Target site search and effective inhibition of leukaemic cell growth by a covalently closed multiple anti-sense oligonucleotide to c-*myb*

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Systematic secondary structure simulation of a target mRNA sequence is shown to be effective for locating a good anti-sense target site. Multiple selected anti-sense sequences were placed in a single molecule. The anti-sense oligonucleotide (oligo) was covalently closed to avoid exonuclease activities and was designated CMAS (covalently closed multiple anti-sense)-oligo. CMAS-oligo was found to be stable, largely preserving its structural integrity after 24 h of incubation in the presence of either exonuclease III or serum. When human c-*myb* mRNA was targeted by the c-*myb* CMAS-oligo, expression of the gene was completely abolished. Further, tumour cell growth was inhibited by 82 ± 3 % as determined by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay and by 90 ± 1 % by [^{3}H]thymidine incorporation. When a leukaemic cell line K562 was treated with CMAS-oligo, colony formation on soft agarose was also decreased by 93 %. In contrast, treatment with a scrambled control oligo did not significantly inhibit leukaemic cell growth. These results suggest that a rational target site search is possible for an anti-sense oligo and that CMAS-oligo can be employed as an effective anti-sense agent with enhanced stability.

Key words: c-*myb*, growth inhibition, oligonucleotide stability.

INTRODUCTION

Anti-sense oligonucleotides (AS-oligos) have been valuable in the functional study of genes by decreasing gene expression in a sequence-specific manner [1–3]. Intense efforts have also been made to develop molecular anti-cancer agents by ablating the aberrant expression of genes involved in tumour initiation and progression [4–10]. To this end, synthetic AS-oligos have been widely used for ease of design and synthesis as well as for their potential specificity. AS-oligos with short lengths (13–30 nt) have been designed to bind a complementary sequence by forming Watson–Crick base pairs, providing specificity and affinity. Inhibition of gene expression is believed to be achieved through either RNase H activity after the formation of a DNA–mRNA duplex or steric hindrance of the binding of the ribosomal complex [11]. The efficacy of AS-oligos has been validated in some animal models as well as in some recent clinical studies [12–15].

However, high expectations of taking advantage of the sequence specificity of an AS-oligo have frequently met with disappointment as the results have not always been unambiguous. Salient problems for an AS-oligo are instability to nucleases, inefficient cellular uptake, inaccessibility to a target site and non-specific activities. These problems need to be addressed adequately before AS-oligos become a consistent method of blocking gene expression. The stability of AS-oligos has been improved to a certain extent by either using modified oligos or adopting a structure resistant to exonucleases [16–19]. Oligos with modified linkages such as phosphorothioate (PS) and methylphosphonate (MP) have been used to augment stability against nucleases. However, each of the modified nucleotides exposed problems of its own, such as a lack of sequence specificity and insensitivity to RNase H. Further, there is potential for the misincorporation of the hydrolysed, modified nucleotide in the genome during DNA replication or repair.

Finding a good target site for an AS-oligo has been largely an empirical process, necessitating time-consuming and often expensive experimentation. AS-oligos selected by rational target site search combined with improved stability might be more effective in achieving the complete ablation of target mRNA. The proto-oncogene c-*myb* is important in the proliferation and differentiation of haemopoietic cells [1,4]. It has often been found to be overexpressed in leukaemic cells. Blockage of c-*myb* expression by an AS-oligo inhibited the growth of leukaemic cell lines [4-7]. However, the AS-oligo used in the experiments was reported to be only partly effective and was used in a rather large quantity.

In the present study we tested the feasibility of a rational target site search method employing secondary structure simulation. On the basis of this approach, eight sites along c-*myb* mRNA were selected for analysis *in vitro*. Four AS sequences from the eight sites were further selected and placed in a combination to construct a large AS molecule. The AS-oligo was then covalently closed to form a covalently closed multiple anti-sense (CMAS)-oligo. The c-*myb* CMAS-oligo was tested for its stability and effectiveness *in vitro* as an AS-oligo.

EXPERIMENTAL

Cell lines and tissue culture

The leukaemic cell lines HL-60 (promyelocyte leukaemic cell line) and K562 (chronic myelogenous leukaemic cell line) were

Abbreviations used: AS, anti-sense; CMAS, covalently closed multiple anti-sense; FBS, fetal bovine serum; MP, methylphosphonate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; oligo, oligonucleotide; PO, phosphodiester; PS, phosphorothioate; RT–PCR, reverse-transcriptase-mediated PCR; SC, scrambled control.

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Table 1 Phosphorothioate-capped linear c-*myb* AS oligos used for the AS effect on c-*myb* expression

AS sequences were from either open regions (MIJ-1 to MIJ-19) or duplexed regions (duplex-1 to duplex-5). Nucleotide numbering is in accordance with [33]. Abbreviation: (s), phosphorothioate modified.

Name	Complementary site	Type	Size (bases)	Sequence
AS-MIJ-1	253–267	AS	15	T(s)CAGTTTTTCATC(s)C(s)T
AS-MIJ-2	401–415	AS	15	T(s)GATCTTCTTCTT(s)T(s)G
AS-MIJ-3	613–627	AS	15	G(s)CTTTGCGATTTC(s)T(s)G
AS-MIJ-4	1545–1559	AS	15	A(s)CCGTATTTAATT(s)T(s)C
AS-MIJ-16	585–602	AS	18	C(s)CCAGTCTCTTGTGT(s)G(s)C
AS-MIJ-17	961–978	AS	18	G(s)CAAGGGGCTCGCC(s)A(s)G
AS-duplex-1	321–335	AS	15	T(s)GTTCGATTCGGG(s)A(s)G
AS-duplex-2	704–718	AS	15	G(s)ACTCCTGCAGAT(s)A(s)A
AS-duplex-3	856–870	AS	15	G(s)TGCTTCAGAAAT(s)G(s)T
AS-duplex-4	1156–1170	AS	15	A(s)GTGGTGTTCTCC(s)C(s)A
AS-duplex-5	1604–1618	AS	15	A(s)TCACATCCTGCA(s)G(s)A
S-MIJ-19	97–114	Sense	18	T(s)GGCGCGGCGGGCGGC(s)G(s)G
SC-MIJ-6	253–267	SC	15	T(s)ATTCTTCTGCTC(s)T(s)A

obtained from A. T. C. C. (Manassas, VA, U.S.A.) and cultured in RPMI 1640 (Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT, U.S.A.) and penicillin/streptomycin (100 i.u., 100 μg/ml). Cells were maintained in a CO_2 incubator at 37 °C. Routine cell-culture practices were strictly followed to keep proper cell density and to avoid cells' being cultured for more than five generations after the thawing of stock vials. Culture media were exchanged 1 day before treatment with AS-oligos.

