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We have discovered that certain G-rich oligonucleotides (GROs) can have potent antiproliferative activity against prostate cancer cells *in vitro*. We had previously shown that growth inhibitory GROs formed specific complexes with nuclear proteins more readily than inactive GROs. Our hypotheses are that the active GROs form structures containing G-quartets, that these structures are recognized by specific cellular proteins, and that binding to these proteins is involved in the growth inhibitory effects. Our overall aims are to assess the specificity of the antiproliferative effects, characterize the structure of GROs, identify GRO-binding proteins, and to design more active GROs by rational and combinatorial methods. A greater understanding of the mechanism of the antiproliferative effects may lead to the identification of new targets and improved therapies for the treatment of prostate cancer. We have now substantially improved our understanding of the growth inhibitory effects. Most significantly, we have now identified a GRO-binding protein, and shown that ability of GROs to bind this protein corresponds to their antiproliferative activity.

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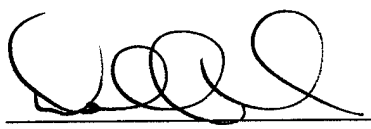

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(5) INTRODUCTION

Oligonucleotides are short fragments of DNA or RNA that can bind to small molecules, nucleic acids and proteins (1-5). We have previously discovered that certain single-stranded guanosine-rich oligonucleotides (GROs) have remarkable antiproliferative activity against prostate cancer cells in culture. Since these oligonucleotides were not designed to be antisense or antigene molecules, we postulated that their target was a cellular protein. In our preliminary data we observed that growth inhibitory oligonucleotides bound to a specific, unknown protein with higher affinity than inactive GROs.

Oligonucleotides that have guanosine-rich sequences are known to be capable of forming G-quartet structures *in vitro*. This type of structure is also thought to be important *in vivo*, and G-quartet forming sequences have been identified in the single-stranded region of telomeres (6). We hypothesized that the antiproliferative GROs would form G-quartets, and suggested telomerase as a potential target protein.

The overal goals of this proposal were to understand the mechanism of these antiproliferative oligonucleotides, to determine their relevance as potential therapeutic agents in the treatment of prostate cancer, and to design more potent and/or selective antiproliferative oligonucleotides. In order to do this, our aims were to identify the target protein(s), characterize the GRO structure, and to use combinatorial and rational design methodologies to identify new therapeutic GROs. We have now made considerable progress in achieving these objectives. We have further demonstrated the relationship between protein binding affinity and antiproliferative activity, and have identified a nucleolar protein, nucleolin, as a potential target protein. We have also confirmed that active GROs form G-quartet structures, and have generated a preliminary model for the most active oligonucleotide.

(6) BODY

6.1 EXPERIMENTAL METHODS

Experimental procedures are explained in detail in the attached manuscript (Bates *et al.*, *J. Biol. Chem.*, **274**, 26369-26377, 1999, Appendix 3) and are described briefly below.

Cell growth assays. Cells were plated at low density (10^2 to 10^3 cells per well, depending on cell line) in the appropriate serum-supplemented medium in 96-well plates. The following day (day 1) oligonucleotide (or water as control) was added to the culture medium to give a final concentration of 15 μM. On days 2, 3 and 4 further oligonucleotide equivalent to half the initial dose was added. Cells were assayed using the MTT assay (7) on days 1, 3, 5, 7 and 9 after plating. The culture medium was not changed throughout the duration of the experiment (which was the time required for untreated cells to grow to confluence). Experiments were performed in triplicate and bars represent the standard error of the data.

Electrophoretic mobility shift assays (EMSAs). Oligonucleotides were 5'-labeled with ^{32}P using T4 kinase. Labeled oligonucleotide (final concentration 1 nM, approximately 50,000 cpm) was pre-incubated for 30 min at 37°C, either alone or in the presence of unlabeled competitor oligonucleotide. Nuclear extracts were added and the sample was incubated for a further 30 min at 37°C. Both the pre-incubation and binding reaction were carried out in Buffer A (20 mM Tris.HCl pH 7.4, 140 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM phenylmethyl sulfonyl fluoride and 8% v/v glycerol). Electrophoresis was carried out using 5% polyacrylamide gels in TBE buffer (90 mM Tris borate, 2 mM

EDTA). For the UV crosslinking experiments, samples were incubated as described then placed on ice and irradiated at 5 cm from the source using the 'autocrosslink' function of a Stratagene UV Stratalinker. Following irradiation, samples were electrophoresed under denaturing conditions on an 8% polyacrylamide-SDS gel.

