
Short Technical Report

Expression of ssDNA in Mammalian Cells

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ABSTRACT

Antisense therapy involves the use of antisense oligonucleotides for altering targeted gene function. However, the low efficiency of cell delivery of antisense oligonucleotides has limited the efficacy of antisense therapeutic approaches. RNA-based antisense or ribozyme oligonucleotides can be either synthesized endogenously (e.g., by a viral vector) or delivered exogenously. However, there is presently no vector delivery system available for DNA-based oligonucleotides. Recently, a novel ssDNA expression vector that can generate intracellularly any ssDNA molecule, such as antisense oligonucleotide or DNA enzyme, has been developed in our laboratory. Here we describe an improved expression vector based on the first-generation two-vector system. To test this new expression vector, we chose to express a single-stranded "10-23" DNA enzyme targeting c-raf mRNA in the human lung carcinoma A549 cell line. After introduction into cells by transient transfection, c-raf-cleaving DNA enzymes produced by this expression vector can significantly suppress the expression of c-raf mRNA. Furthermore, the expressed c-raf DNA enzymes induced cell apoptosis, as indicated by genomic DNA fragmentation assay. Our study further demonstrates the feasibility of using this novel ssDNA expression technology to produce intracellularly any sequence of interest, including antisense oligonucleotides and DNA enzyme molecules.

INTRODUCTION

Substantial advances in antisense therapy have been made during the past several years. Antisense therapy is different from traditional gene therapies in that, instead of giving rise to protein expression, antisense oligonucleotides are designed to inhibit protein translation by specifically binding to the target mRNAs and altering their expression (3). However, the cellular uptake of naked oligonucleotides is generally inefficient since only a small number of oligonucleotide molecules actually gain entry to the cells. Many different strategies have been attempted for the effective delivery of oligonucleotides and have resulted in varying degrees of success (4).

Antisense RNA has been tested mostly using viral expression vectors, which allow antisense RNA molecules to be synthesized in target cells. However, some potential problems with this approach have limited its application, such as the possibility of antisense RNA being translated into an "antisense" protein that has unknown function and is possibly toxic (4).

Another approach is to use ribozymes that catalyze mRNA cleavage. Ribozymes are thought to be more efficient than antisense oligonucleotides because of their catalytic power (15). Ribozymes can be delivered either directly or using expression vectors. However, ribozymes have limited stability because of degradation by RNases in vivo.

Recently, several ssDNAs (DNA enzyme, deoxyribozyme, or DNAzyme) have been demonstrated to catalyze the cleavage of mRNA. These RNA-cleaving DNA enzymes employ distinct cat-

alytic mechanisms and require different kinds of cofactors such as magnesium, histidine, or copper (1). One such enzyme, referred to as "10-23" DNA enzyme (10), has the potential to cleave any RNA targets containing a purine-pyrimidine junction (10,11). This 10-23 DNA enzyme consists of a 15-nucleotide catalytic domain, flanked by two RNA target-binding domains of seven nucleotides each. In contrast to the complicated mechanisms of mRNA down-regulation by antisense oligonucleotides (3), DNA enzyme is responsible for both mRNA targeting and cleavage. DNA enzyme has greater advantages over ribozyme or antisense RNA because of its stability. Several studies have been reported for the potential usage of DNA enzymes (9,12,14).

Although expression vectors have been successfully used for the delivery of antisense RNAs and ribozymes, there is no vector currently available for antisense oligonucleotide delivery. Using a bacterial retron system, Mirochnitchenko et al. (8) were able to generate ssDNA in a multicopy DNA form. 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. We previously reported a two-vector system that allows for the expression of a single-stranded c-raf mRNA-cleaving DNA enzyme (2). In this study, based on our previous two-vector expression system, we constructed a single expression vector that encodes for all the elements necessary for the production of c-raf DNA enzyme.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

