

Potent Growth Inhibition of Leukemic Cells by Novel Ribbon-type Antisense Oligonucleotides to *c-myb1**

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We studied the effects of antisense oligonucleotides (AS oligos) with a novel structure. The AS oligos were covalently closed to avoid exonuclease activities by enzymatic ligation of two identical molecules. The AS oligos of a ribbon type (RiAS oligos) consist of two loops containing multiple antisense sequences and a stem connecting the two loops. Three antisense sequences targeting different binding sites were placed in a loop that was designed to form a minimal secondary structure by itself. RiAS oligos were found to be stable because they largely preserved their structural integrity after 24 h incubation in the presence of either exonuclease III or serums. When a human promyelocytic cell line, HL-60, was treated with RiAS oligos to *c-myb*, *c-myb* expression was effectively ablated. Cell growth was inhibited by >90% determined by both the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay and [³H]thymidine incorporation. Further, when the leukemic cell line K562 was treated with *c-myb* RiAS oligos, colony formation on soft agarose was reduced by 92 ± 2%. These results suggest that RiAS oligos may be employed for developing molecular antisense drugs as well as for the functional study of a gene.

Antisense oligonucleotides (AS oligos)¹ have been valuable in the functional study of gene products by reducing expression of genes in a sequence specific manner (1–3). Intense efforts have also been made to develop molecular anticancer agents by eliminating aberrant expression of genes involved in tumor initiation and progression (4–10). Synthetic AS oligos have been widely utilized for the ease of design and synthesis as well as for potential specificity to genes that cause disease. Inhibition of gene expression is believed to be achieved through either RNaseH activity following formation of DNA-mRNA duplex or steric hindrance of binding of a ribosomal complex (11). There has also been an effort to inhibit gene expression by employing oligos forming triple helix or duplex oligo decoy aimed at or

competing with the promoter region of genomic DNA (12). Efficacy of AS oligos has been validated in animal models as well as several recent clinical studies (13–16). It is also encouraging that the first antisense drug was recently approved for cytomegalovirus retinitis in the United States and Europe.

Expectation for AS oligos has, however, frequently met with disappointment because results have not always been unambiguous. Salient problems for AS oligos have been inaccessibility to a target site (17, 18), instability to nucleases (19–21), poor cellular uptake, and nonsequence specific activities. Stability of AS oligos has been improved to a certain extent by either using modified oligos or adopting a structure resistant to exonucleases (19–21). Modified oligos such as phosphorothioate and methylphosphonate oligos were utilized to augment stability against nucleases. However, each of the modified oligonucleotides exhibited problems of its own, *i.e.* lack of sequence specificity and insensitivity to RNaseH. Further, there is apprehension for recycling of hydrolyzed modified nucleotides. In another effort to enhance stability of AS oligos, a stem-loop structure was reported to be effective in targeting mRNA of Syk kinase (18).

Protooncogene *c-myb* plays an important role in proliferation and differentiation of hematopoietic cells. Hematopoietic cells exhibit differential expression of *c-myb* and show little expression of the gene when differentiated to term (5, 6). *c-myb* has often been found to be overexpressed in leukemic cells. Blockage of *c-myb* expression by AS oligos inhibited growth of a promyelocytic cancer cell line HL-60 and a chronic myelogenous leukemia cell line K562 (2–4, 7). However, the AS oligos used in the experiments were reported to be partially effective, inhibiting tumor cell growth by about 50–60%. *c-myb* AS oligos employed for the experiments were either phosphodiester oligos or phosphorothioate capped oligos (2, 3). These AS oligos were not truly stable, especially the phosphodiester oligos, possibly explaining the partial antisense effect.

In the present study, we devised AS oligos with enhanced stability and antisense effect without problems associated with modified AS oligos. A combination of three antisense sequences to *c-myb* was adopted to construct a large antisense molecule with a loop and stem. Two of the AS oligos were enzymatically joined to form ribbon-type antisense (RiAS) oligos. *c-myb* RiAS oligos were studied for stability and for growth inhibition of leukemic cell lines.

MATERIALS AND METHODS

Cell Lines and Tissue Culture—Leukemic cell lines, HL-60 (promyelocyte leukemic cell line) and K562 (chronic myelogenous leukemic cell line), were obtained from American Type Culture Collection and cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT) and penicillin (100 unit/ml)/streptomycin (100 µg/ml). Cells were maintained in a 5% CO₂

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¹ The abbreviations used are: AS, antisense; RiAS, ribbon-type antisense; oligos, oligonucleotides; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; RT, reverse transcriptase; PCR, polymerase chain reaction; FBS, fetal bovine serum; SC, scrambled; PIPES, 1,4-piperazinediethanesulfonic acid.