Southwestern blotting. Nuclear extracts were electrophoresed on an 8% polyacrylamide-SDS gel and transferred to polyvinylidene difluoride (PVDF) membrane by electroblotting. Immobilized proteins were denatured and renatured by washing for 30 min at 4°C with 6 M guanidine.HCl followed by washes in 1:1, 1:2 and 1:4 dilutions of 6M guanidine in HEPES binding buffer (25 mM HEPES pH 7.9, 4 mM KCl, 3 mM $MgCl_2$). The membrane was then washed for 1 h in a 5% solution of non-fat dried milk (NDM) in binding buffer. Hybridization with labeled oligonucleotide (1-4 x 10^6 cpm) took place for 2 h at 4°C in HEPES binding buffer in the presence of agents to block non-specific binding.

Western blotting. Western blotting was carried out at room temperature in PBS buffer containing Tween 20 at 0.1% v/v (for polyclonal antibody) or 0.05% (monoclonal antibody). PVDF membranes were blocked with PBS-Tween 20 containing 5% NDM for 1 h, washed and incubated for 1 h with a 1:1000 dilution of nucleolin antiserum (a gift from Dr. Marie Wooten, Auburn University) or 1 μg/ml nucleolin monoclonal antibody (MBL Ltd., Japan) in PBS-Tween 20. The membranes were washed 3 times for 5 min with PBS-Tween 20 and incubated for 1 h with secondary antibody diluted in PBS-Tween20 (1:1000 anti-rabbit IgG-HRP or 1:2000 anti-mouse IgG-HRP). After washing as above the blot was visualized using ECL reagent (Amersham) according to the manufacturer's instructions.

Capture of biotinylated oligonucleotide-protein complexes. MDA-MB-231 cells were grown to 50% confluence in 90 mm dishes. The cells were treated by addition of 5'-biotinylated oligonucleotide at a final concentration of 5 μM. After incubation for 2h at 37°C, cells were washed extensively with PBS and lysed by addition of 1ml of lysis buffer followed by incubation at -20°C for 10 min. Lysate was added to streptavidin coated magnetic beads and incubated 10 min at room temperature. Beads were captured and unbound sample was removed. The beads were then washed twice with 1 ml lysis buffer and again with 1 ml Buffer A. Finally, captured proteins were eluted by addition of 50 μl of loading buffer (containing 1% SDS and 5% 2-mercaptoethanol) and incubation for 15 min at 65°C.

Detection of G-quartets by UV spectroscopy. Oligonucleotides were resuspended in Tm buffer (20 mM Tris. HCl pH 8.0, 140 mM KCl, 2.5 mM $MgCl_2$). Samples were annealed by boiling for 5 min and allowing to cool slowly to room. Thermal denaturation/renaturation experiments were carried out using a Pharmacia Biotech Ultrospec 2000 instrument equipped with a Peltier effect heated cuvette holder and temperature controller. Absorbance at 295 nm was monitored over a temperature range of 25-95°C or 20-90°C at a heating/cooling rate of 0.5°C/min.

Oligonucleotide uptake. MDA-MB-231 cells (5 x 10^5 cells/well) were seeded in 24 well plates. After 24 h, 5 nmol of unlabeled oligonucleotide plus 5 x 10^6 cpm (approximately 1 pmol) of ^{32}P-labeled oligonucleotide was added directly to the culture medium. This gave a final concentration of 10 μM. Cells were incubated at 37°C for 10 or 26 h. They were then washed 3 times with PBS, removed from the plate by trypsinization, washed and collected in 100 μl of PBS. A 50 μl aliquot was counted by scintillation counting to assess cell-associated radioactivity.

Molecular modeling. The GRO29A dimer was constructed using a subset of the X-ray crystal structure of d(TGGGGT) to model the G-quartets. The thymine inter-quartet bases were added using standard G-quartet backbone geometry. The loop regions were optimized using molecular dynamics

(Macromodel 6.0, GB/SA continuum solvation using the AMBER* force field). The starting model was fully solvated with TIP3P water molecules in a 10 Å box and equilibrated using standard protocols within AMBER. Molecular dynamics was used to generate the final structure (300ps production with coordinate averaging over the last 50ps).

6.2 RESULTS

Antiproliferative activity of GRO29A. In the preliminary data of the original proposal, we noted that the oligonucleotide GRO29A appeared to be more active than the oligonucleotide which we originally discovered (GRO15A). We have repeated these experiments and confirmed that GRO29A has greater antiproliferative activity than GRO15A when administered to DU145 prostate cancer cells (Figure 1), as measured using the MTT assay. This assay uses colorimetric analysis to determine metabolization of a tetrazolium dye, which is directly proportional to the number of viable cells. The MTT assay is less time-consuming and requires less oligonucleotide (since 96 well plates are used) than the trypan blue assay originally proposed.

