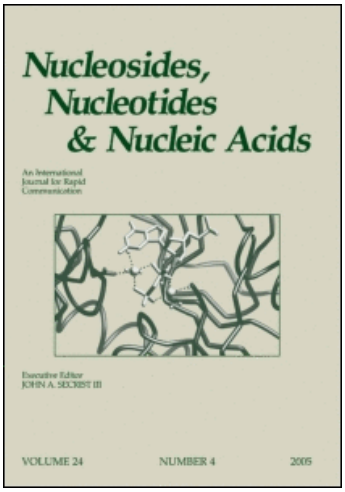

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Inhibition of VEGF mRNA by 2'-*O*,4'-*C*-Ethylene-Bridgednucleicacids (ENA®) Antisense Oligonucleotides and Their Influence on Off-Target Gene Expressions

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INHIBITION OF VEGF mRNA BY 2′-*O*,4′-*C*-ETHYLENE-BRIDGED NUCLEIC ACIDS (ENA®) ANTISENSE OLIGONUCLEOTIDES AND THEIR INFLUENCE ON OFF-TARGET GENE EXPRESSIONS

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□ *We investigated 2′-O,4′-C-ethylene-bridged nucleic acids (ENA) antisense oligonucleotides (AONs) for vascular endothelial growth factor (VEGF) in human lung carcinoma A549 cells. An ENA/DNA gapmer AON with RNase H-mediated activity was virtually stable in rat plasma and exhibited more than 90% inhibition of VEGF mRNA production. Moreover, 22 genes that are likely to bind to the AON were found in the GenBank database by BLAST and CLUSTAL W searches. Three of these genes were actually inhibited by the ENA AON. In shorter ENA AONs with fewer matched sequences of these genes, inhibitiory activities were decreased and off-target effects were improved. These results indicate that ENA AONs act in a sequence-specific manner and could be used as effective antisense drugs.*

Keywords Antisense; 2′,4′-BNA/LNA; ENA; VEGF; RT-PCR; BLAST

INTRODUCTION

Targeting unique mRNA molecules using antisense approaches based on sequence specificity could enable the design of a novel class of drugs against

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This article is dedicated to Professor Eiko Ohtsuka on the occasion of her 70th birthday.
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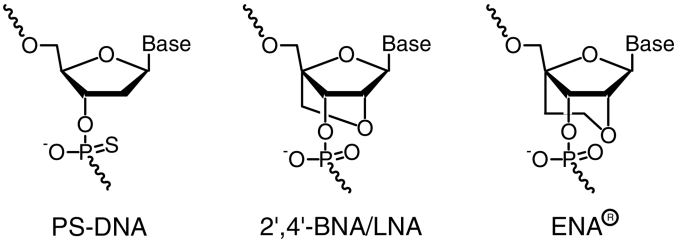

FIGURE 1 Structures of PS-DNA, 2′,4′-BNA/LNA, and ENA.

the expression of disease-related genes. Currently, functional oligonucleotides, namely, antisense oligonucleotides (AONs), small interference RNA (siRNA), and ribozymes, have been developed to knock down the target gene expression.[1] siRNA and ribozymes could offer superior knockdown potency and specificity at low dose *in vitro*, but chemical modifications are limited and systemic *in vivo* activity is required to develop them as nucleic acid drugs. AONs have been evaluated for their therapeutic potential in the treatment of inveterate diseases such as cancer, inflammation, and viral diseases for the last decade. In most clinical trials, phosphorothioated DNA oligonucleotides (PS-DNA, Figure 1) have been used as first-generation oligonucleotides, but revealed inefficiency and toxicity in systemic treatment. These problems are due to their low affinity to the RNA target derived from a mixture of 2^n (n = chain length −1) diastereomers and sequence-nonspecific protein binding that are attributable to the chemical structure of PS, causing inhibition of the blood clotting cascade, activation of the complement cascade and severe hypotension.[2,3] Developing novel modifications for highly effective AONs to overcome these problems could lead to reevaluation of AONs for therapeutic use. Several types of modified oligonucleotides were introduced as second-generation oligonucleotides. For instance, AONs with different types of chemistry like peptide nucleic acids, N3′-P5′-phosphoramidates, morpholino phosphorodiamidates, and 2′-*O*-(2-methoxy)ethyl modifications, are under investigation and some have been applied in clinical trials.[4]

Oligonucleotides composed of conformationally restricted nucleosides whose sugar puckering is fixed in an *N*-conformation as in RNA have recently been developed as an attractive novel type of antisense molecule. It was reported that 2′,4′-bridged nucleic acids/locked nucleic acids (2′,4′-BNA/LNA, Figure 1) with 2′-*O*,4′-*C*-methylene linkage in the sugar backbone exhibited unprecedentedly high binding affinity toward complementary RNA (+4–8°C per modification).[5,6] Conformational restriction of 2′,4′-BNA/LNA led to favorable duplex formation entropically by diminishing the loss of conformational degrees of freedom upon duplex formation.[7] In order to expand the potential of 2′,4′-BNA/LNA, we have reported the synthesis of 2′-*O*,4′-*C*-alkylene nucleosides to optimize the bridging carbon

length of the conformationally restricted nucleosides.[8,9] Consequently, we found that 2′-*O*,4′-*C*-ethylene-bridged nucleic acids (ENA, Figure 1) have both high binding affinity just as 2′,4′-BNA/LNA and much more resistance toward 3′-exonuclease and endonuclease. We have confirmed the intracellular antisense activity of ENA oligonucleotides against organic anion transporting polypeptide (oatp).[10] The ENA oligonucleotides also exhibit excellent triplex formation with dsDNA.[11] They could be used for exon skipping to bind dystrophin pre-mRNA and inhibit its splicing reaction.[12,13] Thus, ENA oligonucleotides are considered to be candidates for the next generation of antisense molecules.[14]

Vascular endothelial growth factor (VEGF) is known as a major inducer of tumor angiogenesis and has been shown to have significant potential as a target for antiangiogenic therapies.[15,16] There have been several reports on the inhibition of VEGF using AONs.[17–20] In the present report, to verify the further potential of ENA oligonucleotides as AONs, we chose VEGF as a target. RNase H activation assay and stability assay in plasma of oligonucleotides differently composed of ENA residues gave us the information to design ENA AONs. Based on these results, we designed ENA AONs targeted to VEGF mRNA and investigated their inhibitory activity of VEGF mRNA.

It is desirable that AONs have a highly selective and sequence-specific potential for inhibiting the expression of an intended gene product. However, AONs would actually have the possibility of hybridizing non-target mRNA and inhibiting their expression (called off-target effect) if they have similar complementary sequences with the non-target mRNA. A careful design of sequences of AONs is needed to maximize gene knockdown and minimize the off-target effect. It is no less important to identify such off-target effects caused by ENA AONs precisely, considering their high binding affinity to mRNA. To check the sequence specificity of ENA AONs and design specific and effective ENA AONs, we utilized a similarity search by BLAST and CLUSTAL W in the GenBank database to select genes that were likely to bind to ENA AONs. Thus, we investigated sequence-related inhibition of VEGF mRNA expression and selected gene expressions in cells treated by ENA AONs targeting VEGF.