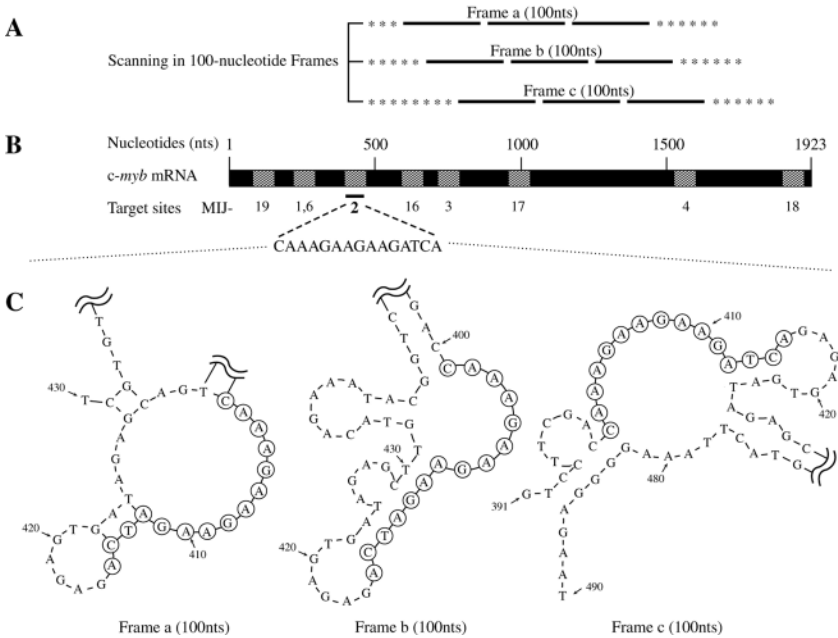
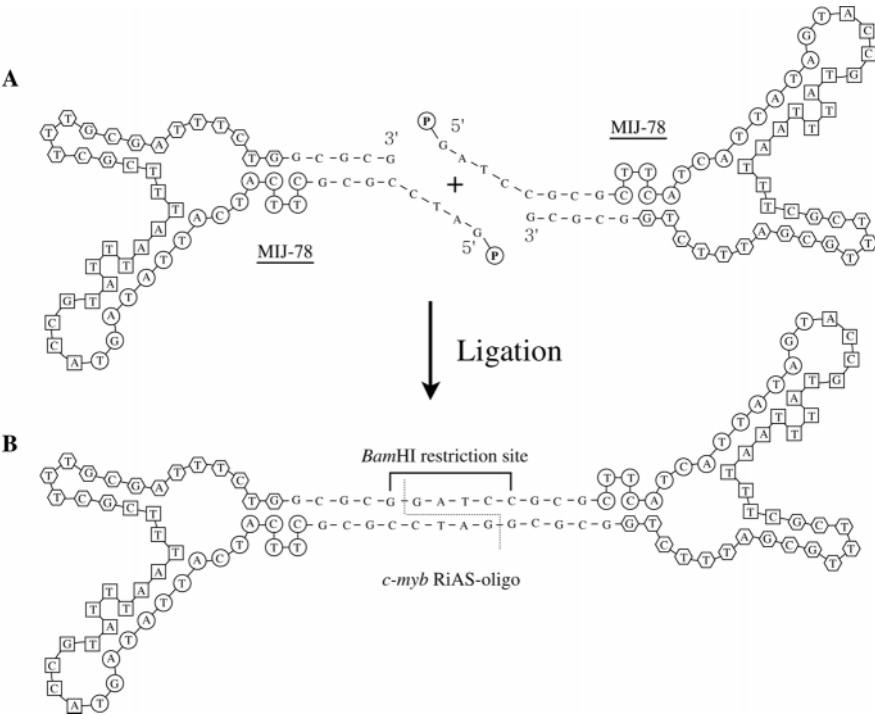


FIG. 1. Selection of target sequences for AS oligos. The cDNA sequence of *c-myb* mRNA is represented by thick horizontal solid bars (22). The entire *c-myb* mRNA sequence was scanned three times with an RNA secondary structure prediction program to find a sequence with a minimal secondary structure. Each scanning was performed 30 bases apart in a 100-nucleotide frame (denoted as *Frames a, b, and c* in panel A) sequentially. Putative secondary structures in the area of *c-myb* mRNA containing the target MIJ-2 sequence are shown in the three frames of 100 nucleotides each (*Frames a, b, and c* in panel C). Thus, a given sequence was scanned for secondary structures in three different frames. The most open sequence 5'-CAAAGAAGAAGATCA-3' (401–415, denoted in circle) in three scanings was chosen as a target sequence (MIJ-2). Other target sites selected similarly are shown (as hatched lines) along the long solid horizontal line representing *c-myb* mRNA (in panel B).

FIG. 2. Scheme for the construction of *c-myb* RiAS oligos. RiAS oligos consist of two molecules of MIJ-78, with two loops and a stem. A, MIJ-78 consists of a loop with three different antisense sequences and a stem. Different antisense sequences are denoted by distinct shapes: circles, squares, and hexagons. MIJ-78 is phosphorylated at the 5' end and harbors complementary sequences at both the 5' and 3' ends with a single-stranded sequence of GATC at the 5' extreme end. B, RiAS oligo molecule covalently ligated from two MIJ-78 molecules. A RiAS oligo, therefore, contains two loops with six antisense sequences and a stem region. The stem has a *Bam*HI restriction site.



incubator at 37 °C. Routine cell culture practices were strictly followed to maintain proper cell density and to avoid culturing cells more than five generations after thawing stock vials. Culture media were exchanged a day before treating with AS oligos.

Selection of Target Sites for AS Oligos—Target sites for AS oligos were selected for eight different regions of *c-myb* mRNA. The method for rational target site search was described previously by us (18). Simulation of secondary structures was carried out with the DNAsis program (Hitach Software, San Bruno, CA). The entire *c-myb* sequence was scanned sequentially for secondary structure formation in contiguous frames of 100 bases. Frames for the simulation of secondary

structures were then staggered down by 30 bases, resulting in an overlap of 60 bases on the 5' side of the next set of downstream frames. Therefore, any given sequence was scanned for its potential secondary structure in three different frames. Eight sequences with a minimal secondary structure (duplex formation) were selected (Fig. 1). Among the eight selected target sites, three sites were further chosen as antisense sequences to the selected site form a minimal intramolecular secondary structure when placed in a single molecule. Three antisense sequences in the AS oligos are as follows: 5'-GCTTTGCGATTCTG-3' (*c-myb* site; 613–627), 5'-CTTCATCATTATAGT-3' (961–977), and 5'-ACCGTATTTAATTTC-3' (1545–1559).