Activity of GROs in other cell lines. To determine the specificity of the antiproliferative activity, we compared the ability of the oligonucleotides to inhibit the growth of prostate cancer cells, other human tumor cell types, and non-malignant fibroblasts. Figure 2 shows the effects of oligonucleotide treatment on MDA-MB-231 (breast cancer), HeLa (cervical cancer) and HS27 (foreskin fibroblasts). GRO29A and GRO15A consistently inhibited the growth of both the breast and cervical cancer cell lines. Although there was also some inhibitory effect on the growth of the non-malignant cell line, HS27, it was encouraging that this effect appeared to be less pronounced than for the tumor cell lines.

GROs bind to a specific cellular protein that also binds the human telomere sequence. At the time of the original proposal, we had shown that radiolabeled GRO15A formed two complexes when incubated with HeLa nuclear proteins. Both of these complexes could be competed for by unlabeled active oligonucleotides (GRO15A, GRO29A), but not by unlabeled inactive oligonucleotides (GRO26A, GRO15B). This suggested the existence of GRO-binding protein(s), and that there was a relationship between antiproliferative activity of GROs and their ability to compete for binding to this protein. We have confirmed this hypothesis by performing a number of further experiments.

First we examined complex formation by using the electrophoretic mobility shift assay (EMSA), as previously. 5'-Radiolabeled GROs were incubated with HeLa nuclear extracts, alone or in the presence of unlabeled competitor. We had hypothesized that G-quartet formation was important for antiproliferative activity and protein binding, and therefore we included a G-quartet-forming oligonucleotide as a competitor in this experiment. This single stranded oligonucleotide, TEL, contains four repeats of the human telomere sequence (5'-TTAGGG), and folds to form intramolecular quartets *in vitro* (8). Figure 3A shows the formation of a stable protein-oligonucleotide complex (marked by '*'). In the absence of competitors, this band was intense when the labeled oligonucleotide was one of the growth inhibitory oligonucleotides, GRO15A or GRO29A (lanes 1 and 5), but the inactive oligonucleotide, GRO26A, formed only a weak complex (lane 9). This experiment also showed that the complex could be effectively competed by either unlabeled antiproliferative oligonucleotide or TEL, but not by the inactive GRO26A.

To confirm that the protein that binds to the GROs also binds directly to the telomere oligonucleotide, we carried out a similar experiment in which TEL was labeled. Labeled TEL formed

two complexes with nuclear extracts in the absence of competitor oligonucleotides (bands A and B, Figure 3B). The slower migrating TEL-protein complex (band A) was competed for by unlabeled growth inhibitory oligonucleotides (GRO15A, GRO29A) but not inactive oligonucleotides (GRO26A, GRO15B). The faster migrating complex (band B) was specific for TEL and was not competed for by G-rich oligonucleotides. Hence binding of competitor GROs was characterized by a decrease in the intensity of band A and an increase in the intensity of band B (due to release of labeled TEL from band A complex). To ensure that competition was due to binding of the GRO to the protein component of complex A, and not a result of interaction between GRO and TEL oligonucleotide, we carried out a mobility shift on a 15% polyacrylamide gel. No shifted bands were observed when labeled TEL was incubated with GROs in the absence of protein (data not shown).

To determine the approximate molecular weight of the protein involved in complex A, and to confirm direct binding of the protein to oligonucleotides, we carried out a UV crosslinking study. 5'-Labeled oligonucleotides and HeLa nuclear extracts were incubated alone or in the presence of unlabeled competitor oligonucleotides. The samples were then irradiated with UV light resulting in crosslink formation between protein residues and thymidines in the oligonucleotide. The protein was thus radiolabeled and could be detected on an SDS-polyacrylamide gel. Figure 3C shows the results of this experiment. Both TEL and GRO15A crosslinked to a protein (marked '*') which was competed for by antiproliferative oligonucleotides and TEL, but not by inactive GRO26A. The most active oligonucleotide, GRO29A, also formed this approximately 110 kDa complex and another complex of higher molecular weight (not shown). Inactive GRO26A produced a barely visible band at ~110 kDa (not shown).