MATERIALS AND METHODS

Synthesis of Oligonucleotides

All oligonucleotides were synthesized by solid-phase phosphoramidite chemistry using an Applied Biosystems model 392 DNA/RNA synthesizer. The synthesis of 2′-*O*,4′-*C*-ethylene nucleoside 3′-*O*-phosphoramidites, 2′-*O*,4′-*C*-methylene nucleoside 3′-*O*-phosphoramidites and the detailed synthetic conditions of modified oligonucleotides were described

previously.[9,21] 2′-*O*,4′-*C*-Ethylene thymidine- or 4,4′-dimethoxytrityl ethylene glycol-attached controlled pore glass (CPG) was used as a polymer support.[9,22] For preparing phosphorothioates, 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent, Sigma-Aldrich) was used as a sulfur-transfer reagent. 5′-Fluorescein-labeled oligonucleotide was synthesized using 6-FAM phosphoramidite (Applied Biosystems). Other reagent solutions were purchased from Applied Biosystems. The oligonucleotides were purified as >90% pure by reverse-phase HPLC and identified by ESI-mass spectroscopy: DNA-1, calcd 2675.80, found 2674.41; DNA-2, calcd 2804.33, found 2802.25; ENA-1, calcd 2885.99, found 2884.46; ENA-2, calcd 2950.25, found 2948.38; ENA-3, calcd 2885.99, found 2885.80; BNA-1, calcd 2880.12, found 2878.30; BNA-2, calcd 2815.85, found 2814.40; PS-SEN, calcd 7084.82, found 7084.43; PS-SCR, calcd 7058.85, found 7058.45; PS-AS, calcd 7058.85, found 7058.77; ENA-AS1, calcd 7492.40, found 7492.36; ENA-AS2, calcd 7334.62, found 7333.89; ENA-4MIS, calcd 7492.40, found 7492.15; ENA-8MIS, calcd 7492.40, found 7492.03; ENA-AS1hp, calcd 7536.10, found 7536.24; ENA-AS3hp, calcd 6503.40, found 6503.01; ENA-AS4hp, calcd 6542.43, found 6542.05; ENA-AS5hp, calcd 5810.93, found 5809.97; ENA-AS6hp, calcd 5509.73, found 5509.90.

RNase H Activation Assay (RNase H-Mediated RNA Cleavage)

The modified oligothymidylate (DNA-1, -2 and ENA-1 to -3) and the complementary 5′-fluorescein-labeled oligoriboadenylate (fluorescein-r$(A)_9$) were annealed at 60°C for 5 min before the reaction. The RNase H-mediated reactions were carried out in a total volume of 20 μl containing 200 pmol oligothymidylate, 200 pmol fluorescein-r$(A)_9$, 40 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 0.8 mM DTT, 4% glycerol, 0.003% BSA, and 0.08 unit of *Escherichia coli* (*E. coli)* RNase H (Takara Bio, Shiga, Japan). All of the reactions were incubated at 37°C. At the time points of 3 and 90 min, 6-μl aliquots were taken and immediately heated at 90°C for 2 min to stop the reaction. Aliquots of 4 μl formamide loading buffer were added to the samples. The sample mixtures were loaded and run on denaturing 15% polyacrylamide gel containing 7 M urea. The gel was exposed to UV light (320 nm) and photographed. The density of individual bands of fluorescein-r$(A)_9$ was quantitated by Scion Image software (Beta 4, Scion Corp., Frederick, MD).

Stability Assay in Plasma

Rat whole blood was obtained from adult male Sprague-Dawley rats (Charles River Laboratories, Yokohama, Japan). Plasma was separated by centrifugation at 3000 rpm for 10 min. A 20-μl aliquot of each oligonucleotide (1 mg/ml) was added to 980 μl of the rat plasma (finally 20 μg/ml) and incubated at 37°C. At the time points of 0, 0.25, 0.5, 1 and 4 h, 100-μl

aliquots were taken and immediately were added with 300 μl methanol containing internal standard compound (5′-XCCCCCCCC-3′: X = 2′-*O*-*tert*-butyldimethylsilyl uridine, C = 2′-deoxycytidine, 5 μg/ml) and centrifuged at 10,000 rpm at 4°C for 2 min to remove the proteins. The supernatants were taken as samples for HPLC analyses. Reverse-phase HPLC analyses were carried out with a gradient of acetonitrile and a constant of 0.1 M triethylammonium acetate (pH 7.0). The average residual amount of oligonucleotides and corresponding standard deviation were obtained from three independent experiments.

Cell Culture and Oligonucleotide Treatment

Human non-small cell lung carcinoma A549 cells (CCL-185; American Type Culture Collection [ATCC]) were maintained in RPMI 1640 (Invitrogen) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere containing 5% CO_2. A549 cells were plated onto collagen type-I coated dishes at 20–40% confluency and cultured overnight. Ethoxylated polyethylenimine (EPEI, Gene Tools, Philomath, OR) was used as a transfection reagent according to the supplier's protocol. A typical transfection experiment was performed as follows: AONs (1 nmol) in 198 μl of H_2O and 2 μl of EPEI (0.4 nmol) were mixed to form cationic complexes, which were then transfected into A549 cells (1×10^5 cells/well) in 12-well dishes with 800 μl of serum-free RPMI 1640 per well. Cells were incubated for 4 h at 37°C and then the medium was exchanged to fresh RPMI 1640 containing 10% FBS and cells were incubated for an additional 20 h.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total mRNAs were isolated at 24 h after transfection using the QuickPrep Micro mRNA Purification Kit (Amersham-Pharmacia, Biotech, Piscataway, NJ). Purified mRNA was then reverse transcribed into cDNA at 42°C for 60 min using Ready-To-Go RT-PCR Beads (Amersham Pharmacia) and oligo $dT_{(12-18)}$ primer. PCR was performed using Takara Taq Premix (Takara Bio, Shiga, Japan) as follows: denaturation at 94°C for 15 s, annealing at 55°C for 30 s, extension at 72°C for 30 s. For each gene, PCR was typically performed for 20–35 cycles, under which conditions the linear concentration-dependent response of PCR was confirmed. The genes (GenBank accession number), the forward and reverse primers, and the corresponding length of the PCR product of each gene are listed as follows: VEGF (AF022375 for $VEGF_{165}$ and AF214570 for $VEGF_{121}$), 5′-tgcattggagccttgccttg-3′, 5′-ctcaccgcctcggcttgt-3′, 554 and 445 bp; β-actin (NM_001101), 5′-atcaccattggcaatgagcg-3′, 5′-ttgaaggtagtttcgtggat-3′, 98 bp; GAPDH (NM_002046), 5′-caactacatggtttacatgttc-3′, 5′-gccagtggactccacgac-3′,

