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Inhibition of c-*myc* Expression in Cells by Targeting an RNA-Protein Interaction Using Antisense Oligonucleotides

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ABSTRACT

Antisense oligodeoxynucleotides (ODNs) are designed to bind to and inhibit a target mRNA. We used a novel approach for the design of ODNs to the c-*myc* mRNA using protein binding sites as targets for ODN action. Our strategy was to identify ODNs that could interfere with the coding region determinant-binding protein (CRD-BP), a protein that binds to the CRD region of the c-*myc* mRNA. Using an in vitro gel shift assay, we show that ODN molecules can occlude the CRD-BP from the mRNA. The best ODN, CRD-ODN4, was able to inhibit RNA binding of the CRD-BP by 75%. This effect was sequence-specific and concentration dependent. K562 cells treated with a 2′-*O*-methyl derivative of CRD-ODN4 showed a concentration-dependent decrease in both c-*myc* mRNA and protein levels, with a maximal 65% inhibition of protein expression at 200 nM CRD-ODN4. In contrast, a 2′-*O*-methyl ODN derivative targeting the translation initiation codon (antimyc-aug) reduced c-*myc* protein but actually increased mRNA levels, an effect resulting at least partly from stabilization of the c-*myc* mRNA. CRD-ODN4 treatment did not alter the c-*myc* mRNA half-life. CRD-ODN4 was more effective in inhibiting K562 cell growth than antimyc-aug, reducing cell number by ≈70% after 48 h of exposure to 750 nM. The correlation between ODN effects on RNA-protein interactions in vitro and those observed in cells supports the hypothesis that CRD-ODN4 inhibits the interaction between the CRD-BP and the c-*myc* mRNA and that disrupting this RNA-protein interaction reduces c-*myc* expression in cells.

Antisense oligodeoxynucleotides (ODNs) are a promising new class of therapeutic reagents. Designed to be complementary to a region of a target mRNA, these short nucleotide sequences of DNA or DNA derivatives inhibit mRNA metabolism and block the flow of information from gene to protein (Crooke, 1993; Stein and Cheng, 1993; Wagner, 1994; Calabretta et al., 1996; Bennett, 1998; Gewirtz et al., 1998). Applications of antisense oligonucleotides include the treatment of diseases like cancer, which often are characterized by overexpression of growth-control genes (Calabretta et al., 1996). Antisense oligonucleotides that target such genes could be valuable for inhibiting cancer cell growth, and this approach is being explored with phosphorothioate ODN derivatives targeted to c-*myc*, c-*myb*, *bcl-2*, and *bcr-abl*. These reagents show promise in animal models, and some are being studied in early clinical trials in humans (Ratajczak et al., 1992; Calabretta et al., 1996; Skorski et al., 1996, 1997; Gewirtz et al., 1998).

The proto-oncogene c-*myc* is a potential target for ODN action in cancer chemotherapy. The c-*myc* gene product belongs to a family of transcriptional regulators and is important for control of cell growth (Cole, 1986). There is an association between changes in c-*myc* regulation and tumor formation (Cole, 1986; Field and Spandidos, 1990), and ODNs that target c-*myc* have been shown to be therapeutically useful either alone or in combination with other drugs (Skorski et al., 1996, 1997; Citro et al., 1998). In addition, the metabolism of c-*myc* mRNA has been well studied relative to most mRNAs (Wisdom and Lee, 1991; Bernstein et al., 1992; Zhang et al., 1993; Herrick and Ross, 1994; Prokipcak et al., 1994). This makes the c-*myc* mRNA an ideal molecule for experimental design of novel ODN reagents, as well as for the assessment of how ODNs function.

The most common antisense oligonucleotide sequence used to inhibit c-*myc* is AACGTTGAGGGGCAT (Kimura et al., 1995) (referred to here as antimyc-aug), which targets the initiation codon (AUG) and the next four codons of the c-*myc* sequence. This oligonucleotide has been synthesized with a phosphorothioate backbone that enhances nuclease resistance in serum and in cells (Crooke, 1993). Antimyc-aug has been used to successfully inhibit cell growth in many cell types in culture and in vivo (Watson et al., 1991; Kimura et al., 1995; Leonetti et al., 1996).

Despite the frequent and successful use of phosphorothio-

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ABBREVIATIONS: ODN, antisense oligodeoxynucleotide; CRD-BP, coding region determinant-binding protein; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RSW, ribosomal salt wash.

ate antimyc-aug, there is no evidence that it is the optimal ODN for inhibiting c-*myc* levels. Phosphorothioate backbones can lead to sequence-independent effects that in part may be attributable to affinity for extracellular proteins such as heparin and fibronectin (Chavany et al., 1995; Stein, 1995; Khaled et al., 1996). Nonspecific activity is enhanced by the presence of two short sequence motifs, the G quartet and CpG, which are both present in antimyc-aug. In addition, despite the widespread use of ODNs that target the translation initiation codon (70% of all ODNs used; Tu et al., 1998), it may not always be the best site. For some mRNAs, sites in the 5′- untranslated, protein coding, and 3′-untranslated regions have been shown to be more sensitive to ODN action than the translation initiation codon (Bacon and Wickstrom, 1991; Chiang et al., 1991; Crooke, 1993; Gewirtz et al., 1998). Antimyc-aug may therefore not be the best candidate for c-*myc* inhibition.

We used a novel approach to identify alternative target sites on the c-*myc* mRNA molecule. Specifically, we assessed the potential of protein binding sites on the c-*myc* mRNA to serve as targets for antisense oligonucleotide action in cells. RNA-binding proteins can influence mRNA transport, localization, translation, and degradation (McCarthy and Kollmus, 1995). Several proteins have been identified that interact with regions of the c-*myc* mRNA and regulate its metabolism (Bernstein et al., 1992; Zhang et al., 1993; Prokipcak et al., 1994). We have previously characterized a 70-kD protein that binds to the 3′ end of the coding region (the coding region determinant) and is called the coding region determinant-binding protein (CRD-BP) (Bernstein et al., 1992; Prokipcak et al., 1994). Our working model for CRD-BP function is that it binds to and protects the c-*myc* mRNA from endonucleolytic cleavage, resulting in enhanced expression of c-*myc*. This model is supported by evidence from an in vitro mRNA decay assay, in which titration of the CRD-BP off the c-*myc* mRNA results in its enhanced degradation (Bernstein et al., 1992). Because CRD-BP homologs have been shown to have a role in mRNA localization (Ross et al., 1997) and in translation (Nielsen et al., 1999), it is possible that the CRD-BP may be able to regulate c-*myc* at more than one level. Whatever the mechanism, the current data support a role for the CRD-BP in up-regulation of c-*myc* (Leeds et al., 1997; Doyle et al., 1998).

If the CRD-BP does function in cells to increase c-*myc* mRNA expression, then disrupting the binding of this protein may decrease c-*myc* mRNA levels. To test this, we designed ODNs to interfere with protein binding to the CRD region of the c-*myc* mRNA. Our data support the hypothesis that ODN molecules are able to occlude the CRD-BP from the mRNA and that disrupting this RNA-protein interaction reduces c-*myc* expression in cells.