Selection of target sites for an AS-oligo

Target sites for AS-oligos were selected for eight different regions of c-*myb* mRNA. The method of searching for rational target sites was as follows. Simulation of secondary structures was performed with the DNAsis program (Hitach Software, San Bruno, CA, U.S.A.). 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 second set of frames. This process was repeated again so that any given sequence was scanned for its potential secondary structure in three different frames. Eight sequences, which had minimal secondary structures in three different frames, were selected from the c-*myb* mRNA sequence. The rational target site search for an AS-oligo was employed to improve the chance of predicting a natural secondary structure (see Figure 1). Table 1 shows eight AS sequences complementary to the selected target sites. Of the eight selected target sites for an AS-oligo, four were finally chosen in a combination that formed a minimal intramolecular secondary structure when converted to a covalently closed molecule.

Construction of CMAS-oligos

Oligos were either made by us or purchased from the Nucleic Acid Core Facility of the University of Pennsylvania (Philadelphia, PA, U.S.A.). One CMAS-oligo harbours four different AS sequences in a combination with the least secondary structure because the AS-oligo is expected to bind to target sites more readily. AS-oligos and scrambled control (SC) oligos were phosphorylated during synthesis at the 5′ end to allow intramolecular covalent ligation. The sequence of the 60-mer AS-oligo was 5′(p)-TCAGTTTTTCATCCTGCTTTGCGATTTCT-GTGATCTTCTTCTTTGACCGTATTTAATTTC-3′ and SC-oligo was 5′(p)-GTTATCGTTTGTCTGATTAATCTCTTTC-TGTAGTTCTCATATGTTCTCTCGTCTCTCT-3′. Both ends of the AS-oligo were joined with a ligation primer that had complementary sequences in both halves to both extreme end sequences (seven bases on each side) of the 60-mer AS-oligo. The sequence of the 14-mer ligation primer was 5′-AAACTGA-GAAATTA-3′. Ligation primer was mixed with an AS-oligo and heated to 85 °C for 2 min followed by gradual cooling to room temperature. T4 DNA ligase (1 unit) was added and incubated for 16 h at 16 °C to generate a covalently closed molecule. The CMAS-oligo was subjected to electrophoresis on a 5% (w/v) Metaphor™ agarose gel (FMC, Rockland, ME, U.S.A.) or on a denaturing 12% (w/v) polyacrylamide gel and identified for its resistance to exonuclease III as well as for gel retardation compared with the linear 60-mer oligos. SC-oligo was also closed covalently. Ligation primer was then degraded with exonuclease III or detached from the CMAS-oligo by running it on a denaturing gel after heating the oligos at 90 °C.

Stability test of linear oligos and CMAS-oligos

Non-specific control phosphodiester (PO)-oligo (linear 60-mer) and a CMAS-oligo (1 μg of each) were incubated with either human serum, fetal bovine serum (FBS) or calf serum (non-heat-inactivated; HyClone) or exonuclease III. Each serum was added to AS-oligos to 50% in a 100 μl reaction volume and incubated for 24 h at 37 °C. AS-oligos were then extracted with phenol/chloroform (1:1, v/v), and examined on a denaturing 15% (w/v) polyacrylamide gel. Exonuclease III (Takara, Otsu, Japan) (160 units/μg of oligo) was added to linear oligos and CMAS-oligos and incubated for 2 h at 37 °C. AS-oligos treated with exonuclease III were extracted and subjected to electrophoresis in the same manner.

Transfection of a CMAS-oligo complexed with cationic liposomes

CMAS-oligo (0.3 μg) and Lipofectin™ (1 μg) (Gibco BRL) were diluted in 20 μl of OPTI-MEM™ (Gibco BRL) separately and