MATERIALS AND METHODS

Vector Construction

The ssDNA expression vector was constructed based on two-vector expression system as described previously (2). pssXA, which contains the Moloney mouse leukemia viral reverse transcriptase gene, was digested with *Xma*I and *Bgl*II, and the resulting *Xma*I-*Bgl*II fragment was replaced with a dsDNA adaptor that was formed by annealing two oligonucleotides, *Xma*I-*Bgl*II-Stop 1 (5'-CCGGATCTAGACCGCAAGCTTCATTAAA-3') and *Xma*I-*Bgl*II-Stop 2 (5'-GATCTTTAATGAAGCTTGCGGTCTCGAT-3'). This adaptor contains a protein translation stop codon and subcloning sites, *Xba*I and *Hind*III. The resulting plasmid was designated as pssXD (Figure 1). *Xba*I-*Hind*III fragments were cleaved from both pssXB and pssXB-II (2) and then cloned into pssXD between *Xba*I and *Hind*III. These DNA fragments contain (i) reverse transcriptase primer binding site, (ii) stem-loop structure, and (iii) random control sequence (derived from pssXB) or *c-raf* DNA enzyme coding sequence (derived from pssXB-II). The resulting plasmids were designated as pssXD-I and pssXD-II, respectively.

Cell Culture and Transfection

A549 human lung carcinoma cell line (ATCC no. CCL-185) used in this study was maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS. A549 cells were transiently transfected with LipofectAMINE™ 2000 (Invitrogen), following the manufacturer's instructions. Genomic DNA and total RNA were prepared from transfected A548 cells. Genomic DNA was isolated using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). Total RNA was purified using TRIzol® reagent (Invitrogen), following the manufacturer's instructions.

Detection of Single-Stranded *c-raf* DNA Enzyme

Previous data have shown ssDNA can be co-purified with total RNA preparation (2,8). Using total RNA fraction, the expression of *c-raf* DNA enzyme was determined by dot-blot analysis as described previously (2). Briefly, 2 µg total RNA, pretreated either with RNase A only or both RNase A and S1 nuclease for 30 min at 37°C, were loaded onto a Hybond®-N+ membrane (Amersham Biosciences, Piscataway, NJ, USA), and fixed by UV ex-

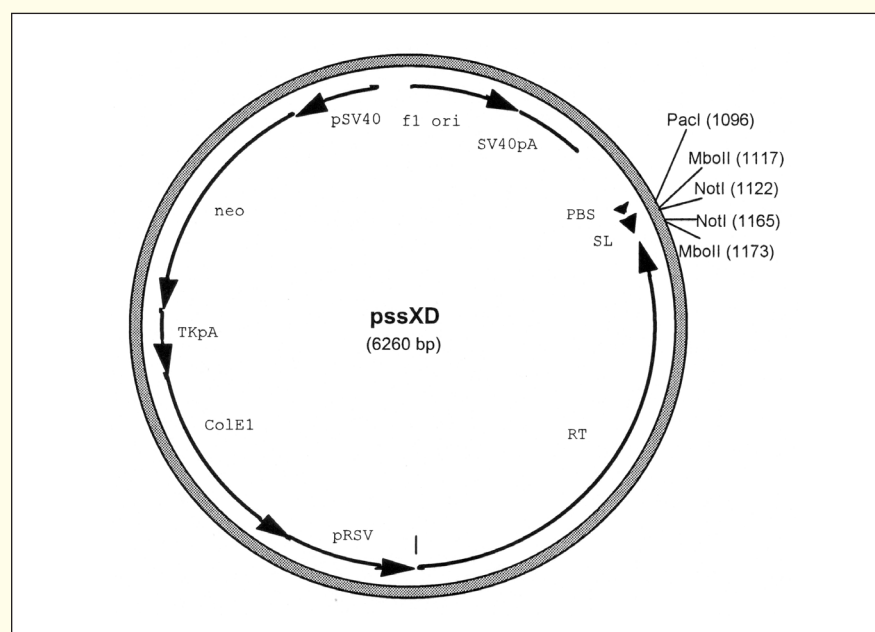


Figure 1. ssDNA expression vector, pssXD. RT, reverse transcriptase gene; SL, stem-loop sequence; and PBS, reverse transcriptase primer binding sequence.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

posure for 3 min. Hybridization and signal detection were performed using the North2South™ Chemiluminescent Nucleic Acid Hybridization and Detection Kit (Pierce Chemical, Rockford, IL, USA). The biotin-labeled *c-raf* oligonucleotide probe (5'-GGCCGCAC-TAATGCATGTCTCGTTGTAGCTA-GCCCAGGCGGGAAGTGC-3') was synthesized by Integrated DNA Technologies (Coralville, IA, USA).