Materials and Methods

Antisense Oligonucleotides. Table 1 shows the sequence of the antisense oligonucleotides used in this study. All ODNs targeting the CRD were 15 nucleotides long and had a 47% G/C content except CRD-ODN2, which was based on a previously published sequence (Gryaznov et al., 1996) and was 27% G/C. Oligonucleotides used for the gel shift assay were standard phosphodiester DNA derivatives. For work in cells, ODNs were synthesized as 2′-*O*-methyl derivatives with a phosphodiester backbone (Monia et al., 1993). For comparison, antimyc-aug, a 15-nucleotide ODN that is complementary to the translation initiation codon of c-*myc*, was used (53% G/C; Table 1); the activity of oligonucleotides corresponding to this region has been characterized previously in several cell types (Watson et al., 1991; Kimura et al., 1995; Leonetti et al., 1996).

Oligonucleotides were synthesized by Dalton Chemical Laboratories (Toronto, Ontario, Canada). Oligonucleotides were resuspended into ddH_2O for gel shift experiments or into RPMI-1640 medium (serum-free) for experiments in cell culture. Actual final concentrations of antisense oligonucleotides are provided in the figure legends.

Cell Culture. The human erythroleukemia cell line K562 was obtained from the American Type Culture Collection (Rockville, MD). K562 cells were grown in suspension culture in RPMI 1640 medium containing 10% FBS (without antibiotics) and maintained in an atmosphere of 5% CO_2/95% room air at 37°C.

Treatment of Cells with Antisense Oligonucleotides. Cells were treated with antisense oligonucleotides with the carrier Superfect (Qiagen, Chatsworth, CA) according to the manufacturer's suggestions. K562 cells were plated into 5 ml of RPMI 1640 with 10% FBS in 60-mm culture dishes at a concentration of 4×10^5 cells/ml. After 24 h, cells were washed once with PBS and resuspended in 5 ml of fresh RPMI 1640 containing 10% FBS. Oligonucleotides were resuspended in 150 μl of RPMI 1640 without serum, and Superfect was added to a final ratio of 6 μl of Superfect/μg oligonucleotide, up to a maximum of 30 μl of Superfect/5 ml of culture. For example, to achieve 100 nM, ≈2.5 μg of ODN in 150 μl of RPMI was complexed with 15 μl of Superfect before addition to the cells. After 5 to 10 min at room temperature, the Superfect-oligonucleotide mixture was added to the cells. Cells were incubated with the mixture for 6, 24, or 48 h before isolation of protein and RNA or assessment of cell number. All experiments were performed with a single application of

TABLE 1
Sequence of antisense oligonucleotides used in this study

Name	Sequence	Location on Human c-*myc* mRNA	Comments
Antimyc-aug	AACGTTGAGGGGCAT	570–584	Targets translation initiation codon
Aug-scram	AGCTGGGGTAGCAAT		Scrambled control for antimyc-aug
CRD-ODN1	TGTTTTCCAACTCCG	1714–1728	Targets CRD region
CRD-ODN2	TTTCATTGTTTTCCA	1720–1738	Targets CRD region (Gryaznov et al., 1996)
CRD-ODN3	GGATAACTACCTTGG	1741–1755	Targets CRD region
CRD-ODN4	ATGTATGCTGTGGCT	1763–1777	Targets CRD region
CRD-ODN5	ACGGACAGGATGTAT	1772–1788	Targets CRD region
CRD-ODN6	AGCTTTTGCTCCTCT	1793–1807	Targets CRD region
CRD-ODN7	GTTTCCGCTTCTTGT	1822–1836	Targets CRD region
CRD-ODN8	TCCGTAGCTGTTCAA	1861–1875	Targets CRD region
ODN5-MM	ACGGAAAGCAGGTAT		Mismatch control for CRD-ODN5
ODN4-sense	AGCCACAGCATACAT	1763–1777	Sense to CRD-ODN4
ODN4-MM	ATGCATACAGTGGCT		Mismatch control for CRD-ODN4
ODN4-scram	TGTCTTGAGAGTGTC		Scrambled control for CRD-ODN4

ODN. Concentrations of ODNs are expressed as final concentrations in the cell culture medium and ranged from 10 to 750 nM. Details are provided in the figures and figure legends.

MTT Assay for Cell Growth. Cell number was assessed with a colorimetric assay based on reduction of the dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO) by living cells (Carmichael et al., 1987). Exponentially growing cells were plated at 3 to 5 $\times$ 10^5 cells/ml in triplicate 96-well microtiter plates (100 μl/well) and were incubated at 37°C for 24 h before the addition of 10 to 750 nM oligonucleotide in 100 μl (complexed with Superfect). Final FBS concentration in all wells was 10%. Three control plates were prepared. In the first, cells were grown in the absence of any treatment. In the second, cells were incubated with Superfect alone at the same concentrations used for the ODN treatments. In the third, oligonucleotide and Superfect were added to plates in the absence of cells. Total time of exposure of the cells to ODN was 48 h. Forty-five hours into this incubation, 20 μl of 0.45 mg/ml MTT was added to each well, followed by an additional 3 h incubation. Plates were then centrifuged at 2500 rpm for 10 min, and 150 μl of medium was removed from each well. MTT Formosan crystals in each well were resolubilized by the addition of 150 μl of the DMSO, and the plates were placed on a shaker for 15 min. Absorbances were measured spectrophotometrically with a Titretek Multiscan automated microplate reader (Labsystems Multiscan MS, Franklin, MA) at a wavelength of 540 nm, and data were collected with Deltasoft (Biometallics Inc., Princeton, NJ). After subtraction of background, absorbance in the wells treated with Superfect and ODN or with Superfect alone were expressed as a percentage of the absorbance from untreated cells within the same plate. Data are expressed as mean absorbance $\pm$ S.E.

RNA and Protein Isolation. Total RNA was isolated from cells via TRIzol reagent (Life Technologies, Paisley, Scotland) according to the manufacturer's instructions. Cells were pelleted by centrifugation and lysed and homogenized in 1 ml of TRIzol reagent per 5 $\times$ 10^6 cells. RNA was isolated as described previously (Andreou and Prokipcak, 1998). Protein was isolated by resuspending K562 cells (1–2 $\times$ 10^8 cells/ml) into lysis buffer (50 mM HEPES-KOH, 1 mM EDTA, 420 mM NaCl, 0.1% Nonidet P-40, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and incubating on ice for 30 min. After centrifugation at 12,000*g* for 10 min at 4°C, the supernatant was stored at −70°C until analysis. Protein concentrations were determined with a commercial kit (Bio-Rad, Richmond, CA).

Northern Blot Analysis. Northern blot analysis was performed essentially as described (Andreou and Prokipcak, 1998). Briefly, total cellular RNA was separated by electrophoresis in a 1 to 2% agarose-2.2 M formaldehyde denaturing gel and transferred to Zeta-probe membranes (Bio-Rad). Blots were prehybridized for 1 h in hybridization buffer (125 mM sodium phosphate, pH 7.2, 1 mM EDTA, 125 mM NaCl, 7% SDS, 50% formamide). Probes for c-*myc* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were prepared as described (Andreou and Prokipcak, 1998). Hybridization was performed in the same buffer containing the radiolabeled probes for 16 to 24 h at 37°C (GAPDH) or 42°C (c-*myc*). Final wash conditions were 1× SSC (150 mM NaCl, 15 mM Na citrate, pH 7) at 37°C for GAPDH and 0.1× SSC at 50°C for c-*myc*. Quantitation of hybridized probes was performed with STORM (Molecular Dynamics, Sunnyvale, CA) after exposure of the blot to a Phosphor screen.