182 bp; CDC25B (NM_021872), 5′-tcacgatgagatcgagaacctcc-3′, 5′-cccacg ctcagatgagaattcac-3′, 352 bp; TAF2C1 (NM_003185), 5′-catctggcaagcagt ctacagag-3′, 5′-gcacaaggtaaggttgaggtgaag-3′, 141 bp; TBL1 (NM_005647), 5′-tttccttttcattcagcccctgccc-3′, 5′-ccgtagaatcaaacgaagcacttgcc-3′, 361 bp; SEC31B-1 (NM_015490), 5′-cattctgtcttctgctcacccc-3′, 5′-accactgcacatcaaag cacc-3′, 389 bp; XB51 (NM_031232), 5′-ggcttgtctcctcctgaacttg-3′, 5′-gacagcag ggaccagaattcag-3′, 186 bp; POLI (NM_007195), 5′-ctgccaaatgtcttgaagcactgg-3′, 5′-tagggcactgacgactctcacg-3′, 354 bp; PLCβ4 (NM_000933), 5′-aatgcccac gagcagcaaac-3′, 5′-ccatctcggcttccaatttcacc-3′, 337 bp; STAT1 (NM_007315), 5′-atctccaacgtcagccagctcc-3′, 5′-tgatgaagcccatgatgcaccc-3′, 376 bp; CCR6 (NM_004367), 5′-ttcctgcactgctgcctgaac-3′, 5′-acctatcagagaccaccccacc-3′, 397 bp; DGUOK (NM_001929), 5′-cgaaaacttacccagaatggcacg-3′, 5′-cccaca ggagaaaagaatgccag-3′, 360 bp; REP1 (NM_000390), 5′-tgacagtgccagca gaggaac-3′, 5′-gggccagagcagacataaacg-3′, 310 bp; ABCA3 (NM_001089), 5′-gcccatcttacatcctctctctcc-3′, 5′-gcctcgctacagttcaacctg-3′, 389 bp; RAF1 (NM_002880), 5′-ccacttttctgctccctttctcc-3′, 5′-actgcctgctaccttacttcctc-3′, 303 bp; GRP58 (NM_005313), 5′-ttttatgccccttggtgtggtc-3′, 5′-tcctcctgtgccttc ttcttcttc-3′, 311 bp; TUSP (NM_020245), 5′-ctctgccatgttacacggtcttc-3′, 5′-tcttagaaccctacccctcccc-3′, 374 bp; NTNG1 (NM_014917), 5′-ccagcc taatgctacagcatcc-3′, 5′-gacactccaaacagaaagacaccc-3′, 337 bp; ELF2 (NM_006874), 5′-tccaacaacagcgacctctcc-3′, 5′-tgccactgcttccgatttaacc-3′, 352 bp; MATK (NM_002378), 5′-aacacgggttcaccagcaag-3′, 5′-cctccgacacc tctttcagtgac-3′, 114 bp; NR2C1 (NM_003297), 5′-cctgatctgtctgcacaacacc-3′, 5′-tgaagcggcacagttggaag-3′, 351 bp; ITGA2 (NM_002203), 5′-cctga tgagaaagccgaagtacc-3′, 5′-caccccccacaatttcttcatcc-3′, 350 bp; TRP7 (NM_020389), 5′-tgcatacattctgccagccaac-3′, 5′-cttccattccacatctgcatcctc-3′, 400 bp; BTK (NM_000061), 5′-acttctcttctccacaagcccc-3′, 5′-aaaacaccctcccctcccatg-3′, 380 bp. PCR products were electrophoresed on 10% non-denaturing polyacrylamide gel. The gel was stained with SYBR Green I (Molecular Probes, Carlsbad, CA) and individual bands were then quantitated on a Molecular Imager FX (Bio-Rad, Hercules, CA). The amount of mRNA in each sample was normalized against the amount of the human β-actin or GAPDH mRNA in the same sample.

Sequence Similarity Search

The database used for the sequence similarity search in this study was the NCBI GenBank human Reference Sequence database (human RefSeq; release 9/21/2001, 14,038 sequences, 35,291,391 nt, http://www.ncbi.nlm.nih.gov/RefSeq/). A BLAST search was performed using standard parameters except for mismatch penalty ($=-1$ or -3). A batch process repeating CLUSTAL W alignment of a query sequence (5′-atgaactttctgctgtcttggg-3′) toward every sequence in the database was also performed using standard parameters of CLUSTAL W.

RESULTS

Induction of RNase H Cleavage by ENA Oligonucleotides

We examined the design of oligonucleotides incorporating ENA residues that can induce RNase H activation effectively. We designed three types of 9-mer chimeric ENA/DNA oligothymidylates, an ENA/DNA/ENA gapmer (ENA-1), an ENA/PS-DNA/ENA gapmer (ENA-2), and an ENA/DNA mixmer (ENA-3). Each oligothymidylate was annealed with equivalent amounts of fluorescein-r$(A)_9$ and incubated with *E. coli* RNase H at 37°C for 3 min or 90 min and then analyzed by electrophoresis (Figure 2). In only 3 min, substantial cleavage mediated by RNase H was observed in ENA-1 and -2, while no cleavage was yet observed in natural DNA or PS-DNA (DNA-1 or -2, respectively). This indicates that ENA-1 and -2 with higher binding ability than DNA-1 and -2 were more efficient substrates for RNase H activation under physiological conditions such as at 37°C. On the other hand, in ENA-3 without a continuous DNA stretch, no cleavage was observed, indicating that ENA residues could not be a substrate for RNase H.

Stability of ENA Oligonucleotides in Rat Plasma

We compared the stability of ENA-2 and -3 with that of DNA-1, -2, 2′,4′-BNA/PS-DNA/2′,4′-BNA gapmer, BNA-1, and 2′,4′-BNA/DNA mixmer, BNA-2, in rat plasma. Each oligonucleotide was incubated with rat plasma (final