Quantitation of *c-raf* mRNA Expression

c-raf mRNA expression was quantitated by RT-PCR as previously described (6). Briefly, 1 µg total RNA was reverse-transcribed using the Reverse Transcription System (Promega). A fraction of the resulting cDNA was used as template for PCR amplification. Primers used for PCR amplification were 5'-TCAGAGAAGCTCTGCTAAG-3' and 5'-CAATGCACTGGACACCTTA-3'. Actin was used as a housekeeping gene control, and primers used for the actin gene amplification were described by Li et al. (6).

Genomic DNA Cleavage Assay

Genomic DNA cleavage was determined using a LM-PCR Ladder Assay Kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Briefly, 0.5 µg genomic DNA were ligated to adaptors, supplied by BD Biosciences Clontech, overnight at 15°C. A fraction of adaptor-ligated DNA was used as template in ligation-mediated PCR (LM-PCR), following the manufacturer's instructions. Twenty-five cycles of PCR (95°C for 1 min and 72°C for 3 min) with an extension of 15 min at 72°C were conducted.

RESULTS AND DISCUSSION

Construction of ssDNA Expression Vector

In our earlier study, we reported a two-vector expression system for expressing ssDNA (2). Based on that expression system, we constructed an ssDNA expression vector, pssXD (Figure 1) by combining the two plasmids,

pssXA and pssXB, as described in the Materials and Methods section. All elements are transcribed into a single mRNA molecule. Newly synthesized reverse transcriptase is used for reverse transcription. Endogenous tRNA^{pro} binds to the PBS on the 3' end of the transcript and is used as the primer for ssDNA synthesis (7). After reverse transcription, ssDNA is released when the template mRNA is degraded either by endogenous RNase H or the RNase H activity of reverse transcriptase (13). A stem-loop structure is designed for the termination of the reverse transcription reaction. The *Mbo*II gene was removed from this version of the expression vector, since the expression of *Mbo*II in cells is a concern because of its nature to digest DNA.

The RNA-cleaving DNA enzyme sequence, which specifically targets the 3' untranslated region of *c-raf* mRNA, was inserted near the stem-loop structure so that only the DNA enzyme and very limited extraneous sequence can be synthesized. The vectors expressing either control sequence or *c-raf* DNA enzyme were designated as pssXD-I and pssXD-II, respectively.

Expression of Single-Stranded *c-raf* DNA Enzyme in A549 Cells

The expression of *c-raf* DNA enzyme in A549 cells was assessed by dot-blot assay. A biotin-labeled *c-raf* oligonucleotide probe was used to detect signals in the RNA samples isolated from A549 cells either transfected with control pssXD-I or pssXD-II containing *c-raf* DNA enzyme coding sequence. To rule out any possible non-specific hybridization to RNA molecules, the RNA samples were pretreated with RNase A, either in the absence or presence of S1 nuclease, for 30 min before hybridization. Figure 2 shows that only sample isolated from cells transfected with pssXD-II displayed a positive signal and that in the presence of S1 nuclease, no detectable signal was observed due to the specific degradation of ssDNA by S1 nuclease.

Suppression of *c-raf* Gene Expression by DNA Enzyme Produced In Vivo

To determine whether ssDNA enzyme expressed in A549 cells altered

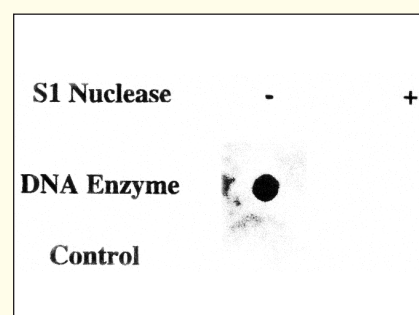


Figure 2. Production of *c-raf* DNA enzyme in A549 cells. To detect *c-raf* DNA enzyme expressed in cells, dot-blot analysis was performed. A549 cells were transiently transfected with control vector, pssXD-I, or pssXD-II containing *c-raf* DNA enzyme coding sequence. Forty-eight hours after transfection, total RNA was isolated according to the Materials and Methods section. Two micrograms of total RNA, isolated from either cells transfected with control vector, pssXD1, or pssXDII containing *c-raf* DNA enzyme coding sequence, was pretreated with RNase A in the presence or absence of S1 nuclease for 30 min at 37°C. The treated samples were then loaded onto the membrane and fixed by UV exposure for 3 min. Hybridization and signal detection were performed according to the Materials and Methods section using a biotin-labeled *c-raf* oligonucleotide probe.