Measurements of mRNA Half-Lives. Cells were treated with 200 nM antisense oligonucleotides for 6 or 24 h. The medium was then replaced with RPMI 1640 with 10% FBS containing 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) at a final concentration of 100 μM in 0.1% DMSF. Treatment times are indicated in the figure legends. Total RNA was isolated from cells via TRIzol reagent (Life Technologies) and analyzed by Northern blot analysis as described above.

Western Blot Analysis. Proteins (50 μg/lane) were separated in SDS-containing 10% polyacrylamide gels (Prokipcak et al., 1994). Separated protein was transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA). The membrane was incubated in 20 mM Tris HCl, 137 mM NaCl, 0.1% Tween 20 containing 5% skim milk for 16 to 18 h. The blots were then incubated with an anti-myc monoclonal antibody (AB-1; Calbiochem Corp., La Jolla, CA) at a 1:1000 dilution for 1 h at room temperature followed by a horseradish peroxidase-conjugated anti-mouse IgG antibody (Amersham Corp., Arlington Heights, IL) at a 1:5000 dilution. The blots were developed with a chemiluminescent substrate (Roche Laboratories, Nutley, NJ). To confirm equal loading of protein, blots were stripped and reprobed with anti-GAPDH monoclonal antibody (Chemicon International, Inc., Temecula, CA) at a 1:500 dilution for 1 h at room temperature followed by a horseradish peroxidase-conjugated anti-mouse IgG antibody (Amersham) at a 1:5000 dilution. Exposed films were scanned with a Super Vista *S*-12 scanner (UMAX Data Systems, Hsinchu, Taiwan), and densitometric quantitation was performed with IPLab Gel (Signal Analytics Corp., Vienna, VA).

Ribosomal Salt Wash (RSW) Preparation. Polysomes and RSW from homogenized K562 cells were isolated by ultracentrifugation as described previously (Prokipcak et al., 1994). Briefly, polysomes were resuspended in 10 mM Tris-HCl, pH 7.6, 1 mM potassium acetate, 1.5 mM magnesium acetate, 2 mM DTT, 10% glycerol (TKMDG). The polysomes were stirred gently and brought to 1.0 M NaCl with dropwise addition of TKMDG plus 4 M NaCl. Soluble material was separated from "washed" polysomes by centrifugation through 30% (w/v) sucrose cushion at 130,000*g*. The supernatant from this spin was called the RSW and stored at −70°C until needed.

Preparation of RNAs by In Vitro Transcription. Templates for in vitro transcription of RNA probes for the gel shift analysis were prepared using PCR with SP6 or T7 promoter sequences incorporated into the 5′-primer (Bernstein et al., 1992; Prokipcak et al., 1994). The full-length c-*myc* CRD template (referred to as CRD) corresponds to c-*myc* sequence 1705–1886; the synthesis of this template has been described previously (Prokipcak et al., 1994). Probe RNAs were labeled with [α-^{32}P]UTP as described (Prokipcak et al., 1994) at $\approx$2 to 4 $\times$ 10^7 cpm/μg and used at 50,000 cpm (1–2 ng)/reaction.

Gel Shift Analysis. The gel shift assay used was a modification of those described previously for the CRD-BP (Prokipcak et al., 1994). ^{32}P-labeled RNA probes (1–2 ng) were incubated with RSW samples in 10-μl reactions containing 5 mM Tris-HCl, pH 7.6, 2 mM DTT, 5% glycerol, 0.5 μg/μl tRNA, and a final concentration of 150 mM NaCl. Reactions were incubated for 10 min at 30°C, followed by addition of heparin to a final concentration of 5 mg/ml and an additional incubation for 10 min at 30°C. In these current experiments with antisense oligonucleotides, the RNase T1 treatment step used previously for CRD-BP analysis (Prokipcak et al., 1994) was omitted. The ODNs and protein were added to the reaction on ice before the addition of the ^{32}P-RNA probe unless otherwise stated. RNA-protein complexes were separated from free RNA by electrophoresis in a 5% nondenaturing polyacrylamide gel, visualized by autoradiography, and quantitated using a PhosphorImager (Molecular Dynamics).

Statistics and Data Analysis. Quantitation of the hybridized ^{32}P-labeled probes was determined with a PhosphorImager or STORM, and data were analyzed subsequently with ImageQuant computer software (Molecular Dynamics). Statistical analysis for data from gel shift experiments was performed using repeat-measures ANOVA. Significantly different groups were identified with a Newman-Keuls test ($P < .01$). Statistical analysis for data from cell culture experiments was performed with Student's t test with a significance level of $P < .05$. For comparison of treatment values with control values from the same set of culture dishes, a paired t test was used ($P < .05$). All mRNA decay curves were analyzed individually via linear regression of a semilog plot of RNA concentration versus time (Ross, 1995). Half-lives obtained from separate experiments then were used to calculate a mean half-life ($\pm$S.E.). Data are represented as the mean $\pm$ S.E. (where $n = 3$) or mean (where $n = 2$).

Results

Mapping of the Core Binding Site for RNA-Protein Interaction via Competition with Antisense Oligonucleotides. The interaction of the CRD-BP with the c-*myc* mRNA was originally localized to the last 180 nucleotides of the coding region in exon 3 (Bernstein et al., 1992). Gel shift reactions with radiolabeled RNAs corresponding to smaller subregions of the c-*myc* CRD sequence showed that the strongest binding is observed with an RNA consisting of nucleotides 1705–1792. Our attempts to observe RNA-protein interactions with RNAs smaller than this 87-nucleotide RNA in the gel shift assay were unsuccessful (Doyle et al., 1998; and data not shown).

To further map the site of protein binding and test the ability of antisense oligonucleotides to inhibit RNA-protein interactions, we prepared ODNs complementary to sections along the 180-nucleotide CRD region. Table 1 and Fig. 1 show the ODN sequences and their positions along the CRD. We performed gel shift analysis in the presence of each ODN molecule at a final concentration of 1 pmol/μl (1 μM; Fig. 1). In these experiments, the ODNs were added to the buffer-RSW mix, and the ^{32}P-labeled RNA was added to the reaction last. Under these conditions, CRD-ODN3, CRD-ODN4, CRD-ODN5, and CRD-ODN8 had the greatest ability to reduce the formation of the RNA-protein complex, presumably by binding to and blocking sites on the RNA that are important for protein binding. The position of the most active ODN (CRD-ODN4) maps to the RNA region (1705–1792) that results in the strongest binding to the CRD-BP (Doyle et al., 1998).

For most of our experiments, the RNA was incubated with the ODN and CRD-BP at 30°C. Under these conditions, the CRD RNA may contain RNA secondary structure that limits the accessibility of the RNA, and the lack of activity of some of the ODNs may be attributable to lack of interaction with the RNA. To test whether RNA-ODN complexes do in fact form, we ran gel shift experiments with the CRD RNA and ODNs only. Each ODN caused a small shift in the mobility of the radiolabeled RNA, suggesting the presence of an RNA-ODN complex (data not shown). We also performed experiments in which the CRD RNA probe was preheated to 85°C for 10 min (to melt any secondary structure) in the presence of each of the ODNs. After a gradual cooling to room temperature, these samples were incubated with the protein samples as described in the legend to Fig. 1. The results obtained were similar with or without the preheating step (data not shown). Overall, these data suggest that altered accessibility (attributable to RNA secondary structure) is not responsible for the different activities of the ODNs.