The molecular weight of the nuclear protein was more accurately determined by southwestern blotting. HeLa nuclear extracts were electrophoresed on a 8% polyacrylamide-SDS gel and transferred to a PVDF membrane. The membrane was blocked and cut into strips. Each strip was incubated at 4°C with a ^{32}P-labeled G-rich oligonucleotide in the presence of unrelated unlabeled double stranded and single stranded DNA to block non-specific binding. Figure 3D shows active oligonucleotides GRO15A and GRO29A hybridized to a single protein band at 106 kDa (the band was exactly adjacent to a 106 kDa molecular weight marker, not shown). Inactive oligonucleotides GRO15B and GRO26A hybridized only weakly to this protein. These experiments also demonstrate that binding of GROs to p106 is highly specific, since only a single protein band is recognized with high affinity (see Figure 3D). This was not simply a result of hybridization to an abundant protein, as India ink staining of immobilized nuclear extracts showed the presence of many other protein bands which were equally or more intense than the band at 106 kDa (data not shown).

For expedience, the experiments shown in Figure 3 used HeLa nuclear extracts (purchased from Promega). EMSA and southwestern blotting experiments carried out using nuclear extracts from DU145 prostate cancer cells showed similar results, indicating the presence of the same ~110 kDa protein that bound selectively to active GROs.

Correlation of growth inhibitory effects of oligonucleotides with protein binding. The data presented in Figures 1-3 suggest a correlation between activity and protein binding, at least for the four oligonucleotides examined.To further investigate the relationship between activity and binding to GRO-binding protein we synthesized four more G-rich oligonucleotides and compared their effects with active (GRO29A) and inactive (GRO15B) oligonucleotides.

Figure 4 shows that the growth inhibitory effect of the oligonucleotides (measured by the MTT assay) correlated with their ability to compete for the GRO-binding protein (measured by EMSA). Three of the new oligonucleotides (GRO14A, GRO25A and GRO28A) displayed a moderate antiproliferative activity, but were not as potent as GRO29A. Oligonucleotide GRO14B showed no antiproliferative activity and was unable to compete for protein binding. Correspondingly, the moderately active oligonucleotides were able to compete with TEL for binding to the nuclear protein, though not as effectively as GRO29A.

Identification of GRO-binding protein. Two previous reports describe binding of the nucleolar protein, nucleolin, to the G-rich telomere sequence. Ishikawa *et al.* (9) identified a 50 kDa protein from HeLa extracts which bound to 5'-$(TTAGGG)_4$-3'. Microsequence determination suggested that this was a proteolytic fragment of nucleolin. Binding of the full length, purified 106 kDa nucleolin protein was demonstrated independently by Dickinson and Kohwi-Shigematsu (10). Since our protein was of the correct molecular weight and also bound to 5'-$(TTAGGG)_4$-3' (TEL), we tested the hypothesis that the G-rich oligonucleotide binding protein was nucleolin.

Nuclear extracts from HeLa cells (purchased from Promega) or MDA-MB-231 breast cancer cells (obtained in our laboratory by standard procedures) were electrophoresed and transferred to PVDF membrane. The immobilized proteins were probed for binding to ^{32}P-labeled GRO15A using the southwestern procedure described, and visualized by overnight exposure to autoradiographic film. The same membrane was stripped of oligonucleotide and western blotted using nucleolin antiserum as primary antibody and a horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody. The blot was visualized by incubation with a chemiluminescence detection reagent followed by a 20 seconds exposure to autoradiographic film. The results are shown in Figure 5. Southwestern blots of nuclear extracts showed an intense band upon hybridization with radiolabeled GRO15A at 106 kDa (HeLa) or 116 kDa (MDA-MB-231). The western blot of MDA-MB-231 nuclear proteins shows one intense band at 116 kDa and weaker bands at about 50 kDa. In HeLa extracts the nucleolin antibody recognizes multiple bands at approximately 50, 75, 106 and 120 kDa. Most importantly, in both cell lines the band that was recognized by GRO15A exactly corresponded to a band recognized when the membrane was stripped and western blotted with nucleolin antibody. Similar results were obtained using DU145 nuclear extracts, identifying a 116 kDa band that bound to both GRO15A and nucleolin antibody. Nucleolin can be phosphorylated in cells by a number of kinases, and is also highly susceptible to proteolysis (11-17). We believe that the difference in the molecular weight of proteins detected in different cell lines may de due to differently phosphorylated or degraded forms of nucleolin being the predominant species. The difference in the intensities of the bands shown in the southwestern blots in Figure 5 may be due to the preferential binding of GRO15A to one form of nucleolin (apparently the 106 kDa species) over others.

Nucleolin is an abundant, multifunctional protein located predominantly in the nucleoli of cells, and there is evidence that the levels of nucleolin are dependent on the rate of cellular proliferation. The characteristics and functions of nucleolin are described in more detail in the 'Conclusions' section.