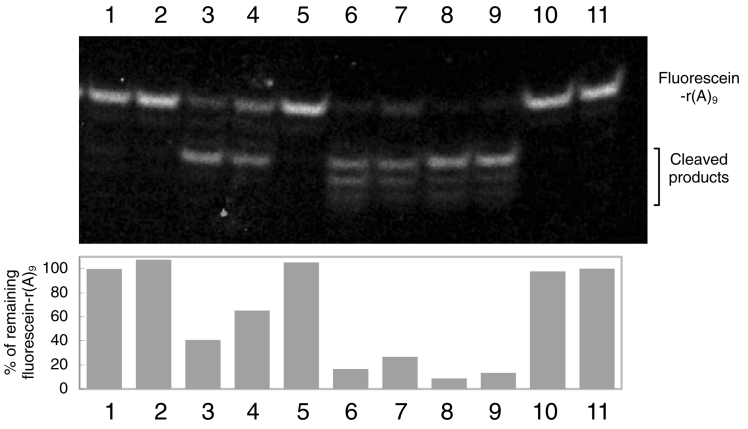

FIGURE 2 RNase H-mediated hydrolysis of oligonucleotides. The fluorescein-labeled oligoriboadenylate (fluorescein-r$(A)_9$) was incubated with equivalent molarity of the following oligothymidylates in the presence of *E. coli* RNase H at 37°C for 3 or 90 min and then analyzed on a 15% polyacrylamide gel. The amount of remaining fluorescein-r$(A)_9$ in each sample was quantitated. Lanes 1 and 6: DNA-1 (natural DNA, 5′-TTTTTTTTT-3′); lanes 2 and 7: DNA-2 (PS-DNA, 5′-TsTsTsTsTsTsTsTsT-3′); lanes 3 and 8: ENA-1 (ENA/DNA/ENA gapmer, 5′-TTTTTTTTT-3′); lanes 4 and 9: ENA-2 (ENA/PS-DNA/ENA gapmer, 5′-TTTsTsTsTsTTT-3′); lanes 5 and 10: ENA-3 (ENA/DNA mixmer, 5′-TTTTTTTTT-3′); underlined: ENA residues; s: phosphorothioate linkages. lane 1 to 5: 3 min of incubation; lane 6 to 10: 90 min of incubation; lane 11: no oligothymidylate.

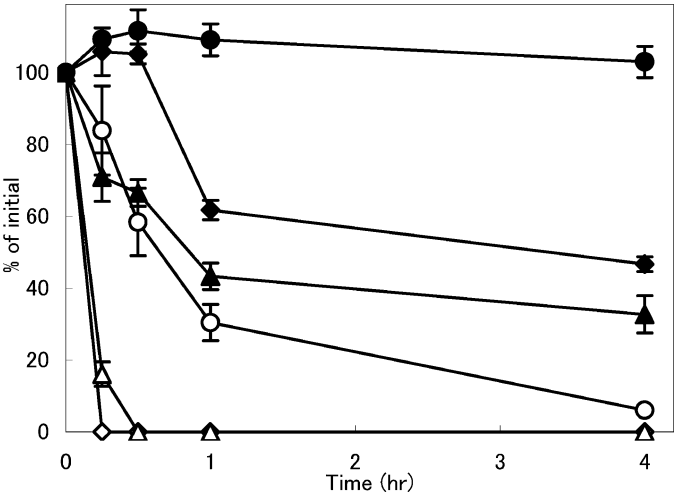

FIGURE 3 Stability of oligonucleotides in rat plasma. Each oligonucleotide was incubated with rat plasma (final conc. 98%). The mixture was analyzed by reverse-phase HPLC. Closed circles: ENA-2 (5′-TTTsTsTsTsTTT-3′), closed diamonds: DNA-2 (5′-TsTsTsTsTsTsTsTsT-3′), closed triangles: BNA-1 (2′,4′-BNA/PS-DNA/2′,4′-BNA gapmer, 5′-TTTsTsTsTsT TT-3′), open circles: ENA-3 (5′-TTTTTTTTT-3′), open triangles: BNA-2 (2′,4′-BNA/DNA mixmer, 5′-TTTTTTTTT-3′), open diamonds: DNA-1 (5′-TTTTTTTTT-3′), underlined: ENA residues; doubly underlined: 2′,4′-BNA/LNA residues; s: phosphorothioate linkages.

conc. 98%) at 37°C and the mixture was analyzed by reverse-phase HPLC. DNA-1 was completely degraded in 15 min. ENA-2 and -3 were more stable than BNA-1 and -2, respectively, as shown in Figure 3. Among the oligonucleotides we tested, ENA-2 showed excellent stability, remaining almost intact, while DNA-2, of which half were degraded in 4 h in rat plasma, did not.

Down-Regulation of VEGF mRNA Expression by ENA AONs

In the present study, we used an antisense sequence targeting the initiation site of VEGF mRNA that had already been reported as a PS AON.[17] Considering the improvement in the effective induction of RNase H cleavage and stability of AONs in plasma as described above, ENA/PS-DNA/ENA gapmers were chosen for AONs targeting VEGF mRNA and the inhibitory activities of the gapmers and PS AON were compared. We prepared two ENA AONs, ENA-AS1 and ENA-AS2, which have the same sequence but whose ENA regions of the gapmer are different (Figure 4A). For the delivery of ENA/PS-DNA/ENA gapmers into cells, we used a cationic polymer, ethoxylated polyethylenimine (EPEI). In a flow cytometry experiment, EPEI showed the highest efficiency of delivering 1 μM of fluorescein-labeled ENA/PS-DNA/ENA gapmer into cells among various transfection reagents of cationic polymers and lipids we tested, with no toxicity (data not shown). The complex of EPEI and each AON (1 μM) was transfected into A549 cells for 4 h. Total mRNAs were

A

PS-SEN	5′–AsTsGsAsAsCsTsTsTsCsTsGsCsTsGsTsCsTsTsGsGsG–3′
PS-SCR	5′–AsCsAsGsTsCsAsGsTsCsAsGsAsAsTsGsAsAsCsAsCsC–3′
PS-AS	5′–CsCsCsAsAsGsAsCsAsGsCsAsGsAsAsAsGsTsTsCsAsT–3′
ENA-AS1	5′–CCCAAGACAsGsCsAsGsAAAGTTCAT–3′
ENA-AS2	5′–CCCAAGsAsCsAsGsCsAsGsAsAsAsGsTTCAT–3′
ENA-4MIS	5′–CCAAAGCCAsGsCsAsGsAACGTTAAT–3′
ENA-8MIS	5′–CCAACGTCAsGsAsCsGsAACGATAAT–3′