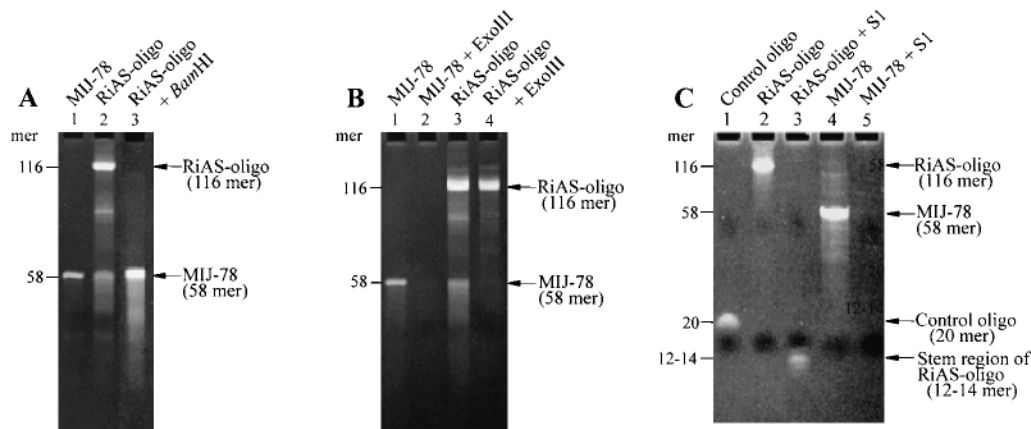


FIG. 3. **Electrophoretic mobility patterns of RiAS oligos.** A, oligos were analyzed on a 15% denaturing polyacrylamide gel. Lane 1, 58-mer MIJ-78 molecules; lane 2, 116-mer RiAS oligos were formed by ligation of two MIJ-78 molecules; lane 3, RiAS oligos were restriction digested by *Bam*HI. RiAS oligos were shown as a major retarded band at the upper region (116-mer) when compared with MIJ-78 (58-mer). B, stability test of MIJ-78 and RiAS oligos upon treatment with exonuclease III. Lanes 1 and 3, samples were not treated with exonuclease III; lanes 2 and 4, samples were treated with exonuclease III. C, digestion of open (single-stranded) regions on MIJ-78 and RiAS oligos upon treatment with S1 nuclease. Lane 1, control oligos (20-mer); lanes 2 and 4, samples were without S1 nuclease; lanes 3 and 5, samples were treated with S1 nuclease.

Construction of RiAS oligos—Oligos were either made by us or purchased from the Life Technologies, Inc. *c-myc* AS oligos (MIJ-78) and control scrambled oligos were phosphorylated at the 5' end. Sequences of the 58-mer MIJ-78 and scrambled oligos are 5'-(p)GATCCGCGCTT-CATCATTATAGTACCGTATTTAATTTTCGCTTTGCGATTCTGGCG-CG-3' and 5'-(p)GATCCGCGCTTACACTATTGATGCACTTATATAT-GTTTCTGCTATGTGTCTGCGCG-3', respectively. Both MIJ-78 and scrambled (SC) oligos were anticipated to form a stem-loop structure. The stem is formed by complementary sequences at both ends of each oligo. The 5' terminus of the stem has 4 bases of a single-stranded sequence of 5'-GATC-3'. Two MIJ-78 molecules were joined by the complementary 4 base sequences at both 5' ends. MIJ-78 molecules were mixed and heated to 85 °C for 2 min followed by gradual cooling to the room temperature. One unit of T4 DNA ligase was added and incubated for 24 h at 16 °C to generate a covalently ligated molecule with diad symmetry. RiAS oligos consist of two loops and one stem connecting the two loops. Each loop contains three different antisense sequences. A combination of three antisense sequences with the least possible secondary structure was chosen, because the AS oligos were expected to bind to target sites more readily. RiAS oligos were electrophoresed on a 15% denaturing polyacrylamide gel and examined for their resistance to exonuclease III as well as for gel retardation. Two of the linear scrambled oligos were also covalently combined to form a ribbon type control oligo, which was denoted SC oligo for convenience.

Characterization and Stability Test of RiAS Oligos—Thermal denaturation of AS oligos was performed in a solution of 100 mM NaCl, 10 mM MgCl₂ and 10 mM sodium PIPES (Sigma). AS oligos of 0.2 μM were heated to 95 °C and allowed to cool slowly to room temperature prior to denaturation experiments. Melting studies were carried out in 1-cm-pathlength quartz cells on a Varian Cary 5G UV-visible NIR spectrophotometer (Varian, Australia) equipped with a thermo-programmer. Absorbance at 260 nm was monitored at every 2 °C increment, whereas temperature was raised from 10 to 96 °C.

To test stability of AS oligos, 1 μg each of the nonligated phosphodiester oligos (MIJ-78) and *c-myc* RiAS oligos were incubated with either human serum, FBS, calf serum, exonuclease III, or S1 nuclease. All serums were used without heat inactivation to preserve DNase activity. Each serum was added to oligos to 50% in a 100-μl reaction volume and incubated for 24 h at 37 °C. Exonuclease III (Takara, Otsu, Japan) at 160 units/μg oligos was added to oligos and incubated for 2 h at 37 °C. S1 nuclease (Takara) at 10 units/μg oligos was added to oligos and incubated for 30 min at 25 °C. The oligos were then extracted with phenol and chloroform and were examined on a 15% denaturing polyacrylamide gel.