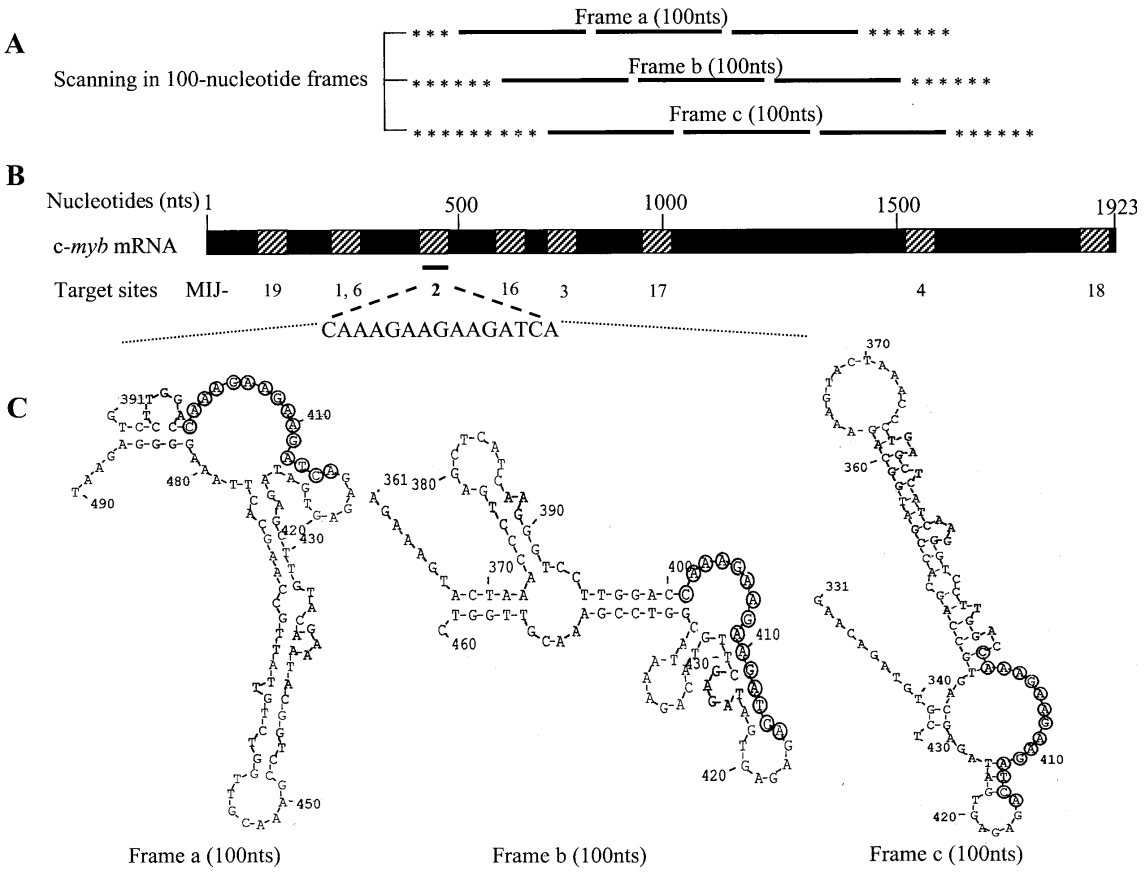


Figure 1 Selection of target sequences for an AS-oligo

(**A**) The cDNA sequence of c-*myb* mRNA is represented by the thick horizontal solid bars [33]. The entire c-*myb* mRNA sequence was scanned three times with an RNA secondary-structure prediction program to find a sequence with minimal secondary structure. Each scan was performed sequentially 30 bases apart in a 100 nt frame (shown as frames a, b and c). (**C**) Putative secondary structures in the area of c-*myb* mRNA containing the target MIJ-2 sequence are shown in the three frames of 100 nt each (frames a, b and c). Thus a given sequence was scanned for secondary structures in three different frames. The most open sequence, 5′-CAAAGAAGAAGATCA-3′ (401–415, encircled), in three scannings was chosen as a target sequence (MIJ-2). (**B**) Other target sites selected similarly are shown (hatched) along the long solid horizontal line representing c-*myb* mRNA.

incubated at room temperature for 40 min. Each of the components was then added to form a complex at room temperature for 15 min. Cells were added with fresh culture medium without antibiotics [RPMI 1640/10% (v/v) FBS] 1 day before the addition of oligos and washed twice with OPTI-MEM before an experiment. The cell density was adjusted to 5×10^5 cells/ml and divided into 100 μl aliquots in a 48-well plate (Falcon, Lincoln Park, NJ, U.S.A.). Liposome–oligo complex (40 μl) was added to cells twice, once on day 0 and once on day 1. Cells treated with oligos were incubated at 37 °C under air/CO_2 (19:1) for 4 h and then added to 100 μl of OPTI-MEM containing 10% (v/v) FBS. The next day, 100 μl of the culture supernatant was carefully removed and replaced with 20 μl of fresh OPTI-MEM containing liposome–oligo complex; 4 h later, cells were added to an additional 100 μl of complete medium and incubated at 37 °C for a further 1 day before assay.

Isolation of total RNA, and reverse-transcriptase-mediated PCR (RT–PCR)

Total RNA was isolated with Tripure™ Isolation Reagent (Boehringer Mannheim, Mannheim, Germany) as recommended by the manufacturer. In brief, cells harvested were added to 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 an Access™ RT–PCR kit (Promega, Madison, WI, U.S.A.). In a PCR tube were added RNA, PCR primers, avian myeloblastosis virus reverse transcriptase (5 units/μl), *Tfl* DNA polymerase (5 units/μl), dNTP (10 mM, 1 μl) and $MgSO_4$ (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, U.K.); 25 cycles of PCR amplification were subsequently performed under the conditions recommended by the manufacturer. Amplified PCR product was confirmed in a 1% (w/v) agarose gel and quantification was done with a gel documentation program (Bio-Rad, Hercules, CA, U.S.A.).

Southern hybridization of RT–PCR fragments

RT–PCR products were subjected to electrophoresis on a 1% (w/v) agarose gel. DNA was transferred to a nylon membrane (New England Biolabs, Beverly, MA, U.S.A.) for 4 h in 0.4 M NaOH. The membrane was hybridized with a 30-mer internal primer, 5′-TGTAACGCTACAGGGTATGGAACATGACT-G-3′, labelled with an enhanced chemiluminescence 3′ end oligo-labelling and detection system (Amersham Life Science, Little Chalfont, Bucks., U.K.). Hybridization was performed at 62 °C for 60 min in 6 ml of buffer containing 5 × SSC (SSC is 0.15 M

NaCl/0.015 M sodium citrate) and 0.02 % SDS. The membrane was washed twice in 5 × SSC containing 0.1 % SDS and washed again twice with 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 by SDS/PAGE [7.5 % (w/v) gel] and transferred to a nitrocellulose membrane. After blocking with PBS containing 3 % (v/v) non-fat milk and 0.05 % (v/v) Tween 20, the membrane was incubated with a mouse monoclonal IgG2aκ antibody specific for mouse or human Myb (Upstate Biotechnology, Lake Placid, NY, U.S.A.) at 1 μg/ml. Horseradish-peroxidase-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO, U.S.A.) was used in the secondary incubation, followed by detection of a reactive band by chemiluminescence (Amersham Life Science). The protein band developed on an X-ray film was quantified by a gel documentation program (Bio-Rad).

