forming oligonucleotides microinjected into mouse cells. *Proc. Natl. Acad. Sci. USA* (2000) **97**:9003-9008.
15. VASQUEZ KM, NARAYANAN L, GLAZER PM: Specific mutations induced by triplex-forming oligonucleotides in mice. *Science* (2000) **290**:530-533.
16. WU W, HENDERSON LE, COPELAND TD, GORELICK RJ, BOSCHE WJ, REIN A *et al*: Human immunodeficiency virus Type 1 nucleocapsid protein reduces reverse transcriptase pausing at a secondary structure near the murine leukemia virus polypurine tract. *J. Virol.* (1996) **70**:7132-7142.
17. WU Y, YU L, MCMAHON R, ROSSI JJ, FORMAN SJ, SNYDER DS: Inhibition of bcr-abl oncogene expression by novel deoxyribozyme (DNAzymes). *Human Gene Ther.* (1999) **10**:2847-2857.
- **This study describes a DNA enzyme with a single nucleotide mutation that can be used as an excellent control.**
18. MAO J, SHIMADA M, INOUE S, INOUE M: Gene regulation by antisense DNA produced *in vivo*. *J. Biol. Chem.* (1995) **270**:19684-19687.
19. MIROCHNITCHENKO O, INOUE S, INOUE M: Production of single-stranded DNA in mammalian cells by means of a bacterial retran. *J. Biol. Chem.* (1994) **269**:2380-2383.

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