Evidence that oligonucleotides bind to GRO-binding protein/ nucleolin inside cells. To determine whether binding of the specific protein occurred within the cell, we used biotinylated G-rich oligonucleotides to treat MDA-MB-231 cells. Streptavidin-coated magnetic beads were then used to capture oligonucleotide-protein complexes after lysing the cells with an immunoprecipitation-type

buffer. This procedure was carried out for cells that were treated with either an active oligonucleotide (5'-Biotin-GRO15A) or an inactive oligonucleotide (5'-Biotin-GRO15B), and untreated cells as a control. Equal volumes of each sample were electrophoresed and transferred to a PVDF membrane. This was analyzed by india ink staining, southwestern blotting with radiolabeled GRO15A, and western blotting with a nucleolin monoclonal antibody. India ink staining of the membrane showed a major protein band at 116 kDa that was present in cells treated with biotinylated GRO15A, but was absent in untreated cells, and of a lower intensity in cells treated with inactive biotinylated GRO15B (data not shown). The southwestern and western blots (Figure 6) confirm that this captured protein binds to both GRO15A and a nucleolin antibody.

This experiment showed that a 116 kDa protein was specifically captured from cells treated with biotinylated GROs and that this protein was recognized also by a nucleolin antibody. Also, that more of this protein was captured by active GRO15A than was captured by the less active GRO15B. While we cannot absolutely exclude that the protein-oligonucleotide association took place during cell lysis or oligonucleotide capture, it is unlikely that the oligonucleotide would exist in a free, uncomplexed state inside the cell. We believe therefore that these results provide strong evidence for binding of oligonucleotide to the 116 kDa protein inside the cell (or possibly at the cell surface).

G-quartet formation by antiproliferative GROs. To investigate the formation of G-quartet structures by the G-rich oligonucleotides, we used a UV melting technique described by Mergny *et al.* (18). This method relies on the fact that dissociation of G-quartets leads to a decrease in absorbance at 295 nm, and is reported (18) to give a more reliable indication of intramolecular G-quartet formation than measurement at 260 nm. As a control for G-quartet formation, we used the single stranded oligonucleotide, which known to form a G-quartet structure *in vitro* (8). Figure 7 shows the annealing curve for this sequence. G-quartet formation is indicated by a clear transition with a melting temperature of 66°C. The transition was reversible and a slight hysteresis was observed between heating and cooling curves (not shown) at 0.5 °C/ min indicating a fairly slow transition. The most active oligonucleotide, GRO29A, showed a similar profile, clearly indicating the presence of G-quartets. The slightly less active oligonucleotide, GRO15A, showed a decrease in absorbance between 20 and 50°C. This is suggestive of G-quartet formation, but a clear transition is not seen since the melting temperature is lower than for TEL or GRO29A. The curves for the two inactive oligonucleotides, GRO15B and GRO26A, showed no transitions characteristic of intramolecular G-quartet formation under these conditions.

Relative uptake of oligonucleotides. To determine if the antiproliferative activity of G-rich oligonucleotides could be explained by their differential uptake into cells, we assessed the cellular uptake of 5'-radiolabeled oligonucleotides. Although this method may underestimate absolute cellular uptake of oligonucleotide due to the action of phosphomonoesterase in removing the 5'-label, it can provide useful information when comparing relative uptake (19,20).

Figure 8 shows the relative uptake of oligonucleotides into cells after 10 h, as measured by cell associated radioactivity. The relative order of uptake was also the same at 26 h. The presence of intact oligonucleotide inside cells was verified by polyacrylamide electrophoresis of cell lysates. Whereas Figure 8 shows that there were differences in the extent of oligonucleotide uptake depending on sequence, these did not correlate with antiproliferative activity. For example, an inactive oligonucleotide, CRO (not shown) was taken up with similar efficiency to the most active

oligonucleotide, GRO29A. Hence, the differential growth inhibitory properties of the oligonucleotides cannot be explained in terms of differences in cell uptake

Molecular modeling studies of GRO29A. We had previously determined that GRO29A migrates on native polyacrylamide gels as two stable species whose mobilities were consistent with monomer and dimer forms. Using this information, we were able to construct a model for GRO29A that was consistent with all of the experimental data. This structure consists of two molecules of GRO29A in a folded, antiparallel chair formation. The structure is stabilized by a total of 8 G-quartets. The antiparallel GGTGG motif is the first structure of this type reported (Figure 9).

6.3 SUMMARY OF PROGRESS IN ACCOMPLISHING STATEMENT OF WORK TASKS

We have made considerable progress in reaching the goals outlined in the statement of work. The accomplishment of many of the tasks has been expedited by the early identification of the GRO-binding protein, and demonstration that it is likely to be involved in the mechanism of action of the antiproliferative oligonucleotides.