Inhibition test of leukaemic cell growth

Growth inhibition of leukaemic cells was measured by three methods: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay, [^{3}H]thymidine incorporation and colony formation on soft agarose.

For the MTT assay, HL-60 cells were washed twice with OPTI-MEM and divided into aliquots in a 96-well plate (4×10^3 cells per well) in a 50 μl volume. Cells were treated with a preformed complex of oligos (0.3 μg/15 μl) and Lipofectin (0.6 μg/15 μl) for 5 h and cultured for 4 days. Cells were then harvested in a 100 μl volume and added to 20 μl (100 μg) of MTT reagent (5 mg/ml in PBS) (Sigma), followed by incubation for 4 h at 37 °C. An equal volume of propan-2-ol containing 0.1 M HCl was added to the cells and incubated for a further 1 h at room temperature. A_{570} was measured with an ELISA reader to determine the number of cells surviving. The percentage growth inhibition was calculated by the following formula: percentage growth inhibition = $100 \times [1 - (A_{570}$ of experimental well$/A_{570}$ of untreated control well)].

For [^{3}H]thymidine incorporation, HL-60 cells were treated with AS-oligo as described above. Cells were added to 0.5 μCi of [^{3}H]thymidine (2.0 Ci/mmol; Amersham) and incubated for 16 h in triplicate. Cells were then harvested on a glass microfibre filter (GF/C; Whatman, Maidstone, Kent, U.K.). The filter was washed with cold PBS, 5 % (w/v) trichloroacetic acid and then with 100 % ethanol. [^{3}H]Thymidine incorporation was measured with a liquid-scintillation counter in a cocktail solution containing toluene, Triton X-100, 2,5-diphenyloxazole and 1,4-bis-[5-(phenyloxazol-2-yl)]benzene. The percentage growth inhibition was calculated as $100 \times [1 - ($c.p.m. in experimental well/c.p.m. in untreated control well)].

Measurement of colony formation on soft agarose was employed as follows. K562 cells were transfected as described above and cultured at 37 °C under air/CO_2 (19:1) for 24 h. An equal volume mixture of 0.8 % low-melting-point agarose (in doubly deionized water) and 2 × RPMI 1640 containing 20 % (v/v) FBS was added to cells and seeded in a six-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 means ± S.D. Statistical significance was determined by using Student's t test. $P \leqslant 0.05$ was considered to be significant.

RESULTS

Rational target site search and decrease in target mRNA by linear AS-oligos

Target site selection for an AS-oligo has been found to be critical for achieving effective ablation of target mRNA but the approach has been somewhat arbitrary. In the present study we scanned the entire sequence of human c-*myb* mRNA for putative secondary structures in multiple overlaps (Figure 1). Eight sites were selected because they were relatively free of RNA secondary

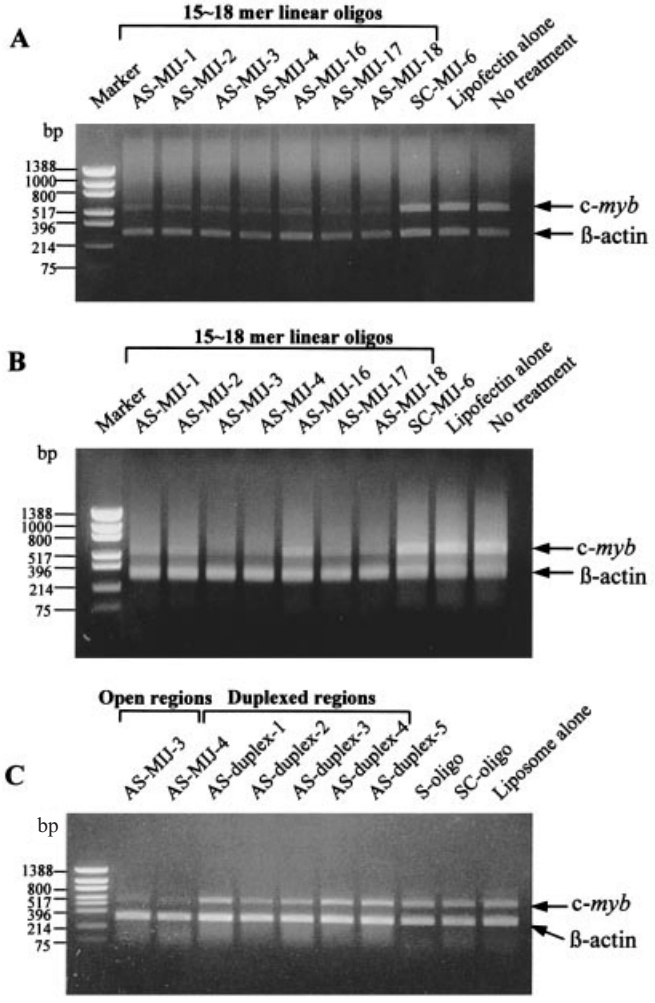
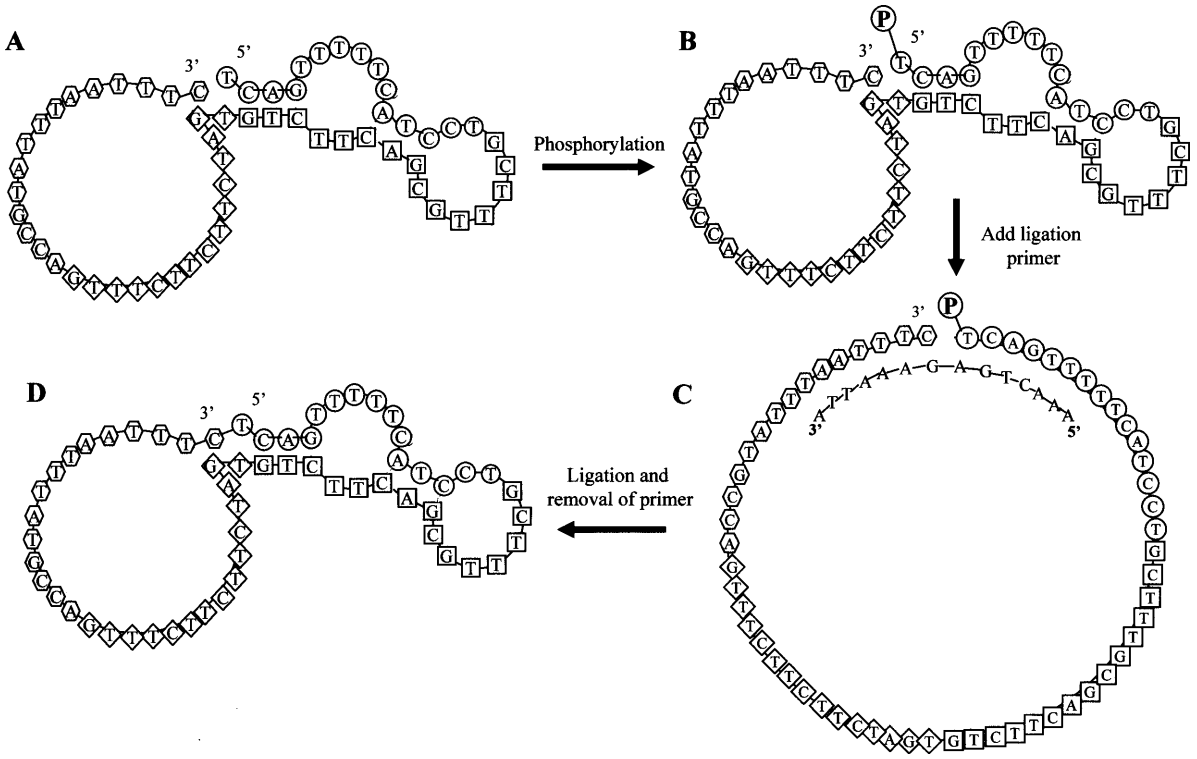