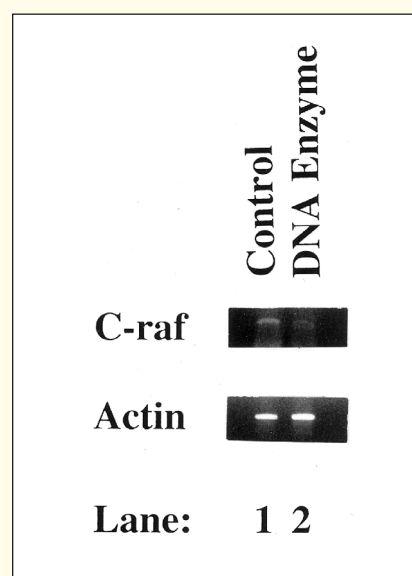


Figure 3. Suppression of *c-raf* mRNA expression by *c-raf* DNA enzyme. Quantitative RT-PCR was conducted using total RNA isolated from cells transfected with control vector, pssXD-I, or pssXD-II containing *c-raf* DNA enzyme coding sequence. Total RNA was isolated from cells 48 h after transfection. Actin gene was used as a control to normalize loading quantities.

c-raf mRNA levels, quantitative RT-PCR was conducted as previously described (6). Total RNA, isolated from cells transfected with either control pssXD-I or pssXD-II, was reverse-transcribed and PCR-amplified using a pair of *c-raf*-specific primers. PCR amplification of actin mRNA was used as a control to normalize loading quantity among different samples. As shown in Figure 3, a significant reduction (67.32%) of *c-raf* mRNA was detected in the cells transfected with pssXD-II compared to that of controls, pssXD-I.

Induction of Cell Apoptosis by *c-raf* DNA Enzyme Produced In Vivo

Our early observation (2), as well as those of others (5), indicates that suppression of *c-raf* gene expression may induce cell apoptosis. To further investigate whether the expression of *c-raf* DNA enzyme could induce A549 cell apoptosis, genomic DNA fragmentation assay was performed. Genomic DNA fragmentation is a hallmark of cell apoptosis.

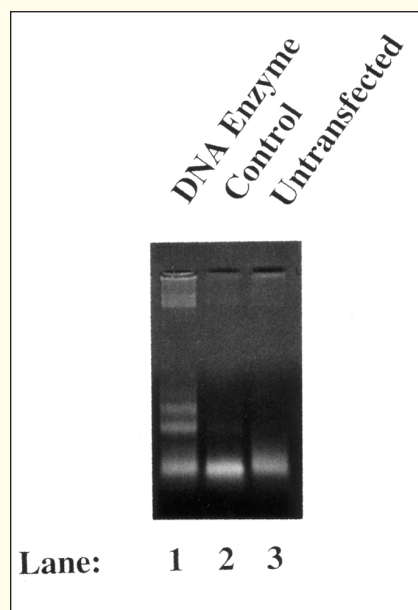


Figure 4. Genomic DNA fragmentation assay. Genomic DNA was isolated from cells transfected with control vector, pssXD-I, or pssXD-II containing *c-raf* DNA enzyme coding sequence, or untransfected cells (48 h after transfection). Genomic DNA (0.5 μ g) was ligated to the adaptor supplied by BD Biosciences Clontech. A fraction of ligated DNAs was then used in LM-PCR. For details about LM-PCR, see the Materials and Methods section.