B

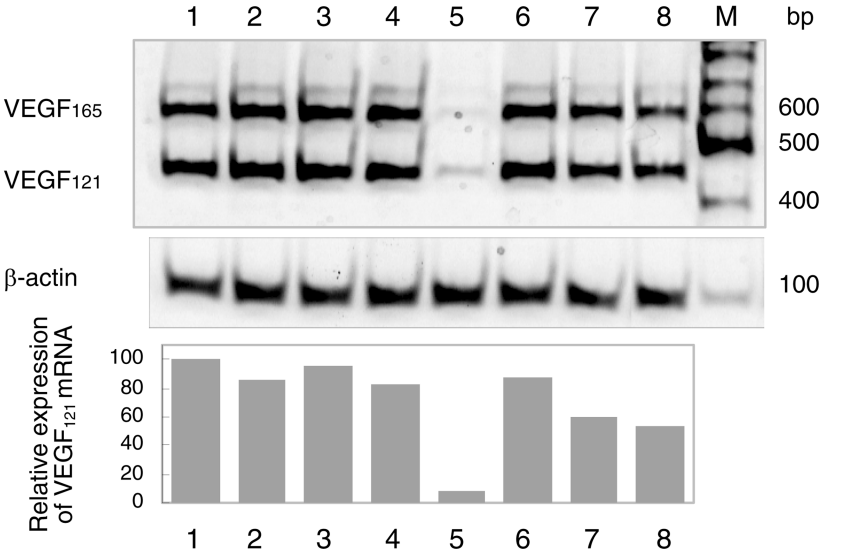

FIGURE 4 (A) Oligonucleotides and their sequences in a VEGF mRNA knockdown assay. Underlined: ENA residues except for 2′-*O*-methylguanosine in the case of G; s: phosphorothioate linkages. (B) Effects of ENA AONs on the expression of VEGF mRNA in human lung carcinoma A549 cells. The A549 cells were treated with a cationic complex of oligonucleotides and EPEI in serum-free medium for a period of 4 h. Transfected cells were then washed and incubated for a further 20 h. VEGF mRNA expression was examined by RT-PCR analysis. Two isoforms, $VEGF_{165}$ and $VEGF_{121}$, were detected in this RT-PCR. Relative expression of $VEGF_{121}$ mRNA normalized to β-actin was quantitated. Lane 1, untreated; lane 2, PS-SEN; lane 3, PS-SCR; lane 4, PS-AS; lane 5, ENA-AS1; lane 6, ENA-4MIS; lane 7, ENA-8MIS; lane 8, ENA-AS2; M, dsDNA molecular marker.

isolated at 24 h after transfection and RT-PCR was performed. While no down-regulation of VEGF mRNA expression by PS oligonucleotides, such as PS-SEN (sense), PS-SCR (scrambled), and PS-AS (antisense), was observed (lanes 2, 3, and 4 in Figure 4B, respectively), ENA-AS1 considerably down-regulated more than 90% of VEGF mRNA production (lane 5 in Figure 4B). In the case of ENA oligonucleotides containing 4 or 8 mismatches, ENA-4MIS or -8MIS, respectively, the expression of VEGF mRNA was not considerably decreased (lanes 6 and 7 in Figure 4B). Inhibitory activity of ENA-AS2, which

is composed of shorter ENA regions and a longer PS-DNA stretch, was significantly weaker than that of ENA-AS1 (lanes 5 and 8 in Figure 4B), indicating that the inhibitory activity of the AONs depended on the higher binding affinity of the relevant oligonucleotide.

We also investigated the expression of VEGF protein by ELISA. At 24 h after transfection, the amounts of VEGF protein inside cells and secreted from cells were measured. In the VEGF protein in the lysate of cells, moderate inhibition of about 40% was observed by the transfection of the ENA-AS1/EPEI complex (data not shown). On the other hand, in the culture medium, no inhibition was observed by the transfection of the ENA-AS1/EPEI complex. By the transfection of the same amount of EPEI only, the amount of secreted VEGF protein was increased by about 40%, while that of lysate VEGF protein was unchanged. The true reduction of the amount of secreted VEGF protein by the treatment with the ENA-AS1/EPEI complex might be concealed by the effect of the transfection increasing the VEGF secretion.

Investigation of Non-Target Gene Expression Other Than VEGF

We verified whether ENA-AS1 exhibited VEGF mRNA-specific inhibition in cells. At first, in order to find genes that were considered likely to bind to ENA-AS1, we performed an alignment search in the NCBI GenBank human RefSeq database (ca. 14,000 genes) using BLAST and CLUSTAL W algorithms with a query for the sequence of ENA-AS1. Consequently, 22 genes, excluding hypothetical proteins, were obtained as candidate genes with sequences similar to ENA-AS1. The sequences of the genes are listed in Table 1. Each gene has 15–20 matched sequences with the sequence of ENA-AS1, and there were 4–10 mismatched sequences such as internal loops and bulge loops. We performed RT-PCR analysis to detect the expression of the 22 genes in the A549 cells. mRNA expression of 13 of the 22 genes was detected and that of the other 9 genes was either not detectable or not expressed (Table 1). Next, we analyzed the expression of these 13 genes in the cells transfected with the ENA-AS1 and ENA-8MIS. Consequently, in 8 genes, namely, polymerase iota (POLI), phospholipase C beta4 (PLCβ4), glucose regulated protein (GRP58), rab escort protein 1 (REP1), tubby superfamily protein (TUSP), deoxyguanosine kinase (DGUOK), E74-like factor 2 (ELF2), and v-raf-1 murine leukemia viral oncogene homolog 1 (RAF1), no down-regulation of mRNA expression was observed (Figure 5B). Inhibition of expression of RNA polymerase II C1 (TAF2C1) and transducin (beta)-like 1 (TBL1) genes was observed; unfortunately, this was also observed in the cells treated with ENA-8MIS (Figure 5C). This indicates that these two genes were inhibited non-sequence specifically, maybe due to a change in gene expression caused by transfection using EPEI. In the three genes of cell division cycle 25B (CDC25B), signal transducer & activator transcription 1

TABLE 1 The List of Genes Obtained by the Search in the NCBI GenBank Human RefSeq Database Using BLAST and CLUSTAL W Alignment

Gene name	Sequence (5′ -3′)	RT-PCR*
Vascular endothelial growth factor (VEGF)	ATGAACTTTCTGCTGTCTTGGG	D(30)
Genes obtained by BLAST		
Cell division cycle 25B (CDC25B)**	CAGAACTTGCTGCTGTCTTGTT	D(25)
RNA polymerase II, C1 (TAF2C1)	AAGCCCTTTCTGCTGTCTCGGC	D(30)
TATA box binding protein-associated factor, transducin (beta)-like 1 (TBL1)**	GTGAACTTTCTGGTGTATCGGT	D(27)
Secretory pathway component Sec31B-1 (SEC31B-1)**	CTCAAGGTTCTGCTGTCTTGGG	N
X11L-binding protein 51 (XB51)	GCTTTCTTCCTGCTGTCTTGGG	N
Polymerase iota (POLI)	ATGAACTTTCTGCGGTGACTGT	D(30)
Phospholipase C, beta4 (PLCβ4)	ATGAACTTTCTGCTGATGACTT	D(27)
Signal transducer and activator of transcription 1 (STAT1)	AAGAACTTTCTGCTGTTACTTT	D(21)
Genes obtained by CLUSTAL W		
Glucose regulated protein (GRP58)	ATGAACTTTCTG*ATTT*TGGCTTGG*A*G	D(26)
Chemokine (C-C motif) receptor 6 (CCR6)	ATCATTTTTC*T*TGCTGTCTTGGG	N
Rab escort protein 1 (REP1)	AAAAACTTTCT*TCA*CTGTCTTGGG	D(28)
Tubby super-family protein (TUSP)	AGGAACTATCTGCTGGC*G*TTGGG	D(28)
Netrin G1 (NTNG1)	AAGAACTTTCT*TCCA*GCTATCATGGG	N
Putative capacitative calcium channel (TRP7)	GTGAAATTCCTG*G*CTGTCTT*T*GG*A*G	N
Deoxyguanosine kinase (DGUOK)	ATGAAC*A*TTCC*A*G*T*GCTG*G*TGTTGGA	D(28)
ATP-binding cassette, sub-family A, member 3 (ABCA3)	ATGAACTTCTT-CT-TCTTGGG	N
E74-like factor 2 (ELF2)	ATGAA*A*TTT*A*TCTTCTGTTTTGGT	D(28)
Nuclear receptor subfamily 2, group C, member 1 (NR2C1)	ATGAACTTTTTTACT*CTTG*GTCTTGCC	D(26)
Integrin α2, CD49B, α2 subunit of VLA-2 receptor (ITGA2)	CTGAGGTTT*GG*C*A*T*A*GCAGT*T*CTTGGG	N
v-raf-1 murine leukemia viral oncogene homolog 1 (RAF1)	GTGAACATTCTGCTTTTTC*A*TGGG	D(28)
Megakaryocyte-associated tyrosine kinase (MATK)	GTGAACTTTCTGCGGACCCGGG	N
Bruton agammaglobulinemia tyrosine kinase (BTK)	ATGGGC*AG*T*A*T*C*TCTGCTG*C*TCTCAGA	N