Figure 2 Effect of AS in HL-60 and K562 cells treated with short linear AS-oligos

Cells were treated with a mixture of linear AS-oligos (15–18-mer, 1 μg) and Lipofectin (1.2 μg) for 2 days before extraction of RNA. Extracted RNA was subjected to RT–PCR for 20 cycles and amplified DNA fragments were run on 1 % (w/v) agarose gels. (**A**) HL-60 cells were treated with various AS-oligos (lanes 2–8) derived from regions free of secondary structures. SC-MIJ-6 denotes an SC-oligo. (**B**) K562 cells were treated with the same set of AS-oligos as those in (**A**). (**C**) HL-60 cells were treated with AS-oligos derived from either open regions (lanes 2 and 3) with minimal secondary structures or duplexed regions of heavy secondary structures (lanes 4–8) of human c-*myb* mRNA.



Scheme 1 Flow diagram for the construction of a c-*myb* CMAS-oligo

(**A**) The 60-mer linear molecule consists of four different AS-oligos. Different AS sequences are denoted by distinct shapes (circles, squares, diamonds and hexagons). Minor secondary structures shown in the figures are unstable at 37 °C. (**B**) 5′ end phosphorylation of the 60-mer linear AS molecule. (**C**) Binding of a ligation primer (14-mer) shown inside the 60-mer AS-oligo molecule. The 60-mer AS-oligo harbours sequences complementary to the ligation primer at both 5′ and 3′ ends. (**D**) A CMAS-oligo molecule containing four AS sequences.

structure (Table 1); they were tested for AS effect in cells expressing the c-*myb* gene. The AS-oligos were complexed with cationic liposomes for enhanced cellular uptake. HL-60 and K562 cells (10^6 cells/ml) in each well of a 48-well plate were transfected with 1 μg each of AS-oligos complexed with 1.2 μg of Lipofectin and were assayed for decrease in c-*myb* mRNA after 2 day culture. Most AS-oligos were able to decrease c-*myb* mRNA by approx. 60 % (43–87 %). In contrast, no significant change in c-*myb* message was detected from the control experiments employing an SC-oligo and Lipofectin alone as well as from mock treatment (Figures 2A and 2B).

We then tested the rationale for the method of secondary structure simulation in locating good AS target sites. HL-60 cells were treated with AS-oligos derived from either regions of minimal secondary structures (open regions) or regions of heavy secondary structures (duplexed regions) of human c-*myb* mRNA. Whereas the AS-oligos (AS-MIJ-3 and AS-MIJ-4) derived from open regions did decrease at least 60 % of the c-*myb* mRNA, the AS-oligos (AS-oligo-1 to AS-oligo-5) from regions of heavy secondary structures exhibited much smaller and varying degrees of target mRNA degradation (Figure 2C). These results demonstrate that secondary structure simulation in multiple overlapping frames could be an effective method for locating target sites.

Construction of a stable CMAS-oligo

AS-oligos with a PO backbone lack the stability essential for successful AS application. Modified oligos, such as a PS-oligo or an MP-oligo, exhibit improved stability but the gain in stability is only partial and can decrease specificity and RNase H activity. Modified oligos can also be associated with the potential hazard of recycling hydrolysed modified nucleotides during DNA replication or repair.

A chemically circularized oligo that forms a triple helix with a target mRNA sequence exhibits excellent stability [20]. In addition, exonuclease activity constitutes most of the nuclease activity in serum and cytoplasm [21]. To avoid exonuclease activity, a 60-mer AS-oligo was closed covalently with a 14-mer ligation primer by enzymic action. Four AS sequences were placed in a combination in the 60-mer AS-oligo (Scheme 1). The four AS sequences were selected from the eight AS-oligos chosen initially, to avoid intramolecular duplex formation of any significance. This AS-oligo was designated CMAS-oligo. CMAS-oligo was found to be approx. 10 % slower than its linear precursor on a denaturing 15 % (w/v) polyacrylamide gel (Figure 3A). CMAS-oligo was resistant to exonuclease III and appeared as multiple bands on a denaturing polyacrylamide gel, with monomers (60-mer) being the most abundant, then dimers (120-mer) and trimers (180-mer). In contrast with a CMAS-oligo, a linear oligo was completely degraded after incubation for 2 h with exonuclease III (Figure 3B).