Task1: To quantitate the antiproliferative activity, induction of apoptosis, reversibility of growth imhibition and selectivity for malignant cells. We have verified and documented our preliminary observation that GRO29A is another antiproliferative G-rich oligonucleotide with greater activity than GRO15A. We have developed an MTT assay to measure antiproliferative activity that requires smaller amounts of oligonucleotide. GRO15A and GRO29A have now been tested in a number of other cell lines, and have been shown to be active against a number of other tumor types. Data also suggest that a non-malignant cell line is less sensitive to the effects of GROs than DU145 prostate cancer cells. We are currently investigating apoptosis and reversibility of the effects.

Task 2: To determine the stability, uptake and intracellular location of GRO15A and GRO29A in serum-containing medium. Preliminary data show that the 3'-amino modified oligonucleotides are stable in serum-containing medium for at least 24 h (not shown). Relative uptake of oligonucleotides has also been determined. Experiments examining intracellular location of oligonucleotides have so far been inconclusive.

Task 3: To characterize the structure of GRO15A and GRO29A and identify the biologically active conformations. We have carried out experiments that indicate G-quartet formation by active oligonucleotides, but not inactive oligonucleotides. A preliminary molecular model for the most active oligonucleotide (GRO29A) has been generated.

Task 4: To identify and characterize proteins binding to GRO15A and GRO29A. We have generated additional data to show that the ability of oligonucleotides to inhibit growth is related to binding to a specific cellular protein (GRO-binding protein). We have now identified this protein as nucleolin, and have also generated evidence that binding of GROs to this protein occurs intracellularly.

Task 5: To use combinatorial methods to identify new therapeutic oligonucleotides which have a similar mechanism of action. This task has not yet been initiated, however, the identification of the target protein will allow the use of the SELEX technique to identify oligonucleotides with strong binding to nucleolin.

Task 6: To characterize the ability of GRO15A and GRO29A to inhibit telomerase. We have shown that the putative target protein that binds to GRO15A and GRO29A, also binds to the human

telomere sequence. Therefore, it is possible that telomerase may bind to G-quartet forming GROs. We are currently developing an assay to measure the effect of GROs on telomerase activity.

6.4 PROBLEMS ENCOUNTERED

We were unable to microsequence the GRO-binding protein band identified by southwestern blotting and isolated from PVDF membrane, probably due to inaccessibility of the N-terminal residues. Partial proteolysis of the band yielded insufficient peptides for sequence analysis. However, we are convinced from our immunoblotting studies that this band was nucleolin.

(7) KEY RESEARCH ACCOMPLISHMENTS

- Extension of correlation between oligonucleotide antiproliferative activity and binding to a specific nuclear protein (GRO-binding protein)
- Demonstration that GRO-binding protein also binds to G-quartet-forming telomere sequence
- Identification of GRO-binding protein as nucleolin
- Demonstration of G-quartet formation by active GROs
- Generation of strong evidence suggesting that GROs are associated with GRO-binding protein/nucleolin inside cells
- Generation of preliminary molecular model for GRO29A
- Measurement of relative uptake of oligonucleotides into cells
- Demonstration of antiproliferative activity in other tumor cell types

(8) REPORTABLE OUTCOMES

Published Manuscript:

1. P. J. Bates, J. B. Kahlon, S. D. Thomas, J. O. Trent and D. M. Miller, "Antiproliferative Activity of G-rich Oligonucleotides Correlates with Protein Binding", *J. Biol. Chem.*, **274**, 26369-26377 (1999).

Patent Filed:

1. D. M. Miller, P. J. Bates and J. O. Trent, "Protein-binding G-rich Oligonucleotides for the treatment of malignancies".

Abstracts:

1. P. J. Bates, J. B. Kahlon, S. D. Thomas, N. Vigneswaren, J. O. Trent and D. M. Miller. "Novel antiproliferative G-rich oligonucleotides that bind to a specific cellular protein". AACR 90th Annual Meeting, April 10-14 1999, Philadelphia.
2. P. J. Bates, S. D. Thomas, J. O. Trent and D. M. Miller. "Inhibition of prostate cancer cell growth by novel G-rich oligonucleotides". AACR 89th Annual Meeting, March 28 - April 1 1998, New Orleans.