The genes are listed in order of increasing number of matched residues which are represented by shading. Gap sequences between VEGF gene and each gene are represented in italics.

*Numbers of PCR cycles in which the genes were detected are shown in parenthesis; D: detected; N: not detected or not expressed.

**Genes also obtained by CLUSTAL W.

(STAT1) and nuclear receptor subfamily 2 group C member 1 (NR2C1), specific inhibition by ENA-AS1 was confirmed (Figure 5D).

If genes are under the influence of VEGF pathways/cascades, those genes may have a possibility of changing their expression level as a result of the knock-down of VEGF. However, we could not observe a decrease in secreted VEGF protein from cells when transfected by ENA-AS1 as described above. Therefore, the inhibition of these three genes seemed to actually occur as sequence-specific off-target effects. The three genes CDC25B, STAT1 and

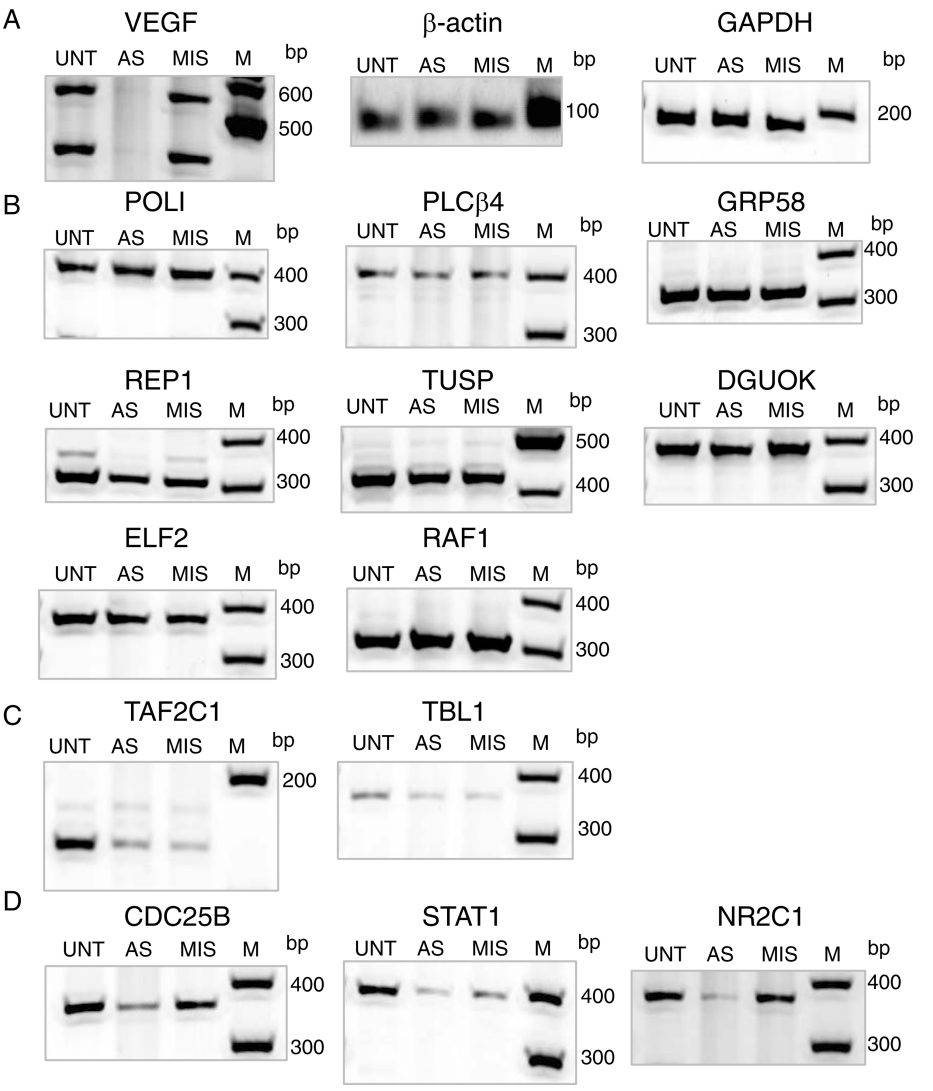

FIGURE 5 RT-PCR analysis of expression of genes with similarity to ENA-AS1. Human lung carcinoma A549 cells were treated with a cationic complex of 1 μM of oligonucleotides (ENA-AS1 or ENA-8MIS) and 0.4 μM of EPEI in serum-free medium for a period of 4 h. Transfected cells were then washed and incubated for a further 20 h. The mRNA expression of the genes was examined by RT-PCR analysis. (A) VEGF and control genes, (B) genes with no change in expression, (C) genes with a decrease in expression by treatment with both ENA-AS1 and ENA-MIS8, (D) genes with increased inhibition of their expression by ENA-AS1. UNT: untreated, AS: ENA-AS1, MIS: ENA-MIS8, M: dsDNA molecular marker.

NR2C1 had 15–17 nucleotides matched with the 22-mer sequence of ENA-AS1, which is likely a length sufficient to bind to ENA-AS1. If an AON that has fewer matched sequence to other genes than VEGF was used, it was considered that the undesired suppression of these other genes would be avoided. To verify this hypothesis, we synthesized newly designed ENA AONs,