In competing with the protein for the RNA, it would be expected that the ODN molecules would increase the concentration of free RNA in the gel shift experiments. In practice, the presence of free RNA is sometimes difficult to detect in RNA gel shift assays, an observation that is not unique to the current experiments with antisense oligonucleotides. We are using a fairly crude cell extract (RSW) as a source of the CRD-BP, and this RSW has been shown to contain abundant RNases (Ross et al., 1987; Lee et al., 1998). Although RNA that is bound to the CRD-BP is protected from degradation, it is likely that any free RNA not bound to protein will be rapidly degraded in the incubation time before gel electrophoresis. This hypothesis is supported by the increased presence of free RNA in gel shifts with more purified material, where the CRD-BP has been separated from endogenous nuclease activity (Prokipcak et al., 1994; and data not shown).

Dose-Dependence and Sequence Specificity of the Effects of ODNs on CRD-BP Binding. From experiments similar to those shown in Fig. 1, we determined that CRD-ODN4 had the most significant and consistent effect on CRD-BP binding to the RNA. To characterize this effect more thoroughly, we examined the influence of ODN concentration on ability to inhibit protein binding. CRD-ODN5 was used as a comparison because it overlaps with CRD-ODN4 by 6 nt. For CRD-ODN4, inhibition was observed at concentrations as low as 10 nM and maximal inhibition of 75% occurred at 1 μM (Fig. 2). The concentration-response curve for CRD-ODN5 was shifted to the right, with 100 nM required to observe an effect (Fig. 2). The activity of both CRD-ODN4 and CRD-ODN5 was sequence-specific; neither a sense oligonucleotide (ODN4-sense) nor oligonucleotides with three mismatches (ODN4-MM and ODN5-MM) were able to inhibit RNA-protein interactions (Fig. 3).

For most of our experiments, we added the ODN to the RSW first, so that the RNA was exposed to the ODN and

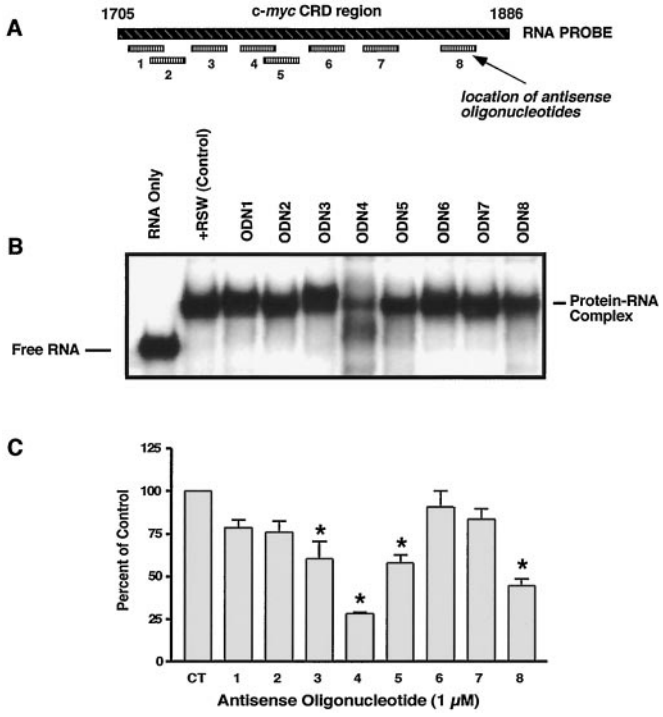

Fig. 1. Effect of antisense oligonucleotides on CRD-BP binding to its RNA. RSW from human K562 cells was incubated with ^{32}P-labeled CRD RNA in the presence of 1.0 pmol/μl (1 μM) of the indicated ODN molecule. ODNs used for all gel shift analyses were phosphodiester derivatives. A, schematic showing the relative positions of the indicated ODN molecules along the CRD sequence. Numbers 1 through 8 correspond to CRD-ODN1 through CRD-ODN8. B, autoradiograph of a representative gel shift experiment showing the CRD-BP-RNA complex in the absence and presence of the indicated CRD-ODNs. First lane, ^{32}P-labeled CRD RNA probe alone. Second lane, RNA probe in the presence of the RSW containing the CRD-BP. Lanes 3 through 10, RNA probe and RSW in the presence of CRD-ODN1 through CRD-ODN8. C, data from three separate gel shift experiments carried out as described in B were quantitated with a PhosphorImager, and the signal intensity was expressed as a percentage of the control (lane 2). Values shown are mean ± S.E. for the three experiments. The numbers 1 through 8 correspond to CRD-ODN1 through CRD-ODN8; CT, control. *$P < .01$, significantly different from control.

CRD-BP at the same time. For CRD-ODN4 to be active in cells, it must be able to compete for RNA-protein complexes that have already formed. To test this, we preincubated the RNA probe with the protein sample before adding the ODN competitors. We found a similar pattern of inhibition of protein binding as when the ODN was added to the protein first (data not shown). This suggests that there is sufficient "breathing" of the RNA-protein complex to allow for access of the ODN.

Effect of CRD Antisense Oligonucleotides on c-*myc* mRNA and Protein Levels in K562 Cells. From the gel shift data, it appeared that CRD-ODN4 might be a good candidate for inhibition of CRD-BP binding in cells. To test this, we first obtained 2′-*O*-methyl derivatives of the ODNs to enhance intracellular stability of the oligonucleotides. We chose 2′-*O*-methyl derivatives instead of phosphorothioates because they do not target the RNA for degradation by RNase H (Monia et al., 1993). We reasoned that this would increase our chances of observing effects directly attributable to removal of the CRD-BP from the c-*myc* mRNA rather than to RNase H cleavage. Preliminary experiments with the 2′-*O*-methyl derivatives of CRD-ODN3, CRD-ODN4, and CRD-ODN5 in the gel shift assay confirmed that these derivatives could also block RNA-protein complexes in vitro and that the rank order of activity observed with the phosphodiester versions was maintained with the 2′-*O*-methyl-modified oligonucleotides (data not shown).

ODNs were added to K562 cells with Superfect as a carrier molecule. Protocols from the manufacturer suggested a constant ratio of Superfect to oligonucleotide. Because of the observed toxicity of Superfect to K562 cells at concentrations higher than 30 μl/5-ml culture (data not shown), we limited our initial characterizations to ODN concentrations of $\leq$200 nM. Protein and RNA were isolated from treated cells 24 h after ODN addition, and samples were analyzed for c-*myc* mRNA and protein levels.

Treatment of cells with 100 nM CRD-ODN4 resulted in a 45% drop in c-*myc* mRNA levels compared with cells treated with Superfect alone (Fig. 4A). The specificity of this effect was tested with 100 nM ODN4-MM, which is CRD-ODN4 with three mismatches. ODN4-MM had a small effect (15%) that was not statistically different from the control. When the effect on c-*myc* protein levels was determined, CRD-ODN4 treatment was found to result in a 55% reduction, compared with only 5% for ODN4-MM (Fig. 4B). At 100 nM, CRD-ODN3 and CRD-ODN5 were found to have intermediate effects on c-*myc* mRNA and protein, paralleling the intermediate effects of these different ODNs in the gel shift assay.