Transfection of RiAS Oligos Combined with Cationic Liposomes—Cells (HL-60 and K562) were fed with fresh culture medium without antibiotics (RPMI 1640 containing 10% FBS) 1 day prior to adding oligos and washed twice with Opti-MEM (Life Technologies, Inc.) prior to an experiment. Cell density was adjusted to 5×10^5 cells/ml, and the cell suspension was aliquoted in 100 μl each in a 48-well plate (Falcon, Lincoln Park, NJ). Cells were transfected with 0.2 μg of AS oligos combined with 0.8 μg of Lipofectin™ (Life Technologies, Inc.). Lipofec-

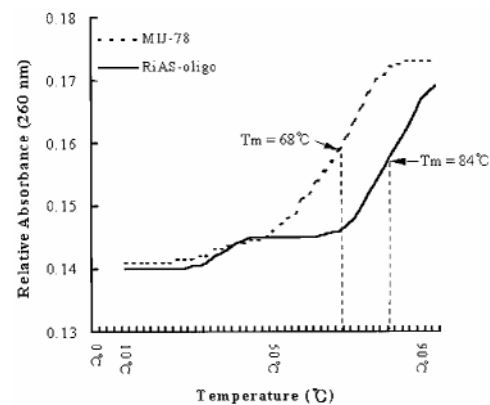


FIG. 4. **Thermal melting profiles for AS oligos.** Absorbance was monitored at every 2 °C while temperature was raised from 10 to 96 °C. The solid line denotes the melting curve of RiAS oligos, and the dotted line represents that of MIJ-78 (the precursor antisense molecules of RiAS oligos).

tin was added to a tube containing 30 μl of Opti-MEM and incubated for 40 min at room temperature. Oligos (0.2 μg of oligos in 30 μl of Opti-MEM) were added to a tube containing liposomes and mixed to form the oligo-liposome complex for 15 min. 60 μl of oligo-liposome complex was added to cells. Cells treated with oligos were incubated at 37 °C and 5% CO₂ for 5 h. Cells were then added with 100 μl of Opti-MEM with 10% FBS and incubated further at 37 °C for 24 h before assay.

Isolation of RNA and RT-PCR—Total RNA was isolated with Tri-pure™ Isolation Reagent (Roche Molecular Biochemicals) according to the procedures recommended by the manufacturer. Briefly, cells harvested were added with 0.4 ml of Tripure reagent, 10 μg of glycogen, and 80 μl of chloroform to obtain total RNA. RT-PCR was performed in a single reaction tube with Access™ RT-PCR kit (Promega, Madison, WI). In a PCR tube were added RNA, PCR primers, avian myeloblast virus reverse transcriptase (5 units/μl), *Tfl* DNA polymerase (5 units/μl), dNTP (10 mM, 1 μl), and MgSO₄ (25 mM, 2.5 μl). Synthesis of the first strand cDNA was done at 48 °C for 45 min in a DNA thermal cycler (Hybaid, Teddington, United Kingdom). Twenty cycles of PCR amplification were subsequently carried out with the recommended conditions by the manufacturer. Amplified PCR product was confirmed on a 1% agarose gel, and quantification was done with a gel documentation program (Bio-Rad).

Southern Hybridization of RT-PCR Fragments—RT-PCR products were electrophoresed on a 1% agarose gel. DNA was transferred onto a nylon membrane (New England Biolabs) for 4 h in 0.4 M NaOH. The membrane was hybridized with a 30-mer internal primer (5'-TGTA-ACGCTACAGGGT ATGGAACATGACTG-3') labeled with the ECL 3' end oligo-labeling and detection system (Amersham Pharmacia Bio-

tech). Hybridization was carried out at 62 °C for 60 min in 6 ml of buffer containing 5× SSC, 0.02% SDS. The membrane was washed twice in 5× SSC containing 0.1% SDS and washed again twice with 1× SSC containing 0.1% SDS at 58 °C for 15 min. The membrane was blocked with a blocking solution and then treated with anti-fluorescein horseradish peroxidase-conjugated antibody for 30 min before autoradiography.

Western Blot Analysis—Total cellular proteins prepared from cells were separated on a 7.5% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. After blocking with phosphate-buffered saline containing 3% nonfat milk and 0.05% Tween 20, the membrane was incubated with a mouse monoclonal IgG2a κ antibody specific for mouse and human Myb (Upstate Biotechnology, Lake Placid, NY) at a concentration of 1 μ g/ml. Horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) was used in the secondary incubation, followed by detection of reactive bands by chemiluminescence (Amersham Pharmacia Biotech).

Inhibition Test of Leukemic Cell Growth—Growth inhibition of leukemic cells was measured with three methods, MTT assay, [³H]thymidine incorporation, and colony formation on soft agarose. For the MTT assay, HL-60 cells were washed twice with Opti-MEM and aliquoted in a 96-well plate (5 × 10³ cells/well) in a 50- μ l volume. Cells were treated with preformed complex of oligos (0.2 μ g/15 μ l) and Lipofectin (0.6 μ g/15 μ l) for 5 h and cultured for 5 days. Cells were then harvested in a 100- μ l volume and added with 20 μ l (100 μ g) of the MTT reagent (5 mg/ml in phosphate-buffered saline; Sigma), followed by 4 h incubation at 37 °C. An equal volume of isopropanol containing 0.1 N HCl was added to the cells and incubated for one more hour at room temperature. Absorbance was measured at 570 nm with an enzyme-linked immunosorbent assay reader to score the amount of cells that survived. The percentage of growth inhibition was calculated by the following formula: percentage of growth inhibition = [1 - (absorbance of an experimental well/absorbance of a sham-treated control well)] × 100.