(9) CONCLUSIONS

One of the major accomplishments has been the identification of nucleolin as a GRO-binding protein. We believe that the strong correlation between nucleolin binding and antiproliferative activity of oligonucleotides is highly suggestive that this protein may be the target for the action of the GROs. Nucleolin is an abundant multifunctional 110 kDa phosphoprotein, thought to be located predominantly in the nucleolus of proliferating cells (for reviews, see 21,22). It has been reported to have many

functions, including ribosome biogenesis (21-23) and as a cytoplasmic-nucleolar shuttle protein (24-26). Perhaps most importantly, levels of nucleolin are known to relate to the rate of cellular proliferation (27,28), being elevated in rapidly proliferating cells, such as malignant cells, and lower in more slowly dividing cells. For this reason, nucleolin may be an attractive therapeutic target for the treatment of malignant disease. Our findings that GRO29A has a lesser effect in non-malignant fibroblasts, but is able to inhibit other tumor cell types, indicate that the antiproliferative effects, while not specific for prostate cancer cells, may be selective for rapidly dividing cancer cells.

In support of our findings that nucleolin binds selectively to G-rich oligonucleotides which form stable G-quartet structures, Maizels *et al.* (29,30) have recently demonstrated binding of purified nucleolin to G-quartet forming DNA sequences from immunoglobulin switch regions and ribosomal DNA. It is likely that nucleolin has currently undefined functions *in vivo* that depend on recognition of G-quartet forming sequences in ribosomal DNA, switch region sequences or telomeres.

In order to develop more potent growth inhibitory GROs that work by a similar mechanisms, we have developed several assays to screen for active and nucleolin-binding oligonucleotides. The MTT assay allows us to screen for antiproliferative oligonucleotides in a 96-well format without requiring trypsinization of monolayer cells. We have also developed an EMSA screen that allows us to easily determine relative nucleolin binding affinities by measuring the abilities of GROs to compete for a complex formed by TEL and prostate cancer cell extracts (or other tumor cell extracts).

Our future studies will address the remaining tasks proposed. We will further investigate the structure of the active oligonucleotides by HPLC isolation of monomer and dimer species. Guanosines involved in G-quartets will be identified by DMS footprinting and by 7-deazaguanosine substitution. The model of the dimer is being used to develop and refine a structure-activity relationship that will aid in the design of oligonucleotides with greater activity. Based on this model, we have initiated structure-activity studies to determine the importance of various structural features, such as G-quartets, and loop and tail regions. We aim to define the minimum sequence required for antiproliferative activity, which will facilitate NMR studies of the GROs.

The proposed BIAcore studies would be simplified by the availablity of purified nucleolin (GRO-binding protein), and we will therefore attempt this purification which has been described previously in the literature. We will then used this purified nucleolin (or a synthetic peptide fragment of nucleolin) to study affinitiy and kinetics of binding, and in SELEX studies to identify more potent oligonucleotides.

The new oligonucleotides designed by both rational and SELEX approaches will be tested for antiproliferative activity (MTT assay), protein binding (EMSA) and G-quartet formation (UV melting assay). We envisage that these studies will translate to *in vivo* testing of the therapeutic potential of the most active GROs, initially in animal models of prostate cancer, and eventually in human trials.

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APPENDIX 1: FIGURE LEGENDS

Figure 1: (A) MTT assays showing the growth of DU145 tumor cells treated with 3'-protected G-rich oligonucleotides or water as a control. The cell type used is shown in the top left corner of each curve. The OD 595 nm value is proportional to the number of viable cells in the sample. (B) Sequences of oligonucleotides used in this and subsequent experiments.

Figure 2: MTT assays showing the growth of MDA-MB-231 (breast cancer), HeLa (cervical cancer) and HS27 (foreskin fibroblasts) treated with GROs or water as a control.

Figure 3: (A) Electrophoretic mobility shift assay (EMSA) showing binding of ^{32}P-labeled G-rich oligonucleotides to 5 μg HeLa nuclear extracts and competition by unlabeled competitor oligonucleotides (100-fold molar excess over labeled oligonucleotide). Competitor oligonucleotides are abbreviated to T (TEL), 29 (GRO29A), 26 (GRO26A) and 15A (GRO15A). (B) EMSA showing complexes formed between ^{32}P-labeled TEL oligonucleotide (1 nM) and 5 μg HeLa nuclear extracts, and competition by unlabeled competitor G-rich oligonucleotides (10 or 100 nM). (C) SDS-polyacrylamide gel showing complexes formed by UV crosslinking of labeled oligonucleotides and HeLa nuclear extracts incubated in the absence or presence of unlabeled competitor (100-fold molar excess). (D) Southwestern blots of HeLa nuclear extracts probed with ^{32}P labeled G-rich oligonucleotides (2 x 10^6 cpm, approximately 0.75 nmol). The probe GRO is indicated at the top of each strip.

Figure 4: (A) MTT assay of MDA-MB-231 cells treated with a single 10 μM dose of G-rich oligonucelotide, or phosphate buffered saline (PBS) as a control. The assay was performed on day 9 (oligonucleotide added on day 1). (B) EMSA showing complex formed by binding of 5 μg MDA-MB-231 nuclear extracts to ^{32}P-labeled TEL oligonucleotide, and competition by unlabeled G-rich oligonucleotides (10-fold molar excess).