We then proceeded to characterize the dose response of the ODNs by treating K562 cells with 10 to 200 nM ODNs. For these experiments, we focused on CRD-ODN4 and compared its effects with those of a 2′-*O*-methyl derivative of antimyc-aug (Table 1). Both CRD-ODN4 and antimyc-aug inhibited c-*myc* protein levels. At 200 nM, a maximum effect of 65% inhibition of c-*myc* protein levels was observed for CRD-ODN4, with antimyc-aug having a 45% inhibition at this concentration. CRD-ODN4, therefore, was at least as effec-

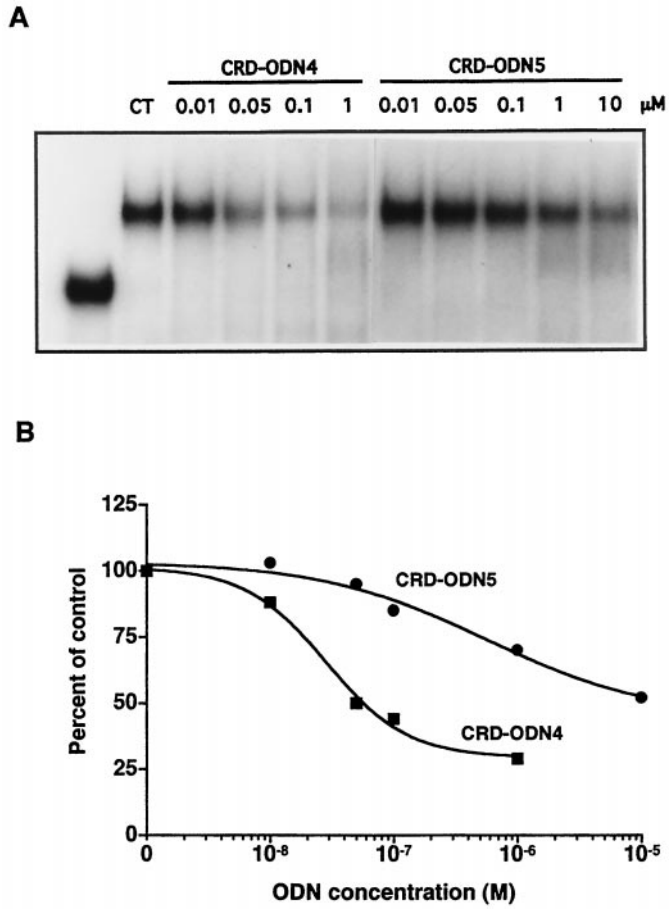

Fig. 2. Inhibition of RNA-protein interaction by antisense oligonucleotides is concentration dependent. RSW from human K562 cells was incubated with ^{32}P-labeled CRD RNA in the presence of increasing concentrations of CRD-ODN4 or CRD-ODN5. RNA-protein complexes were separated by gel electrophoresis, and the radioactivity associated with the complex was quantitated with a PhosphorImager. A, autoradiograph of a gel shift experiment showing the CRD-BP-RNA complex in the absence (lane 2, CT) and presence of 0.01, 0.05, 0.1, and 1 μM CRD-ODN4 or 0.01, 0.05, 0.1, 1, and 10 μM CRD-ODN5. B, the data from the gel shift were quantified after exposure to a Phosphor screen. Complex formation after addition of CRD-ODN4 (■) or CRD-ODN5 (●) are plotted as a percentage of control (CT). Data shown are representative of three separate experiments.

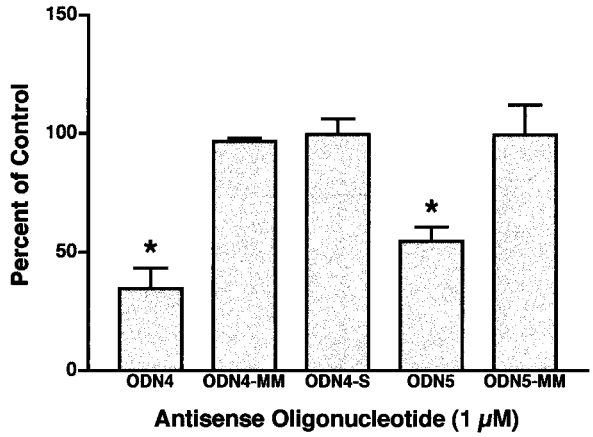

Fig. 3. Sequence specificity of the inhibition of RNA-protein interaction by ODNs. RSW from human K562 cells was incubated with ^{32}P-labeled CRD RNA in the presence of 1 μM of the indicated ODN molecule. RNA-protein complexes were separated by gel electrophoresis, and the radioactivity associated with the complex was quantitated with a PhosphorImager and plotted as a percentage of control. ODN4-S corresponds to ODN4-SENSE. Data shown are mean ± S.E. for three separate experiments. *$P < .01$, significantly different from control.