For [³H]thymidine incorporation, HL-60 cells were treated with AS oligos as described above. Cells were added with 0.5 μ Ci of [³H]thymidine (2.0 Ci/mmol; Amersham Pharmacia Biotech) and incubated for 16 h in triplicate. Cells were then harvested on a glass microfiber filter (GF/C Whatman, Maidstone, Kent, UK). The filter was washed with cold phosphate-buffered saline, and then 5% trichloroacetic acid and absolute ethanol. [³H]Thymidine incorporation was measured by a liquid scintillation counter in a mixture solution containing toluene, Triton X-100, 2,5-diphenyloxazole, and 1,4-bis[2-(5-phenyloxazoly)]benzene (percentage of growth inhibition = [1 - (cpm of an experimental well/cpm of a sham-treated control well)] × 100).

Colony formation on soft agarose was determined as follows. An equal volume of 0.8% low melting agarose (in double distilled H₂O) and 2× RPMI 1640 containing 20% FBS were added to transfected cells. The mixture was then placed in a 6-well plate to solidify. The plate was cooled to 4 °C for 5 min and incubated for 15 days. Colonies containing more than 20 cells were scored as positive.

Statistical Analysis—All determinations were made in triplicate, and the results were expressed as the means \pm S.D. Statistical significance was determined by using the Student's *t* test. A *p* value of 0.05 or less was considered to be of statistical significance.

RESULTS

Construction of Stable RiAS Oligos—Oligos modified with phosphorothioate or methylphosphonate exhibit improved stability, but the gain in stability is only partial and bears the potential hazard of misincorporation of hydrolyzed modified nucleotides during DNA replication or repair. We previously reported stem-loop oligos combined with cationic liposomes with partial improvement of stability (18). Because stability of AS oligos still remains a major concern, our goal was to develop improved AS oligos with better stability.

It has been reported that exonuclease activity constitutes most of the nuclease activity in the cytoplasm and serum (19). To avoid exonuclease activity, two identical AS oligos of stem-loop structure (MIJ-78) were enzymatically ligated to form a ribbon-typed molecule termed RiAS oligo. The RiAS oligos (116-mer) consist of two loops containing antisense sequences and one stem connecting the two loops. The stem was designed to harbor a restriction site for *Bam*HI in the middle junction to help confirm covalent ligation of two antisense molecules (Fig. 2). Three antisense sequences were placed in tandem to in-

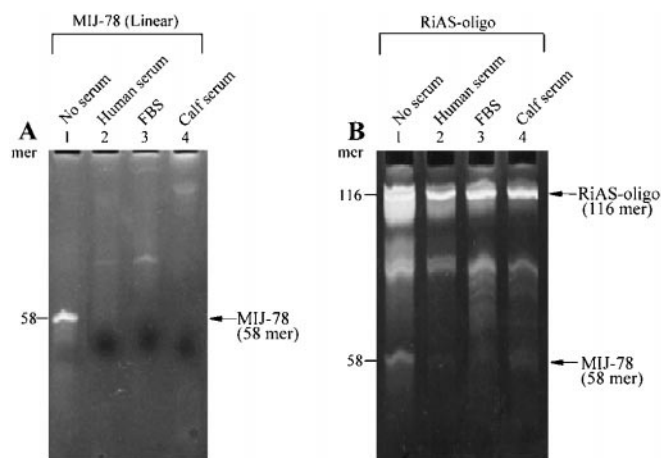


FIG. 5. Degradation patterns of linear and RiAS oligos in the presence of serum. A, stability test of MIJ-78 molecules. Lane 1, oligos were not treated with serum (negative control); lanes 2–4, treated with 50% human serum, FBS and calf serum for 24 h respectively. B, stability test of RiAS oligos. Lane 1, AS oligos were not treated with serum (negative control); lanes 2–4, treated with 50% human serum, FBS and calf serum for 24 h, respectively.

crease the length of the loop. Consequently, two sets of three different antisense sequences (total of six antisense sequences) were placed in a RiAS oligo. This enlarged length (45 nucleotides) of the loop in RiAS oligos was necessary to have effective antisense activity as a loop with less than 30 nucleotides was not active (result not shown). RiAS oligos were found to be slowed markedly compared with MIJ-78 (linear precursor) on a denaturing polyacrylamide gel. *Bam*HI was able to cut RiAS oligos, generating two 58-mer oligos (Fig. 3A). RiAS oligos were, as expected, resistant to exonuclease III and were observed as a major band (116-mer) on gel electrophoresis. In contrast to RiAS oligos, MIJ-78 was completely degraded after 2 h of incubation with exonuclease III (Fig. 3B). These results demonstrate the covalent closure of RiAS oligos. We further examined molecular characteristics of RiAS oligos employing two different approaches to confirm the stem loop structure of RiAS oligos. The oligos were incubated with S1 nuclease, which digests a single-stranded loop region in a DNA molecule. The stem of RiAS oligos was the only region found to be protected from S1 nuclease, shown as a DNA band of 12–14 bases on a denaturing gel (Fig. 3C). The presence of a stem in RiAS oligos was examined again by measuring the melting temperature. When absorbance at 260 nm was monitored for RiAS oligos while temperature was raised, a typical chromatic change was detected around 84 °C, indicating the denaturation of a duplexed stem region. When the melting temperature of RiAS oligos was compared with that of MIJ-78, RiAS oligos showed a higher melting temperature than MIJ-78, 84 °C versus 68 °C (Fig. 4). These results support the concept that RiAS oligos are indeed a ribbon-shaped molecule with a stem and two loops.