CMAS-oligo was then tested for its stability by incubation with sera that were not heat-inactivated, to maintain nuclease activities. Oligos were treated with 50 % human serum, FBS or calf serum for more than 24 h. The linear 60-mer oligo was completely digested after incubation for 24 h in the presence of serum (Figure 3C). CMAS-oligo, however, remained mostly

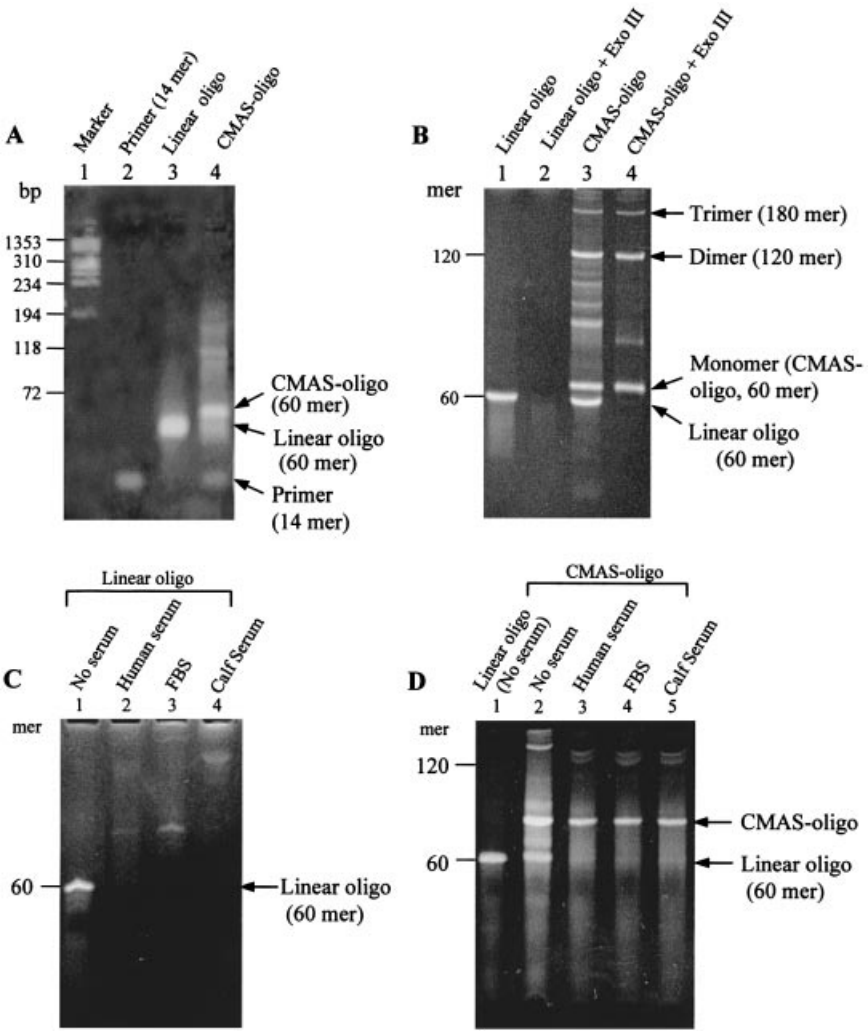


Figure 3 Electrophoretic mobility patterns of a CMAS-oligo

(**A**) Oligos were analysed on a 5% (w/v) Metaphor agarose gel; lane 1, size marker; lane 2, 14-mer primer that was the ligation primer; lane 3, linear 60-mer oligo; lane 4, CMAS-oligo. 60-mer CMAS-oligo was retarded in comparison with the linear 60-mer oligo. (**B**) Stability of linear and covalently closed oligos on treatment with Exonuclease III. AS-oligos are shown on a denaturing 12% (w/v) polyacrylamide gel. Both linear and covalently closed oligos were treated with Exonuclease III (160 units/μg of oligo) at 37 °C for 2 h. Lanes 1 and 3, samples not treated with exonuclease III; lanes 2 and 4, samples treated with exonuclease III. (**C**) Stability of linear AS-oligos: lane 1, oligos not treated with serum (negative control); lanes 2, 3 and 4, oligos treated with 50% (v/v) human serum, FBS and calf serum respectively for 24 h. (**D**) Stability of CMAS-oligos: lane 1, negative control; lanes 2, 3 and 4, oligos treated with 50% (v/v) human serum, FBS and calf serum respectively for 24 h.

intact after incubation for 24 h with human serum, FBS and calf serum, exhibiting significantly improved stability over the linear oligos to nucleases (Figure 3D).

Specific decrease in c-*myb* mRNA and Myb protein content by the c-*myb* CMAS-oligo

Encouraged by the enhanced stability of a CMAS-oligo, we examined whether this AS-oligo functioned well in eliminating the target mRNA in a sequence-specific manner. CMAS-oligo was delivered into cells after formation of a complex with Lipofectin. Lipofectin was employed because it was found to be less toxic to cells and yielded consistent results. MIJ-5 (78 nM), a CMAS-oligo to human c-*myb*, was complexed with 1 μg of Lipofectin for transfection into HL-60. MIJ-5 decreased c-*myb* mRNA content by at least 95% in comparison with a covalently closed SC-oligo. Meanwhile the linear counterpart of MIJ-5, MIJ-5A, decreased 65% of c-*myb* mRNA content (Figure 4A). The RT–PCR results were confirmed by Southern hybridization (Figure 4B) with an internal primer designed to bind the PCR-amplified DNA fragment. In contrast, β-actin expression (Figure 4B, bottom panel) was not affected by the treatment of MIJ-5. Analogously, Myb protein was also largely eliminated from the cells treated with the c-*myb* CMAS-oligo (Figure 4C). These results indicate that a CMAS-oligo is superior to a linear oligo in ablating target mRNA even when used in a smaller amount.