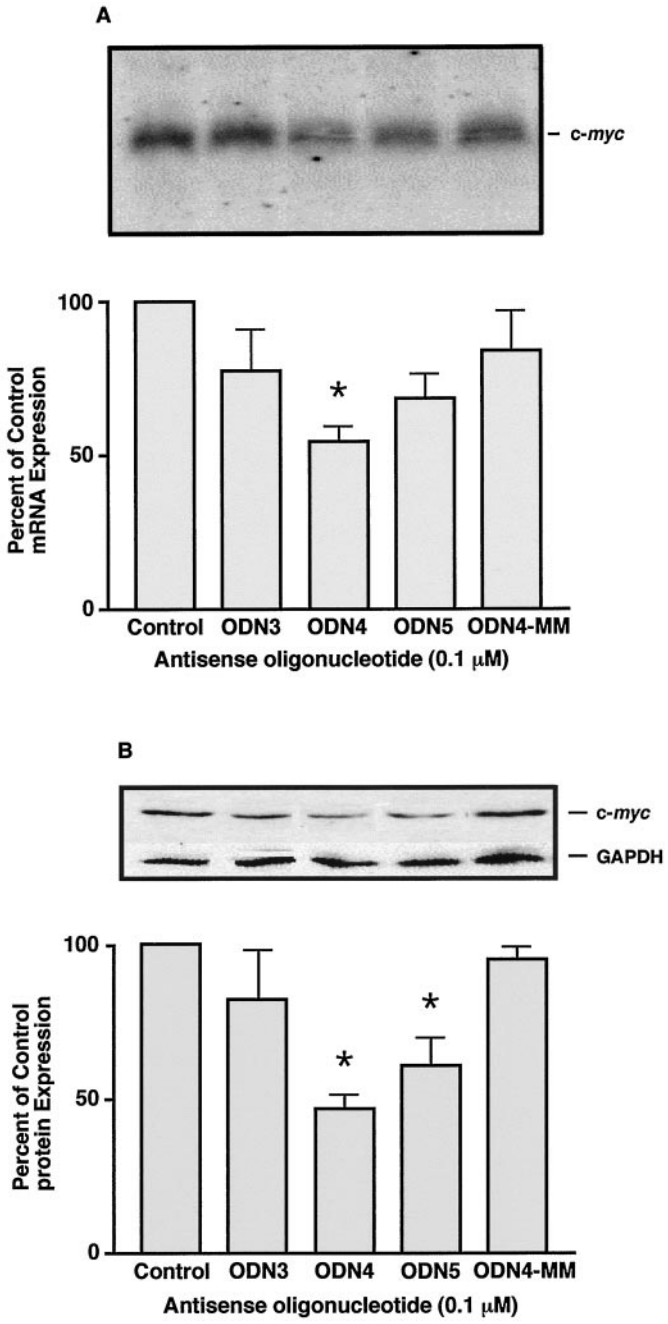

Fig. 4. Effect of ODNs on c-*myc* mRNA and protein levels in cells. K562 cells were treated for 24 h with 100 nM 2′-*O*-methyl derivatives of the indicated ODN, and RNA and protein were isolated as described in *Materials and Methods*. A, RNA (20 μg/lane) was separated by gel electrophoresis, transferred to Zetaprobe membrane, and probed for c-*myc* and GAPDH mRNAs as described in *Materials and Methods*. Radioactive signals were detected by autoradiography (top). c-*myc* mRNA levels were quantitated by use of STORM and normalized to GAPDH (to adjust for loading). Levels were expressed as a percentage of the Superfect control (bottom). Data shown are mean ± S.E. for three separate experiments. B, protein (50 μg/lane) was separated by SDS-polyacrylamide gel electrophoresis, transferred to Immobilon-P membrane, and probed for c-*myc* ($M_r \approx 67{,}000$) and GAPDH ($M_r \approx 37{,}000$) proteins. Bound antibody was detected by chemiluminescence (top). c-*myc* protein levels were quantitated by densitometry and normalized to GAPDH (to adjust for loading). Levels were expressed as a percentage of the Superfect control (bottom). Data shown are mean ± S.E. for three separate experiments. *$P < .05$, significantly different from control (paired *t* test).

tive as antimyc-aug in reducing c-*myc* protein levels (Fig. 5B). In contrast to this similar effect on c-*myc* protein levels, the effect of CRD-ODN4 and antimyc-aug on c-*myc* mRNA levels was dramatically different. Whereas CRD-ODN4 inhibited c-*myc* mRNA levels (50% at 200 nM; Fig. 5A), antimyc-aug treatment actually increased the c-*myc* mRNA levels 2-fold (Fig. 5A).

Effect of CRD-ODN4 and Antimyc-aug on c-*myc* mRNA Stability. The different response of c-*myc* mRNA to treatment with CRD-ODN4 and antimyc-aug suggests that these ODN molecules are acting through different mechanisms. From the results in Fig. 5A, it seemed possible that inhibiting CRD-BP binding with CRD-ODN4 resulted in enhanced degradation of the c-*myc* mRNA (e.g., because of endonucleolytic cleavage) (Bernstein et al., 1992; Lee et al., 1998). Antimyc-aug, on the other hand, might lead to an increase in c-*myc* mRNA stability as a secondary effect of translational inhibition (Brewer and Ross, 1989; Baker et al., 1997). To test this, we determined the half-life of the c-*myc* mRNA after treatment with CRD-ODN4 or antimyc-aug. Cells were treated with the antisense oligonucleotides or the Superfect carrier alone as a control. After 6 h of treatment, the ODNs were removed, and cells were treated with the transcriptional inhibitor DRB. RNA samples isolated at increasing time after DRB addition were analyzed by Northern blot (Fig. 6). In cells treated with Superfect alone, c-*myc* decays with a half-life of 33 ± 2 min ($n = 4$). After 6 h of pretreatment with antimyc-aug, the half-life increased to 70 ± 6 min ($n = 3$), which was significantly different from the control ($P < .05$). The half-life of c-*myc* mRNA after CRD-ODN4 treatment was 31 ± 5 min ($n = 3$), which was not significantly different from the control (Fig. 6).

The half-life of c-*myc* mRNA was also measured after a 24-h treatment with the ODNs. The decay patterns were similar to those observed after 6 h, except that the effect of antimyc-aug was less pronounced (data not shown), perhaps because of ODN degradation over time.

Comparison of the Effect of Antimyc-aug and CRD-ODN4 on K562 Cell Growth. To determine whether the reduction in c-*myc* expression results in an inhibition of cell growth, we used an MTT assay to assess cell number in treated and untreated cells. Cell number is an indirect measure of effects of the ODNs on cell growth and/or cell death. We treated cells with scrambled versions of the ODNs as controls (Table 1) and cells with Superfect alone. For these experiments, we used concentrations of ODNs up to 750 nM and an incubation time of 48 h. Superfect was used at a constant ratio (6 μl/μg ODN) up to 200 nM, after which it was used at the constant level of 30 μl/5-ml culture. Uptake of the ODNs therefore may not be directly proportional to amount added at concentrations >200 nM.

Cell number was decreased by both antimyc-aug and CRD-ODN4 (Fig. 7). Although we limited the concentration of Superfect to no more than 30 μl/5-ml culture, this concentration still had some effect on cell number, which likely contributes to some of the effect observed with the ODNs (Fig. 7). The maximum reduction in cell number (70%) was observed with 750 nM CRD-ODN4. This effect was significantly greater than the effect of antimyc-aug at the same concentration (50% inhibition) (Fig. 7). Both ODNs had a greater effect on cell growth than did their corresponding scrambled controls or the Superfect alone (Fig. 7).