RiAS oligos were tested for their stability by incubation with serums that were not heat-inactivated to maintain nuclease activity. Oligos were treated with 50% human serum, FBS, or calf serum for 24 h. Linear 58-mers were completely hydrolyzed after 24 h of incubation in the presence of each serum (Fig. 5A). RiAS oligos, however, remained largely intact after 24 h of incubation with these different serums, exhibiting significantly improved stability compared with the linear AS oligos (Fig. 5B).

Specific Reduction of *c-myc* mRNA and Myb Proteins by *c-myc* RiAS Oligos—We next examined whether RiAS oligos function well in eliminating target mRNA in a sequence-specific manner. HL-60 cells were transfected with *c-myc* RiAS

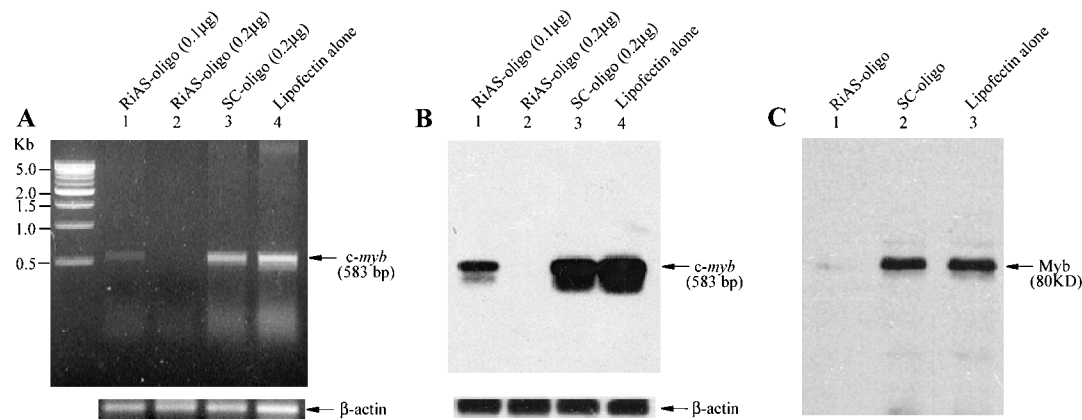


FIG. 6. **Effect of *c-myb* RiAS oligos on *c-myb* mRNA and Myb protein expression in HL-60.** A, RT-PCR was performed with total RNA using two *c-myb* primers. Amplified PCR fragments were run on a 1% agarose gel and visualized with ethidium bromide staining. Cells were treated with RiAS oligos (0.1 μ g, 14 nM) + Lipofectin (0.8 μ g) (lane 1), RiAS oligos (0.2 μ g, 28 nM) + Lipofectin (0.8 μ g) (lane 2), SC oligos (0.2 μ g) + Lipofectin (0.8 μ g) (lane 3), and Lipofectin (0.8 μ g) alone (lane 4). B, PCR products were analyzed by Southern hybridization, and hybridized bands were visualized with the ECL 3' oligo labeling and detection system. The upper panel shows the hybridized RT-PCR bands of *c-myb*, and the lower panel shows the hybridized RT-PCR bands of β -actin. C, Western blot analysis of Myb protein levels of HL-60 cells with treatment of RiAS oligos. Analysis was performed at day 2 before the onset of cell death caused by AS oligo treatment.

oligos and SC oligos, as well as Lipofectin alone. *c-myb* RiAS oligos were delivered into cells after forming a complex with Lipofectin. The RiAS oligos (0.1 or 0.2 μ g) were combined with 0.8 μ g of Lipofectin for transfection into HL-60 cells. Total RNA was isolated from transfected cells and *c-myb* message was amplified by RT-PCR. RiAS oligos at 0.2 μ g (28 nM) were able to completely ablate *c-myb* mRNA. In addition, 0.1 μ g (14 nM) of *c-myb* RiAS oligos decreased about 70% of *c-myb* mRNA (Fig. 6A). In contrast, SC oligos exhibited only a mild reduction of *c-myb* mRNA when compared with Lipofectin treatment alone. However, β -actin expression shown in the bottom panel was not affected by the treatment of *c-myb* RiAS oligos. The antisense effect of *c-myb* RiAS oligos was examined again by Southern blotting of the PCR product. *c-myb* message amplified by RT-PCR was detected with a labeled internal hybridization oligos (30-mer) (Fig. 6B). The results confirmed that the amplified message was indeed *c-myb*-derived, with the total elimination of the message by treatment with 0.2 μ g (28 nM) of *c-myb* RiAS oligos. Myb proteins were also shown to be largely eliminated by *c-myb* RiAS oligos. In contrast, Myb proteins were only slightly reduced when cells were treated with the control SC oligos (Fig. 6C). These results indicate that RiAS oligos are effective in ablating target mRNA even when used in lesser doses.

