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 sera. When a human promyelocytic cell line, HL-60, was treated with RiAS oligos to c-myc, c-myc 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-myc 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) 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-myc plays an important role in proliferation and differentiation of hematopoietic cells. Hematopoietic cells exhibit differential expression of c-myc and show little expression of the gene when differentiated to term (5, 6). c-myc has often been found to be overexpressed in leukemic cells. Blockage of c-myc 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-myc 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-myc 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-myc 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 (promyelocytic leukemia cell line) and K562 (chronic myelogenous leukemia 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
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 mRNA sequence was scanned sequentially for secondary structure formation in contiguous 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'-CAAAGAGAGATCA-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).

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 to form a minimal intramolecular secondary structure when placed in a single molecule. Three antisense sequences in the AS oligos are as follows: 5'-GCTTTGCGATTTCTG-3' (c-myb site; 613–627), 5'-CTTCATCATTATAGT-3' (961–977), and 5'-ACCGTATTTAATTTC-3' (1545–1559).
Construction of RiAS oligos—Oligos were either made by us or purchased from the Life Technologies, Inc. c-myb AS oligos (MJ-78) and control scrambled oligos were phosphorylated at the 5' end. Sequences of the 58-mer MJ-78 and scrambled oligos are 5'-p(GATCCGGCTTCTTACACTATTGATGCACTTATATATGTTCGCTATGTTGTCTGCGC-3') and 5'-p(GATCTCCGGCTTACACTATTGATGCACTTATATATGTTCGCTATGTTGTCTGCGC-3'), respectively. Both MJ-78 and scrambled (SC) oligos were anticipated to form a stem-loop structure. The 5' terminus of the stem has 4 bases of a single-stranded oligo. MJ-78 molecules were restriction digested with BamHI. MJ-78 oligos were shown as a major retarded band at the upper region (116-mer) when compared with MJ-78 (58-mer). B, stability test of MJ-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 MJ-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.
In the 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 antibody specific for mouse and human Myb (Upstate Biotechnology, Lake Placid, NY) at a concentration of 1 μg/ml. Horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) was used in the secondary incubation, followed by detection of reactive bands by chemiluminescence (Amer sham 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 × 10^3 cells/well) in a 50-μl volume. Cells were treated with preformed complex of oligos (0.2 μg/15 μl) and Lipofectin (0.6 μg/15 μl) for 5 h and cultured for 5 days. Cells were then harvested in a 100-μl volume and added with 20 μl (100 μg) of the MT reagent (5 mg/ml in phosphate-buffered saline; Sigma), followed by a 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 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 a sham-treated control well)] × 100.

For thymidine incorporation, HL-60 cells were treated with AS oligos as described above. Cells were added with 0.5 μCi of [3H]thymidine (2.0 Ci/mmol; Amer sham Pharmacia Biotech) and incubated for 16 h in triplicate. Cells were then harvested on a glass microfiber filter (GF/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 ± 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 (MJL-78) were enzymatically ligated to form a ribbon-shaped 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 BamHI in the middle junction to help confirm covalent ligation of two antisense molecules (Fig. 2). Three antisense sequences were placed in tandem to increase 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 MJL-78 (linear precursor) on a denaturing polyacrylamide gel. BamHI 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, MJL-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 not observed, indicating the denaturation of a duplexed stem region. When the melting temperature of RiAS oligos was compared with that of MJL-78, RiAS oligos showed a higher melting temperature than MJL-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 sera 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 sera, exhibiting significantly improved stability compared with the linear AS oligos (Fig. 5B).

Specific Reduction of c-myb mRNA and Myb Proteins by c-myb 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-myb RiAS
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 μg) were combined with 0.8 μg 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 μg (28 nM) were able to completely ablate c-myb mRNA. In addition, 0.1 μg (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 μg (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 μg of c-myb RiAS oligos or SC oligos combined with 0.8 μg 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 ± 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 [3H]thymidine incorporation. c-myb RiAS oligos (0.2 μg) inhibited cell growth by 93 ± 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 oligos, 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...
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. $c\text{-}myb$ RiAS oligos were also noted to be effective in ablating $c\text{-}myb$ 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 $3'\text{-}$side of the translational start codon of $c\text{-}myb$ 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 $c\text{-}myb$ 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-
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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