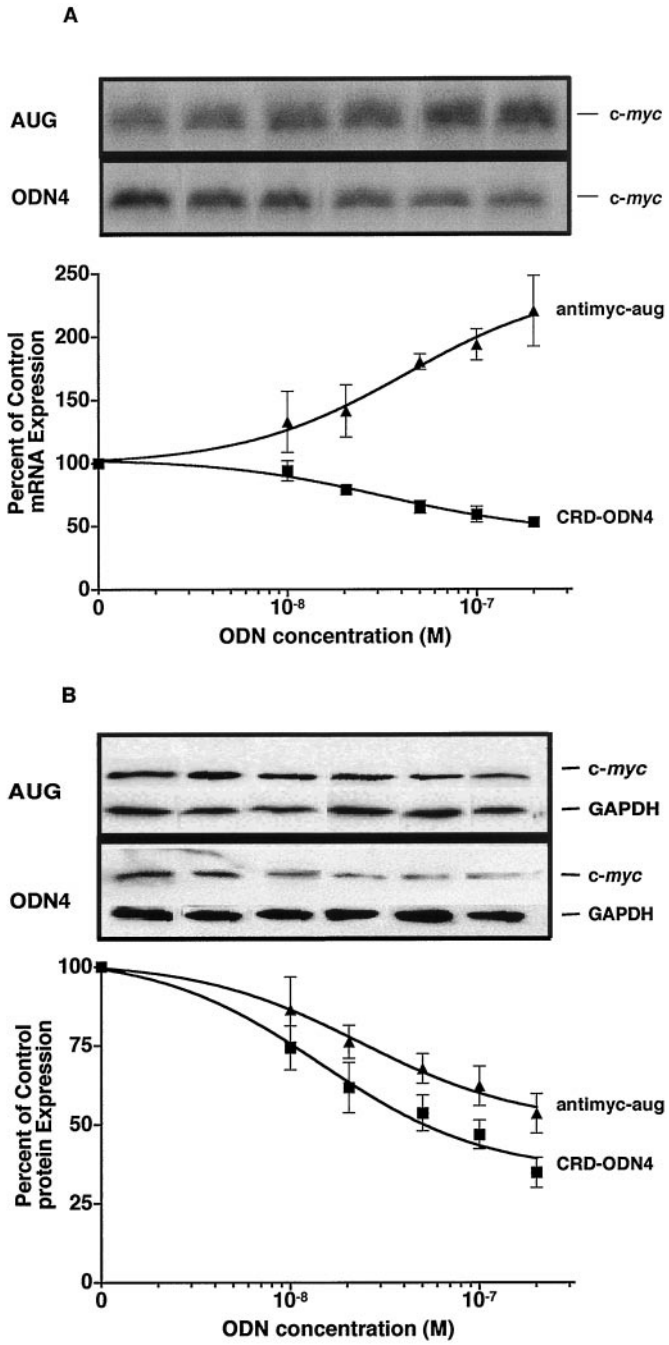

Fig. 5. Concentration-dependent effect of CRD-ODN4 and antimyc-aug on c-*myc* protein and mRNA in K562 cells. K562 cells were treated for 24 h with 0, 10, 20, 50, 100, or 200 nM either a 2′-*O*-methyl derivative of CRD-ODN4 (ODN4; ■) or a 2′-*O*-methyl derivative of antimyc-aug (AUG; ▲) followed by isolation of RNA and protein as described in *Materials and Methods*. A, RNA (20 μg/lane) was separated by gel electrophoresis, transferred to Zetaprobe membrane, and probed for c-*myc* and GAPDH mRNAs as described in *Materials and Methods*. Radioactive signals were detected by autoradiography (top) and quantitated with STORM, and c-*myc* mRNA levels were normalized to GAPDH (to adjust for loading). Levels were expressed as a percentage of the Superfect control (bottom). Data shown are mean ± S.E. for three separate experiments. B, protein (50 μg/lane) was separated by SDS-polyacrylamide gel electrophoresis, transferred to Immobilon-P membrane, and probed for c-*myc* and GAPDH proteins. Bound antibody was detected by chemiluminescence (top). c-*myc* protein levels were quantitated by densitometry and normalized to GAPDH (to adjust for loading). Levels were expressed as a percentage of the Superfect control (bottom). Data shown are mean ± S.E. for three separate experiments.

Overall, CRD-ODN4 had a more potent effect on cell number than antimyc-aug. For both ODNs, the dose response for effect on K562 cell number was shifted to the right when compared with concentrations required for inhibition of c-*myc* expression. Higher concentrations may be needed to maintain ODN concentrations in the cell for the longer time frames (48 h) required to see an effect on growth.

Discussion

Within the cell, mRNAs are associated with proteins that have functions in mRNA processing, transport, localization, stability, and translation (McCarthy and Kollmus, 1995). We are among the first to consider protein binding sites on mRNAs as targets for therapeutic ODNs. A lack of effort in this area may be related to concerns that proteins might prevent or reduce ODN binding, leading to reduced activity

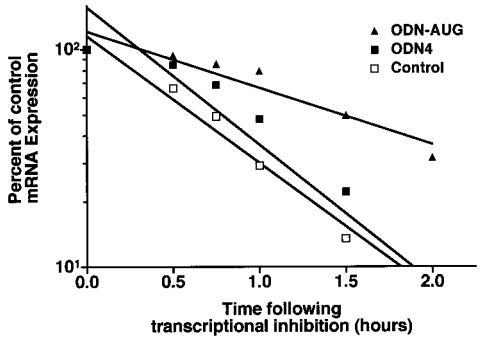

Fig. 6. c-*myc* mRNA decay in the presence and absence of CRD-ODN4 or antimyc-aug. K562 cells were treated for 6 h with 200 nM 2′-*O*-methyl derivatives of CRD-ODN4 (ODN4; ■), antimyc-aug (AUG; ▲), or Superfect alone (control; □). Cells were then treated with RPMI 1640 medium containing 100 μM DRB. RNA was isolated at the indicated times as described in *Materials and Methods*. RNA (15 μg/lane) was separated by gel electrophoresis, transferred to Zetaprobe membrane, and probed for c-*myc* and GAPDH mRNAs as described in *Materials and Methods*. c-*myc* mRNA levels were quantitated by using STORM and normalized to GAPDH (to adjust for loading). Data shown are representative of three separate experiments.

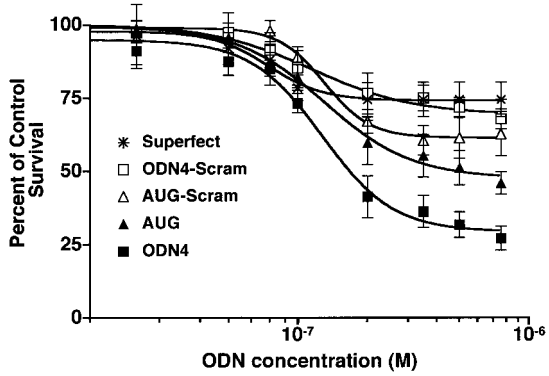

Fig. 7. Comparison of the effect of CRD-ODN4 and antimyc-aug on K562 cell growth. K562 cells were treated for 48 h with 10 to 750 nM 2′-*O*-methyl derivatives of CRD-ODN4, antimyc-aug, or the corresponding scrambled controls. In the last 3 h of the incubation, MTT was added to the wells. Analysis of reduced MTT dye was carried out as described in *Materials and Methods*. Absorbance was corrected for background and expressed as a percentage of untreated cells. Eight replicate wells of each concentration curve were analyzed for each ODN, and the complete experiment was repeated twice (ODN4-scram and aug-scram) or three times (CRD-ODN4, antimyc-aug, or Superfect alone). Data shown are mean ± S.E. ■, CRD-ODN4; ▲, antimyc-aug; □, ODN4-scram; △, aug-scram; ∗, Superfect alone.

(Stein and Cheng, 1993; Gewirtz et al., 1998). There is evidence, however, that these regions are in fact accessible (Politz et al., 1995), possibly because of frequent "breathing" of the RNA-protein complexes. We set out to test whether a specific RNA-protein interaction could be inhibited by an ODN and whether this inhibition could disrupt function and lead to a biological effect.

We started with a gel shift assay established previously for the characterization of the c-*myc* RNA-binding protein, CRD-BP (Prokipcak et al., 1994). We tested for direct competition between ODN molecules and the CRD-BP for the RNA in vitro (Figs. 1 and 2). The greatest inhibition of binding (70–75%) was achieved with CRD-ODN4, with intermediate inhibition observed for CRD-ODN3, CRD-ODN5, and CRD-ODN8. This inhibition of RNA-protein interaction was sequence-specific, because neither sense oligonucleotides nor those containing three mismatches were able to influence binding. Comparison of CRD-ODN4 and CRD-ODN5 provides additional evidence of a strong sequence dependence; although these ODNs overlapped by six nucleotides, there was a distinct shift in the dose response, with CRD-ODN4 being more effective at lower concentrations (Fig. 2).

To test our ODNs in cells, we used 2′-*O*-methyl derivatives, which, in addition to being resistant to RNase H, have a higher affinity for RNA than phosphorothioate oligonucleotides (Chiang et al., 1991; Monia et al., 1993). This characteristic may be required for efficient competition with the protein for the c-*myc* mRNA. When these oligonucleotides were transfected into the human erythroleukemia cell line K562, the relative ability of the 2′-*O*-methyl ODNs to inhibit c-*myc* protein and mRNA levels paralleled their activity in the gel shift assay, with CRD-ODN4 having the greatest activity, followed by CRD-ODN5 and CRD-ODN3 (Fig. 4). This correlation between in vitro effects and those observed in cells supports the hypothesis that the biological activity of the CRD-ODN molecules results from their influence on the interaction between the CRD-BP and the c-*myc* mRNA in cells.