Effective Growth Inhibition of Leukemic Cells by *c-myb* RiAS Oligos—*c-myb* plays an important role in the proliferation of leukocytes. AS oligos to *c-myb* have been reported to block leukemic cell growth preferentially (2, 3). *c-myb* RiAS oligos were tested for their ability to inhibit leukemic cell growth. A leukemic cell line, HL-60, was treated with 0.2 μ g of *c-myb* RiAS oligos or SC oligos combined with 0.6 μ g of Lipofectin or with Lipofectin alone. Cells were incubated for 5 days and subjected to an MTT assay to determine the index of cell growth. Cell growth was observed to be inhibited by $91 \pm 4\%$ with *c-myb* RiAS oligos ($p < 0.001$) (Fig. 7A). In contrast, SC oligos and Lipofectin alone did not significantly inhibit cell growth when compared with that of the sham-treated control. These results indicate that *c-myb* RiAS oligos are an effective antisense agent for inhibition of leukemic cell growth.

Growth inhibition of leukemic cells was also measured by [3 H]thymidine incorporation. *c-myb* RiAS oligos (0.2 μ g) inhibited cell growth by $93 \pm 2\%$ ($p < 0.001$) compared with the sham-treated control, whereas SC oligos and the liposome control did not significantly inhibit cell growth (Fig. 7B). On a microscopic observation, after treatment with *c-myb* RiAS oli-

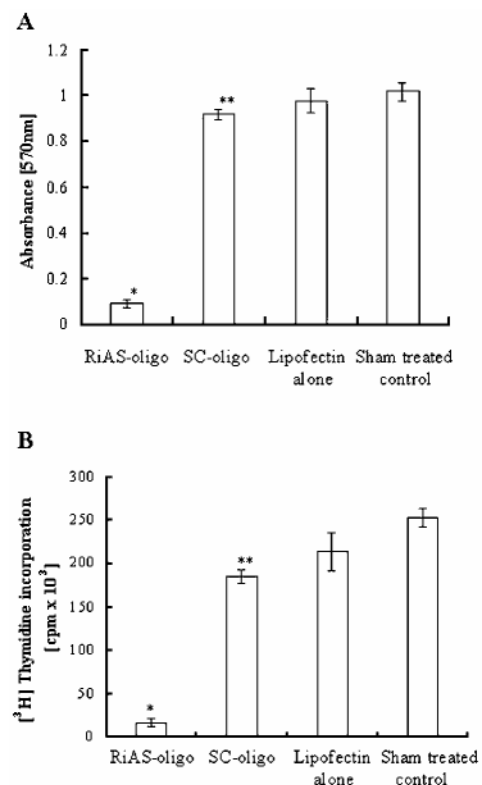


FIG. 7. **Effect of *c-myb* RiAS oligos on proliferation of HL-60 cells.** HL-60 cells were treated with complexes containing 0.2 μ g of oligos and 0.6 μ g of Lipofectin. Each bar is shown for growth inhibition of HL-60 cells. SC oligos, Lipofectin alone, and sham controls were treated in the same fashion. The results show growth inhibition by *c-myb* RiAS oligos examined with the MTT assay (A) and [3 H]thymidine incorporation (B). Each bar value represents the mean \pm S.D. of three experiments. *, $p < 0.001$; **, $p < 0.05$ versus sham-treated control.

gos, HL-60 cell growth was markedly inhibited when compared with cells treated with SC oligos and Lipofectin alone (Fig. 8).

Inhibition of Colony Formation of Leukemic Cells on Soft Agarose—Inhibition of tumor cell growth was also examined for colony formation on soft agarose. K562 was employed for colony formation on soft agarose because the cells form colonies readily and distinctively. Cells transfected with *c-myb* RiAS oligos were seeded in 0.4% agarose and incubated for 15 days before scoring for colonies formed. *c-myb* RiAS oligos were able

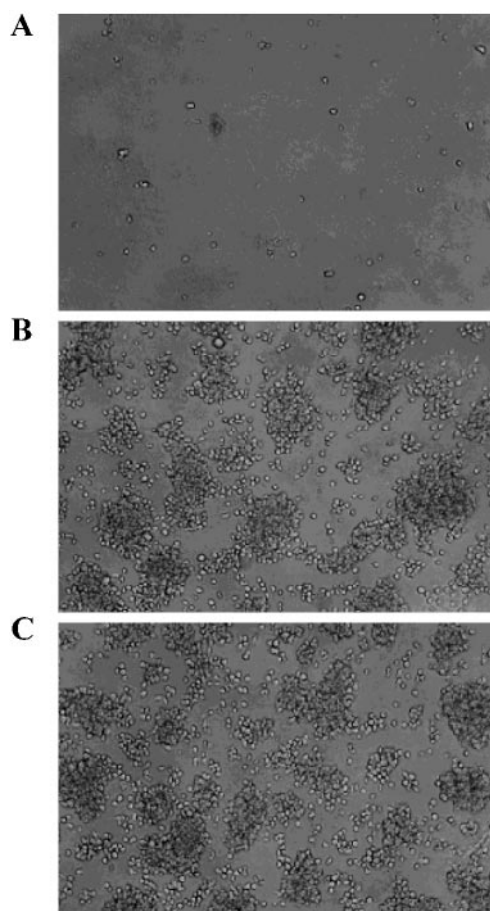


FIG. 8. **Photomicrograph for inhibition on proliferation of HL-60 cells with RiAS oligo.** HL-60 cells were treated with complexes of oligos (0.2 μ g) and Lipofectin (0.6 μ g) and incubated for 5 days. Each photomicrograph represents the effect of growth inhibition after treatment with RiAS oligos (A), SC oligos (B), and Lipofectin alone (C). Original magnification, 100 \times .

to reduce the number of colonies formed by $92 \pm 2\%$ ($p < 0.001$) when compared with sham-treated controls (Table I). In contrast, SC oligos and Lipofectin alone failed to demonstrate any significant reduction of colonies.