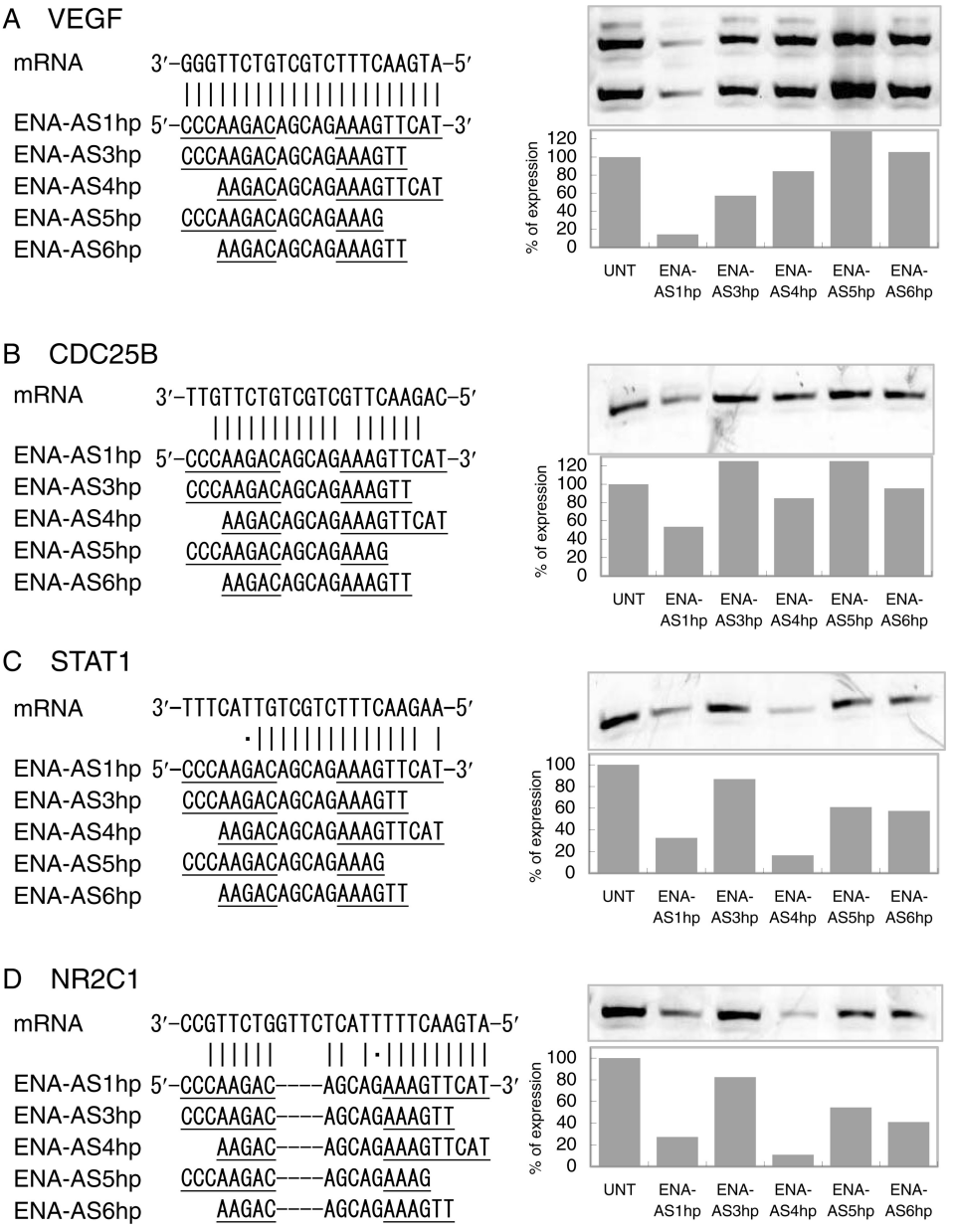

FIGURE 6 Effects of ENA-AS1hp and -AS3hp to -AS6hp on mRNA expression of (A) VEGF, (B) CDC25B, (C) STAT1 and (D) NR2C1. Human lung carcinoma A549 cells were treated with a cationic complex of 1 μM of each oligonucleotide (ENA-AS1hp and -AS3hp to -AS6hp) and 0.4 μM of EPEI in serum-free medium for a period of 4 h. Cells were then washed and incubated for a further 20 h. The mRNA expression of VEGF, CDC25B, STAT1 and NR2C1 was examined by RT-PCR analysis. UNT: untreated. |: Watson-Crick base-pairing, • : wobble base-pairing.

ENA-AS3hp, -AS4hp, and -AS5hp, which were shorter either from the 3′ or 5′ end than ENA-AS1, and ENA-AS6hp that was shorter both at the 3′ and 5′ ends. These AONs were attached with a 2-hydroxyethyl phosphate group (hp) at the 3′ end to easily synthesize ENA AONs with various sequences at the 3′ end and stabilize them against 3′-exonuclease, as reported previously.[22] We treated the A549 cells with these newly designed ENA AONs and compared their inhibitory activity with that of ENA-AS1hp, which had the same sequence as ENA-AS1, as shown in Figure 6. ENA-AS3hp inhibited approximately 40% of VEGF mRNA expression, which was weaker than that of the 22-mer ENA-AS1hp, while ENA-AS4hp, -AS5hp, and -AS6hp almost lost their inhibition activity of VEGF mRNA expression (Figure 6A). The mRNA expression of CDC25B was recovered with ENA-AS3hp, -AS4hp, -AS5hp, and -AS6hp, maybe due to a decrease in the number of matched sequences with CDC25B (Figure 6B). In STAT1 and NR2C1, inhibition of mRNA expression by ENA-AS3hp, -AS5hp, and -AS6hp was improved (Figures 6C and 6D). ENA-AS4hp continued to inhibit expression of STAT1 and NR2C1 because the extent of the matched sequence of ENA-AS4hp with STAT1 and NR2C1 was still the same as that of ENA-AS1hp (Figures 6C and 6D). These results demonstrate that these ENA AONs acted in a sequence-specific manner.

DISCUSSION

AONs act via two main mechanisms for their inhibitory activity: functional steric block of the ribosomal translation and induction of cleavage of the target mRNA by RNase H.[1] Utilizing RNase H-mediated hydrolysis of target mRNA is one of the advantages of using AONs because of the reduction of the amount of the mRNA. Oligonucleotides fully modified with 2′-*O*-alkylated ribose derivates, including 2′,4′-BNA/LNA residues, show enhanced hybridization affinity.[5,6,23,24] On the other hand, their duplexes with complementary RNA are not recognized by RNase H.[23,24] Gapmers, which contain a DNA stretch in the centre flanked by regions with 2′-*O*-alkylated nucleosides, are now considered to be a feasible design for AONs, because they are stable against nucleases and have increased affinity to the target mRNA, and could activate RNase H as well, compared to other designs of mixmer oligonucleotides.