Growth inhibition of leukaemic cells by the c-*myb* CMAS-oligo

C-*myb* is important in the proliferation of leukocytes. AS-oligos to c-*myb* have been observed to inhibit leukaemic cell growth [2–7]. We examined the inhibition of leukaemic cell growth by the c-*myb* CMAS-oligo. A leukaemic cell line, HL-60, was treated with either the c-*myb* CMAS-oligo (85 nM) or the SC-

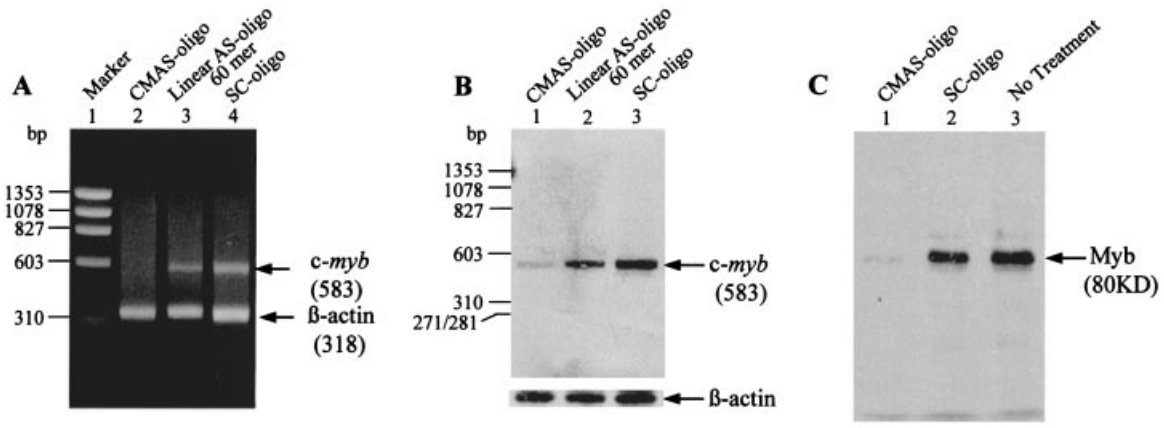


Figure 4 Effect of c-*myb* CMAS-oligos on c-*myb* mRNA and Myb protein expression in HL-60

(**A**) RT–PCR was performed with total RNA and two c-*myb* primers (described in the Experimental section). Cells were treated with 60-mer CMAS-oligo (0.3 μg, 78 nM) plus Lipofectin (1 μg) (lane 2), 60-mer linear oligos (0.3 μg) plus Lipofectin (1 μg) (lane 3) and covalently closed SC-oligo (0.3 μg) plus Lipofectin (1 μg) (lane 4). (**B**) PCR products were analysed by Southern hybridization; hybridized bands were detected with the ECL 3′ oligolabelling and detection system (described in the Experimental section). The upper panel shows the hybridized RT–PCR bands of c-*myb* mRNA and the lower panel the hybridized RT–PCR bands of β-actin mRNA. (**C**) Western blot analysis of Myb protein levels of HL-60 cells with treatment of c-*myb* CMAS-oligo. Analysis was performed at day 2 before the onset of massive cell death caused by the treatment with AS-oligo.

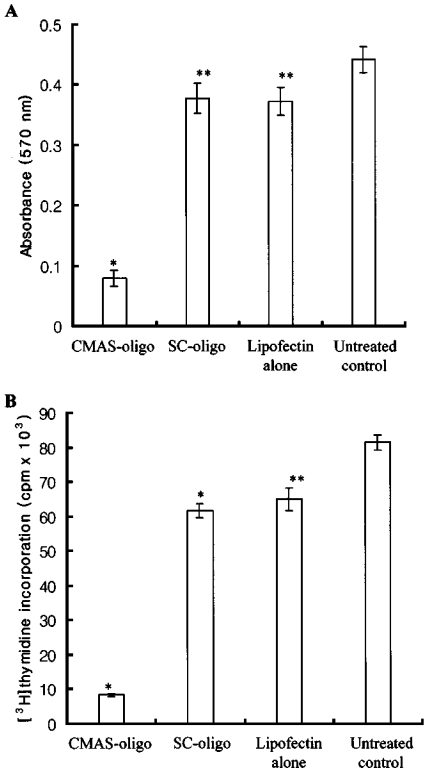


Figure 5 Effect of c-*myb* CMAS-oligo on proliferation of HL-60

HL-60 cells were treated with complexes containing 0.3 μg of oligos (85 nM) and 0.6 μg of Lipofectin. Each bar is shown for growth inhibition of HL-60 cells. SC-oligo, Lipofectin alone and untreated controls were treated in the same manner. The results show growth inhibition with c-*myb* CMAS-oligos determined by MTT assay (**A**) and [^{3}H]thymidine incorporation (**B**). CMAS-oligo, but not SC-oligo, inhibited tumour cell growth. Results are means $\pm$ S.D. for three experiments (*$P < 0.0001$; **$P < 0.05$ compared with untreated control).

oligo complexed with an equal amount of Lipofectin or with Lipofectin alone. Cells were incubated for 4 days and measured for absorbance by the MTT assay as an index of cell growth. Cell growth was inhibited by $82 \pm 3\%$ with the CMAS-oligo ($P < 0.0001$ compared with untreated control) (Figure 5A). In contrast, the SC-oligo and Lipofectin alone did not significantly inhibit cell growth in comparison with that of the untreated control. Similarly when leukaemic cell growth was measured by [^{3}H]thymidine incorporation, the CMAS-oligo inhibited cell growth by $90 \pm 1\%$ ($P < 0.0001$ compared with untreated control) (Figure 5B). Inhibition values are means $\pm$ S.D.