DISCUSSION

In the present study, we devised novel RiAS oligos to improve stability against nucleases. RiAS oligos contain two sets of three antisense sequences without an open end, allowing multiple targeting of a target mRNA or targeting more than one mRNA. RiAS oligos were observed to be exceptionally stable in the presence of serum. *c-myb* RiAS oligos were also noted to be effective in ablating *c-myb* mRNA and in blocking leukemic cell growth.

In many antisense studies, a region encompassing the translational start site has been adopted for a target site (2, 23). However, a quartet of G residues is found in the 3' side of the translational start codon of *c-myb* mRNA. Because there has been some controversy over the nonspecific effect of these G residues (24), this region was avoided as a target site. To date, antisense to *c-myb* have been studied in phosphodiester or phosphorothioate oligo forms as well as in expressed antisense molecules (25). That RiAS oligos contain a covalently closed molecule with loops is supported by both resistance of the whole molecule to exonuclease III and resistance of the stem region to S1 nuclease. The data from the S1 nuclease assay also showed the presence of a stem in RiAS oligos, and the result was reinforced by the detection of a typical melting tempera-

TABLE I
Effects of *c-myb* RiAS-oligos on colony formation of K562 cells

K562 cells were treated with different oligos for 24 h, and the cells were seeded on a 0.4% LMP-agarose gel. Transfection was performed with the treatment of complexes containing 0.8 μ g of Lipofectin and 0.2 μ g of RiAS or SC oligos and incubated for 24 h. Colonies arising in 6-well plates containing cells treated with *c-myb* RiAS oligos and SC oligos were enumerated. Colonies in controls (Lipofectin alone or sham-treated) are shown in the bottom two lines.

Oligos	Size	Number of colonies	Colonies formed ^a
	<i>mer</i>		%
RiAS oligos	116	$7.6 \pm 1.5^{b,c}$	7.8
SC oligos	116	90.5 ± 2.1	92.1
Lipofectin TM alone		91.3 ± 4.1	92.9
Sham-treated control		98.3 ± 4.0	100.0

^a number of colonies (oligo-treated)

^a $\frac{\text{number of colonies (oligo-treated)}}{\text{number of colonies (sham-treated control)}} \times 100$

^b mean \pm standard deviation of three experiments.

^c $p < 0.001$ versus sham-treated control.

ture curve obtained with RiAS oligos. That the melting temperature of RiAS oligos at 84 $^{\circ}$ C was 16 $^{\circ}$ C higher than that of MIJ-78 (68 $^{\circ}$ C) demonstrates the enhanced thermal stability of RiAS oligos and is in agreement with similar data obtained with nicked or closed duplex molecules (26).

The merits of RiAS oligos other than their exceptional stability include the following: 1) RiAS oligos have a better chance to find a target site because they contain three different antisense sequences, 2) RiAS oligos can bind to multiple targets, either many sites on the same molecule or multiple molecules, and 3) RiAS oligos may be used in a smaller quantity because the molecule is much more stable and can avoid sequence independent side effects. In a RiAS oligo, two AS oligo molecules are joined together in a fashion of diad symmetry, negating the need for a ligation primer. When *c-myb* RiAS oligos were added to HL-60 and K562 cells at a concentration of 0.2 μ g/ml (28 nM), the level of *c-myb* mRNA as well as colony formation was markedly reduced. In other reports, much greater amounts of AS oligos ranging from 20 to 200 μ g/ml have been used to obtain biological effects that might not be entirely sequence-dependent (2, 3, 19, 23, 27, 28).

AS oligos have been modified to enhance stability against nucleases. Two modified oligos that have been frequently used are phosphorothioate and methylphosphonate oligos. These oligos were shown to be more stable than regular phosphodiester oligos but had only a partial gain in stability (29). Furthermore, phosphorothioate oligos have nonspecific activity, and methylphosphonate oligos have low solubility and show poor sensitivity to RNaseH (30). RiAS oligos would be expected to have normal sequence-specificity and susceptibility to RNaseH activity because the oligos bear no modified oligo at the present structure. Additional potential merit of RiAS oligos is that it is unlikely to introduce undesired mutations in the genomic DNA during DNA replication or repair upon recycling of hydrolyzed nucleotides.

We used cationic liposomes to enhance the cellular uptake of RiAS oligos. From the experience of our own and other groups, a meaningful level of AS oligo uptake should be consistently obtainable when carried into cells by liposomes, regardless of the size of AS oligos (31, 32). Therefore, the relatively large size of RiAS oligos should not pose a problem for efficient cellular uptake.

In conclusion, RiAS oligos are found to have a markedly enhanced stability and excellent sequence-specific antisense effect. We have some desirable preliminary data from other RiAS oligos against other gene targets and are also examining synergistic effects of RiAS oligos targeted oncogenes with anti-neoplastic chemical drugs. RiAS oligos require studies on bio-

logical effects, pharmacokinetics, and pharmacodynamics *in vivo*. They may provide a novel therapeutic modality for human disorders in which inhibition of gene expression would be advantageous.

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**Potent Growth Inhibition of Leukemic Cells by Novel Ribbon-type Antisense
Oligonucleotides to c- *myb*1**

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