CRD-ODN4 had a significant effect on K562 cells growth (70% reduction at 750 nM), being more effective at growth inhibition than a 2′-*O*-methyl derivative targeting the translation initiation codon (antimyc-aug). Although the effects we observed were substantial, we believe that this system has even greater potential for decreasing c-*myc* and inhibiting cell growth. In designing these experiments, we chose ODN sequences along the CRD to be equivalent in their G/C content (Table 1) to be able to compare the activities of the different oligonucleotides without the complication of the influence of altered melting temperatures. This constraint likely resulted in ODN molecules that were not ideal. Possible improvements to CRD-ODN4 include increasing ODN length and/or shifting its position slightly along the CRD sequence. Increasing the intracellular stability of the ODN by altering the backbone structure also would likely improve activity (Baker et al., 1997). Because we are not limited to antisense derivatives that work through RNase H degradation, we can take advantage of novel ODN derivatives that have been developed that possess greater stability, reduced binding to extracellular proteins, and greater affinity for RNA (Gryaznov et al., 1996; Baker et al., 1997).

How does CRD-ODN4 influence c-*myc* expression? Treatment of cells with CRD-ODN4 resulted in a decrease in both c-*myc* protein and mRNA levels. The effect on mRNA was particularly striking because 2-*O*-methyl derivatives do not target mRNAs for degradation by RNase H, and their use is not usually associated with decreased mRNA levels (Chiang et al., 1991; Monia et al., 1993). This suggests that the effect of CRD-ODN4 is related to its sequence rather than to the chemistry of the oligonucleotide. Based on our model of CRD-BP function, we would predict that the CRD-BP is protecting the c-*myc* mRNA from degradation by an endonuclease and that its removal would enhance the rate of decay (Bernstein et al., 1992; Lee et al., 1998). However, when we measured the decay rate of the c-*myc* mRNA in the presence of CRD-ODN4, we were unable to detect an alteration in its half-life. This could indicate that the lowered c-*myc* steady-state mRNA levels are attributable to disruption of alternative functions of the CRD-BP, for example, in c-*myc* mRNA splicing, transport, localization, or translation (Ross et al., 1997; Nielsen et al., 1999) (see below). Alternatively, it is possible that there is an effect of CRD-ODN4 on c-*myc* mRNA decay but that we cannot detect this experimentally. Because the c-*myc* mRNA is very short-lived even in untreated cells ($t_{1/2} \approx 30$ min), detecting an increased decay is technically challenging. Additional approaches, such as analyzing for possible c-*myc* mRNA endonucleolytic cleavage products, will be required for deciphering any potential effect of CRD-ODN4 on c-*myc* mRNA decay.

Unlike CRD-ODN4, antimyc-aug treatment did not cause a drop in c-*myc* mRNA levels. There was instead an increase in c-*myc* mRNA levels that was associated with an increase in the half-life of c-*myc* mRNA. This ability of antimyc-aug to stabilize the c-*myc* mRNA is likely an indirect effect of translational inhibition. Translational inhibitors such as cycloheximide have been shown to stabilize the c-*myc* mRNA (Brewer and Ross, 1989), and ODNs targeting sites important in translation initiation have been shown to increase mRNA levels (Baker et al., 1997).

The cDNA for the CRD-BP has been recently cloned and sequenced (Doyle et al., 1998). In addition, RNA-binding proteins have been isolated that are closely related or identical with the CRD-BP. Two are involved in mRNA localization: the chicken β-actin zip code binding protein (Ross et al., 1997) and the *Xenopus* Vera (Deshler et al., 1998) or Vg1 RBP (Havin et al., 1998). The third is a family of proteins called IMPs that play a role in controlling translation of insulin-like growth factor II mRNAs during development (Nielsen et al., 1999). In the light of the multiple functions proposed for these isolated proteins, the CRD-BP may influence c-*myc* mRNA localization or translation in addition to a potential role in regulating stability. Interestingly, the proper localization of the β-actin mRNA can be inhibited in intact cells with ODNs complementary to the localization signal (Kislauskis et al., 1994), providing additional evidence that disruption of RNA-protein complexes in intact cells is possible and may be a useful strategy to disrupt function.

The use of the ODN molecules in the gel shift assay allowed us to define more closely the binding site for the CRD-BP within the c-*myc* CRD region. The most active ODN, CRD-ODN4, binds to a region spanning 1763–1777 on the c-*myc* mRNA. The location of this ODN is consistent with previous data that identified the region 1705–1792 as the segment of the CRD most important for protein binding (Doyle et al., 1998). The sequence that CRD-ODN4 interacts

with is AGCCACAGCAUACAU. The actual nucleotides that are critical for CRD-BP binding are not known and may include residues outside this region. The sequence reported to be important for binding of the β-actin zip code binding protein to its RNA substrate is ACACCC (Ross et al., 1997), and for IMPs the sequence is thought to be UUCACGUUCAC (Nielsen et al., 1999). Although there are some similarities between these recognition sequences, the identity is not absolute. This suggests some flexibility in the ability of the CRD-BP to recognize RNA sequences, which may be related to the CRD-BP having multiple RNA-binding motifs (Doyle et al., 1998; Nielsen et al., 1999). We also cannot rule out a role for RNA structure in CRD-BP interaction with the RNA.

Recently, the purification of a nuclease that preferentially cleaves the c-*myc* mRNA in the CRD region has been reported (Lee et al., 1998). The cleavage sites are located through the region 1727–1736, which is ≈30 nucleotides 5′ to the region we have identified as being the most important for interaction with the CRD-BP. CRD-ODN2 (Table 1) interacts with nucleotides 1720–1738, which overlaps this proposed nuclease cleavage site (Lee et al., 1998). In our experiments, CRD-ODN2 has no ability to inhibit CRD-BP binding in vitro (Fig. 1). However, previous use of this sequence, synthesized as an N3 ′-P5′ phosphoramidate derivative, has shown it has the ability to inhibit expression of c-*myc* in HL60 cells but not in U87 glioblastoma cells (Gryaznov et al., 1996). It is unknown whether this ODN can influence the nuclease cleavage of c-*myc* mRNA or whether differences in expression of the nuclease or CRD-BP explain its cell-type specific activity.

If disruption of the CRD-BP is necessary for the change in c-*myc* expression that we observe with CRD-ODN4, then we would predict that CRD-ODN4 would have greater biological activity in cells expressing the CRD-BP. Previous work has shown that the CRD-BP is expressed abundantly in transformed cells and in fetal tissues but is undetectable in adult tissues, suggesting it is an oncofetal protein (Leeds et al., 1997; Nielsen et al., 1999). This relationship might enhance the selectivity of CRD-ODN4 for cancer cells. Additional work is required to confirm the importance of CRD-BP expression to CRD-ODN4 activity.

Overall, our data support a role for the CRD-BP in regulating c-*myc* mRNA levels in cells. They also suggest that targeting RNA-protein interactions may be a generally useful strategy for antisense action and that gel shift assays can be convenient screens for activity of these molecules in cells. Antisense molecules identified with this strategy have potential as therapeutic agents and may be useful as probes for studying the function of RNA-protein interactions in cells.

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