Inhibition of tumour cell growth was also examined for colony formation on soft agarose. K562 cells were treated with CMAS-oligo (MIJ-5) because the cells formed more distinctive colonies on soft agarose. Cells transfected with the c-*myb* CMAS-oligo were seeded in a 0.4% agarose gel and incubated for 15 days before being scored for colonies formed. CMAS-oligo was able to decrease the number of colonies formed by 93% ($P < 0.0001$ compared with untreated control) compared with the untreated control (Table 2). MIJ-5A (60-mer linear AS-oligo) and MIJ-1 (15-mer linear AS-oligo) also decreased the number of colonies formed but to a smaller extent. In contrast, a sense oligo and SC-oligos did not significantly decrease the number of colonies. These results indicate that the c-*myb* CMAS-oligo is an effective AS agent for inhibition of leukaemic cell growth.

DISCUSSION

In the present study we devised a CMAS-oligo to improve the stability against nucleases. CMAS-oligo contains four AS sequences in tandem without an open end, allowing multiple targeting of a target mRNA or targeting of more than one mRNA. CMAS-oligo was found to be exceptionally stable in the presence of serum that readily degrades a regular PO-oligo. The c-*myb* CMAS-oligo was shown to be effective in ablating c-*myb* mRNA and in inhibiting leukaemic cell growth.

One of the critical factors for a maximal AS effect is the selection of a right target site. However, finding a good target site has been a process of trial and error, in need of a better method. mRNA forms extensive secondary and tertiary structures in the cytoplasm. It has been reported that a 10-mer AS-oligo against Ha-*ras* binds a single-stranded loop region with an affinity six orders of magnitude higher than that to the flanking double-

Table 2 Effect of c-*myb* 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% low-melting-point agarose gel. Transfection was performed with two treatments of complexes containing 0.8 μg of Lipofectin and 2 μg/0.2 ml (2.2 μM) of linear phosphorothioate oligos (15-mer) and 0.5 μg/0.2 ml (130 nM) of 60-mer linear or CMAS-oligos for 24 h. Colonies arising in six-well plates containing cells treated with sense (S), AS, SC and CMAS oligos were enumerated. Colonies in controls (Lipofectin alone or untreated) are shown in the bottom two lines. Experiments were repeated twice and similar results were obtained. $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$ compared with untreated control.

Oligos				
Structure	Size (bases)	Type	Number of colonies	Colonies formed (% of control)
Linear	15	AS (MIJ-1)	$55 \pm 9.4^{**}$	44
Linear	15	S (MIJ-19)	110 ± 9.5	89
Linear	15	SC (MIJ-6)	$84 \pm 7.2^{*}$	68
Circular	60	CMAS (MIJ-5)	$9 \pm 2.6^{***}$	7
Linear	60	AS (MIJ-5A)	$39 \pm 5.6^{***}$	32
Linear	60	SC (MIJ-5B)	$92 \pm 8.6^{*}$	74
Lipofectin™ alone			109 ± 11.8	88
Untreated control			124 ± 9.0	100

stranded region [22,23]. To avoid regions rich in secondary structures, a systematic target site search was performed to scan the entire sequence of human c-*myb* mRNA. Simulation of secondary structures of a given sequence was done in multiple staggered frames to improve the likelihood of predicting an actual secondary structure. The single-stranded regions with a G + C content of more than 30% were selected as target sites for AS-oligos. We had applied a similar approach to finding target sites for the ablation of Syk mRNA [19]. The result shown here demonstrates the rationale for this approach in locating a good target site.

In many anti-sense studies, the region encompassing the translational start site has been adopted for a target site [2,24]. However, in the c-*myb* mRNA is a quartet of G residues on the 3′ side of the translational start codon. Because there has been some controversy over the non-specific effect of these G residues [25], the region was avoided as a target site. So far, AS-oligos to c-*myb* have been studied in PO-oligo or PS-oligo forms as well as in an expressed AS molecule [4]. The c-*myb* CMAS-oligo is a closed molecule that can target four different sites along c-*myb* mRNA. The potential merits of a CMAS-oligo, other than its exceptional stability, are as follows: (1) the AS-oligo has a greater likelihood of finding a target site because it contains four different AS sequences; (2) one AS molecule can bind multiple targets, either many sites on the same molecule or on multiple molecules; and (3) the AS-oligo can be used in a smaller quantity because the molecule is much more stable and therefore avoids sequence-independent side effects. In fact, when CMAS-oligo was added to HL-60 cells at 78 nM (1.5 μg/ml), cell growth was decreased by more than 80%. However, in previous studies, much larger amounts of AS-oligos ranging from 20 to 200 μg/ml were used to obtain biological effects that might not have been entirely sequence-dependent [2,3,16,24,27,28].

AS-oligos have been modified to enhance stability against nucleases. Two modified oligos that have been used frequently are PS-oligos and MP-oligos. These oligos have been shown to be more stable than regular PO-oligos, but only slightly so [29]. Furthermore, PS-oligos have sequence-independent activity and MP-oligos have low solubility and show decreased sensitivity to RNase H [30]. CMAS-oligo is likely to have normal sequence specificity and sensitivity to RNase H because the oligo bears no modified nucleotide in its present structure. It is possible to incorporate modified nucleotides in a CMAS-oligo to enhance binding affinity to a target site. An additional potential merit of a CMAS-oligo is that it is unlikely to introduce undesired mutations in the genomic DNA during DNA replication or repair by recycling of hydrolysed nucleotides.

We used cationic liposomes to enhance the cellular uptake of a CMAS-oligo. From our experience and that of other workers, a meaningful cellular level of an AS-oligo can be consistently obtained with liposomes, regardless of the size of an AS-oligo [19,31,32]. The relatively large size of a CMAS-oligo would therefore not pose a problem for efficient cellular uptake. The synthesis of a 60-mer oligo would require some caution but we routinely obtained approx. 60% recovery of the final product. In addition, we are currently studying an AS-oligo molecule that is shorter and does not require a ligation primer for covalent ligation.

In conclusion, CMAS-oligo is observed to have a markedly enhanced stability and provides an excellent sequence-specific AS effect. CMAS-oligos require more extensive studies for biological effects in animals. Once the molecular and pharmacological properties of a CMAS-oligo are fully known, these AS-oligos might prove valuable as a molecular therapeutic agent.

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