Genomic DNA, isolated from cells transiently transfected with either control pssXD-I or pssXD-II, was ligated to an adaptor from LM-PCR Ladder Assay Kit (see Materials and Methods section). Subsequently, LM-PCR was carried out according to the manufacturer's instructions. As shown in Figure 4, there was a significant increase in fragmented genomic DNA in cells transfected with pssXD-II (Figure 4, lane 1) compared to cells transfected with control pssXD-I (lane 2) or untransfected cells (lane 3).

In conclusion, our study demonstrated that *c-raf*-cleaving DNA enzyme can be produced intracellularly and the expressed DNA enzymes can significantly suppress the expression of *c-raf* mRNA (67%). Since RNA samples used for quantification of gene down-regulation were isolated from cells, including those that were not transfected with our expression vector, it is likely that any improvement of transfection efficiency will lead to increased activity.

The expression systems presented here and previously (2) are the first systems that can produce ssDNA such as antisense oligonucleotides and DNA enzymes with limited extraneous vector sequences in mammalian cells. An expression vector has potential advantages over the direct antisense oligonucleotide or DNA enzyme delivery in that the vector can continuously produce oligonucleotides once it is introduced into the cells and result in a longer duration of action. Furthermore, the addition of a cell-specific promoter to the vector may allow oligonucleotide production in specific target cells.

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REFERENCES

1. Breaker, R.R. 1999. Catalytic DNA: in training and seeking employment. *Nat. Biotechnol.* 17:422-423.
2. Chen, Y., Y. Ji, R. Roxby, and C. Conrad. 2000. *In vivo* expression of single-stranded

DNA in mammalian cells with DNA enzyme sequences targeted to C-raf. *Antisense Nucleic Acid Drug Dev.* 10:415-422.

3. Crooke, S.T. 1999. Molecular mechanisms of action of antisense drugs. *Biochim. Biophys. Acta* 1489:31-44.
4. Jain, K.K. 2000. *Antisense Therapy: Technical Aspects and Commercial Opportunities.* Datamonitor PLC, London.
5. Lau, Q.C., S. Brusselbach, and R. Muller. 1998. Abrogation of C-raf expression induces apoptosis in tumor cells. *Oncogene* 16:1899-1902.
6. Li, Y., S.E. Starr, J. Lisziewicz, and W.Z. Ho. 2000. Inhibition of HIV-1 replication in chronically infected cell lines and peripheral blood mononuclear cells by retrovirus-mediated anti-tat gene transfer. *Gene Ther.* 7:321-328.
7. Marquet, R., C. Isel, C. Ehresmann, and B. Ehresmann. 1995. tRNAs as primer of reverse transcriptase. *Biochimie* 77:113-124.
8. Mirochnitchenko, O., S. Inouye, and M. Inouye. 1994. Production of single-stranded DNA in mammalian cells by means of a bacterial retron. *J. Biol. Chem.* 269:2380-2383.
9. Santiago, F.S., H.C. Lowe, M.M. Kavurma, C.N. Chesterman, A. Baker, D.G. Atkins, and L.M. Khachigian. 1999. New DNA enzyme targeting Egr-1 mRNA inhibits vascular smooth muscle proliferation and regrowth after injury. *Nat. Biotechnol.* 5:1264-1269.
10. Santoro, S.W. and G.F. Joyce. 1997. A general purpose RNA-cleaving DNA enzyme. *Proc. Natl. Acad. Sci. USA* 94:4262-4266.
11. Santoro, S.W. and G.F. Joyce. 1998. Mechanism and utility of an RNA-cleaving DNA enzyme. *Biochemistry* 37:13330-13342.
12. Sun, L.Q., M.J. Cairns, W.L. Gerlach, C. Witherington, L. Wang, and A. King. 1999. Suppression of smooth muscle cell proliferation by a *c-myc* RNA-cleaving deoxyribozyme. *J. Biol. Chem.* 274:17236-17241.
13. Tanase, N., and S.P. Goff. 1988. 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* 85:1777-1781.
14. Warashina, M., T. Kuwabara, Y. Nakamatsu, and K. Taira. 1999. Extremely high and specific activity of DNA enzymes in cells with a Philadelphia chromosome. *Chem. Biol.* 6:237-249.
15. Woolf, T.M. 1995. To cleave or not to cleave: ribozymes and antisense. *Antisense Res. Dev.* 5:227-232.

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