Kurreck et al.[24] reported that a DNA stretch of more than 6 nucleotides was necessary to recruit *E. coli* RNase H for a gapmer including 2′,4′-BNA/LNA residues. However, our result (Figure 2) was rather similar to that of the other reports of 2′-*O*-methyl nucleosides in which a minimal stretch of at least four DNA residues was enough for efficient activation of *E. coli* RNase H.[25] As well, in the VEGF mRNA inhibition assay using human lung carcinoma A549 cells, ENA-AS1 worked well, indicating that a DNA

stretch of 5 nucleotides was sufficient in ENA/PS-DNA/ENA gapmers for human RNase H just as reported previously for 2′-*O*-methyl nucleosides.[23]

Stability in biological media is one of the desired requirements for AONs. In our previous reports, we demonstrated that oligonucleotides containing ENA residues were more stable against snake venom phosphodiesterase (3′-exonuclease) and nuclease P1 (endonuclease) than those containing 2′,4′-BNA/LNA.[8] Also, the incorporation of continuous ENA residues in oligonucleotides rendered phosphodiester linkage more nuclease-resistant than PS-DNA.[9] Here we studied the stability of oligonucleotides composed of ENA residues in rat plasma. Consequently, an ENA/PS-DNA/ENA gapmer was found to be much more stable than a PS-DNA and an 2′,4′-BNA/PS-DNA/2′,4′-BNA gapmer, and was virtually intact after 4 h of incubation in rat plasma. ENA/DNA/ENA gapmers, which have no PS linkage, were previously used to design oatp AONs.[10] We demonstrated that increase in the number of ENA residues in these oatp AONs enhanced their inhibitory activity by injection into *Xenopus* oocytes.[10] However, an ENA/DNA/ENA gapmer was less stable than an ENA/PS-DNA/ENA gapmer in rat plasma (data not shown). Although ENA/DNA/ENA gapmers would be sufficiently stable inside cells or oocytes, introduction of PS linkages in the DNA stretch would be suitable for *in vivo* systemic administration of the gapmers.

The three factors for finding specific and effective sequences of AONs are considered to be as follows: (a) antisense sequences with high accessibility to targeted mRNA; (b) antisense sequences with less similarity to non-target genes, especially sequences of DNA stretch of gapmers concerned with RNase H-mediated cleavage; and (c) relative high expression of the target gene compared with non-target genes with similar sequence. In other words, if there is more abundant expression of non-target mRNA than target mRNA, the AONs would not be able to inhibit the target mRNA because AONs are subject to being trapped by non-target mRNA.

Accessibility to target mRNA is one of the factors that determine the effects of AONs, as described in the previous paragraph. If we selected antisense sequences in the stem regions of target mRNA, AONs with those sequences would not hydridize mRNA.[26] The antisense sequence used in this study was the initiation site of VEGF mRNA and seemed to have a secondary structure that was hard to access. Using ENA/PS-DNA/ENA gapmers as AONs, we successfully inhibited the expression of human VEGF mRNA in A549 cells using 1 μM of ENA-AS1 (Figure 4B). On the other hand, PS-AS actually exhibited no antisense effect on 1 μM (Figure 4B), while 10 μM of the same PS-DNA did in Nomura′s experiment using human umbilical vascular endothelial cells (HUVEC).[17] The high binding ability of ENA-AS1 and ENA-AS1hp, which includes 15 ENA residues, was quite effective in targeting this site. ENA-AS2, with the same sequence but with 10 fewer ENA residues, was not so effective. In ENA-AS1hp and -AS3hp to -AS6hp, the inhibitory activities of VEGF mRNA expression were well correlated with their hybridizing

ability to mRNA (Figure 6A). In this study, we did not know the secondary structure of VEGF mRNA, however, we would be able to predict accessible sequences in mRNA using an array analysis method and predictive computer programs.[26–28]

As for the second factor for choosing effective antisense sequences described above, it would be important to understand the occurrence of inhibitory activities on other genes, if possible. If such inhibitions are based on an antisense mechanism of sequence specificity, it occurred to us that the sequence information of inhibited genes could be used in the redesign of AONs. To find genes with similar sequences to ENA-AS1 in the GenBank database, two alignment algorithms of BLAST and CLUSTAL W were applied in this study. In the search of short sequences such as 22-mer, BLAST, which is generally used for gene analysis, provides a quick and powerful similarity search for sequences with nearly exact matches. However, it has a drawback in finding sequences inserted by a number of short unmatched gaps. To complement BLAST, a search using the CLUSTAL W alignment algorithm was also performed. The search by CLUSTAL W was highly sensitive to the detection of sequences having several discontinuous gaps and mismatches. The combination of both algorithms would be suitable to find similar sequences for AONs in a gene database. Thus, from the GenBank database, 22 human sequences were hit as genes with the possibility of hybridizing ENA-AS1 (Table 1).

In the cell culture assay, we found that the mRNA expression of three genes, CDC25B, STAT1, and NR2C1, of the 22 genes was inhibited by ENA-AS1 (Figure 5D). The genes having matches with the DNA stretch of gapmer AONs would have the possibility of their expression being inhibited because of the RNase H-mediated cleavage. This is in fact the case for CDC25B and STAT1 (Figure 6B, C). In NR2C1 (Figure 6D), there are exceptional structures of an internal loop of 4 nucleotides, a mismatch and a wobble base-pairing when forming a duplex with ENA-AS1. Lima and Crooke[29] demonstrated that even the introduction of a mismatch at the center of the 5 DNA nucleotide stretch was recognized by RNase H and resulted in only a slight reduction in the cleavage in 2′-*O*-methyl nucleosides/DNA gapmers. There is the possibility that NR2C1 mRNA would be inhibited by an RNase H-mediated mechanism. There are no reports yet concerning internal loop structure and RNase H cleavage as far as we know. Further experiments would be necessary to investigate whether a heteroduplex with a loop structure adjacent to the DNA stretch could be recognized by RNase H. As well, the mRNA expression of the three genes was observed to be relatively high in comparison with that of VEGF in semi-quantitative RT-PCR experiments (Table 1), reflecting the third factor for finding specific and effective AONs we presented. When using ENA-AS1hp and -AS3hp to -AS6hp, the inhibitory activities on CDC25B, STAT1, and NR2C1 were changed in direct proportion to the number of matched sequences, demonstrating that ENA AONs

act in a sequence-specific manner. VEGF-specific inhibition was improved in ENA-AS3hp. These results indicate the possibility that we could decrease the undesirable non-target effects as much as possible by a series of processes of similarity search, RT-PCR experiments and redesign of AONs, to eventually optimize specific and effective AONs for targeted mRNA.

Microarray technology has been used to examine off-target effects.[30–33] In Cho's study,[30] an AON was targeted to only one mRNA in a sequence-specific manner and some off-target activities were considered to represent genuine physiological effects of specific target knockdown in certain biochemical pathways/cascades. However, careful microarray analysis by the same authors showed expression change of off-target genes that were not related to the target gene.[31] Fisher et al.[32] reported that an AON changed the expression level of non-target genes in addition to decrease of target mRNA levels. Also, Bilanges and Stokoe[33] demonstrated that a comparison of the specificity of down-regulation by AONs and siRNAs showed their non-specific alternation of many gene expressions, while they utilized control AONs and siRNAs to avoid misinterpretation of the effect of the phenotype of cells. Considering the findings from these studies and our results, we should take care in the design to ensure obtaining effective AONs and should conduct experiments with appropriate control AONs.

In conclusion, ENA/PS-DNA/ENA gapmers targeted to VEGF mRNA exhibited high inhibition activity and acted in a sequence-specific manner. Besides, the extreme stability of ENA in plasma would make them ideal AONs for *in vivo* systemic use. Further antisense studies of ENA including *in vivo* use will be reported elsewhere.

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