Figure 5: Southwestern (SW) and Western (W) blots probed respectively with ^{32}P-labeled active G-rich oligonucleotide (GRO15A) or nucleolin antiserum. Left panel shows MDA-MB-231 nuclear extracts (5 μg/lane); right panel shows HeLa nuclear extracts (Promega Inc., 5μg/lane).

Figure 6: Southwestern and Western blots of proteins captured from the lysates of MDA-MB-231 cells which had been treated with no oligonucleotide (none), active G-rich oligonucleotide (15A) or less active G-rich oligonucleotide (15B).

Figure 7: UV thermal renaturation curves to assess G-quartet formation by G-rich oligonucleotides. The oligonucleotide name is shown in the top right corner of each graph. Experiments were carried out in Tm buffer (20 mM Tris. HCl pH 8.0, 140 mM KCl, 2.5 mM $MgCl_2$).

Figure 8: Relative uptake of G-rich oligonucleotide by MDA-MB-231 breast cancer cells. A mixture of unlabeled and 5'-^{32}P-labeled oligonucleotide was added to the medium of cells to give a final concentration

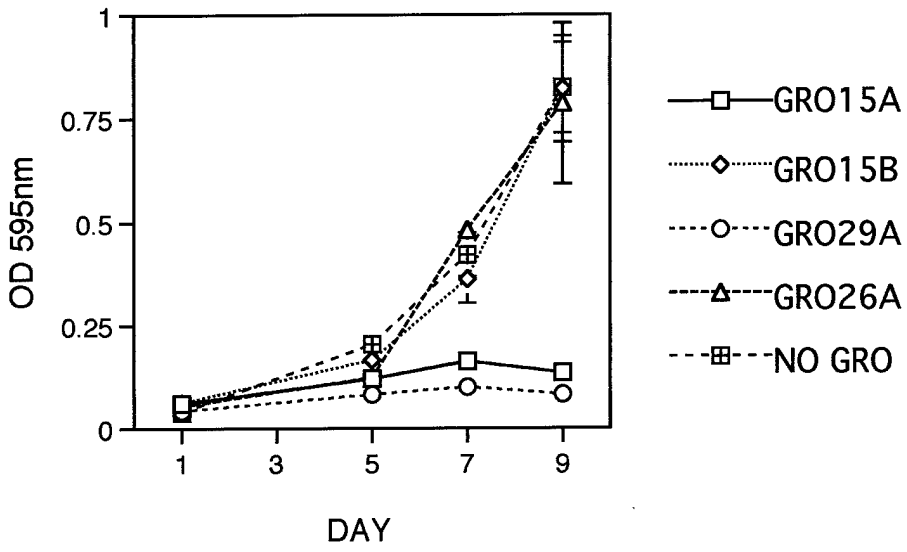
of 10 μM. After incubation for 10 h, cells were washed extensively to remove extracellular oligonucleotide and uptake was assessed by counting cell-associated radioactivity.

Figure 9: (A) Schematic representation of GRO29A dimer showing eight G-quartets with intermediate thymines (orange). The tail sequence is dTTT and loop regions are dTTGT. (B) Model of GRO29A dimer generated by molecular modeling and dynamics showing G-quartets in white and inter-quartet thymine in orange. One molecule of GRO29A is indicated in red, the other in blue.

APPENDIX 2: FIGURES

Figure 1: Antiproliferative activity of GRO29A against DU145 prostate cancer cells

A



B **Oligonucleotide Sequences**

3'-Aminoalkyl modified:

GRO15A: 5'-GTTGTTTGGGGTGGT
GRO15B: 5'-TTGGGGGGGGTGGGT
GRO26A: 5'-GGTTGGGGTGGGTGGGGTGGGTGGGG
GRO29A: 5'-TTTGGTGGTGGTGGTTGTGGTGGTGGTGG

GRO14A: 5'-GTTGTTTGGGGTGG
GRO14B: 5'-TTGGGGGGGGTGGG
GRO25A: 5'-GGTTGGGGTGGGTGGGGTGGGTGGG
GRO28A: 5'-TTTGGTGGTGGTGGTTGTGGTGGTGGTG
CRO: 5'-TTTCCTCCTCCTCCTTCTCCTCCTCCTCC
MIX1: 5'-GACTGTACCGAGGTGCAAGTACTCTAT

Unmodified oligonucleotides:

TEL: 5'-TTAGGGTTAGGGTTAGGGTTAGGG

Figure 2: Activity of GROs in other cell lines

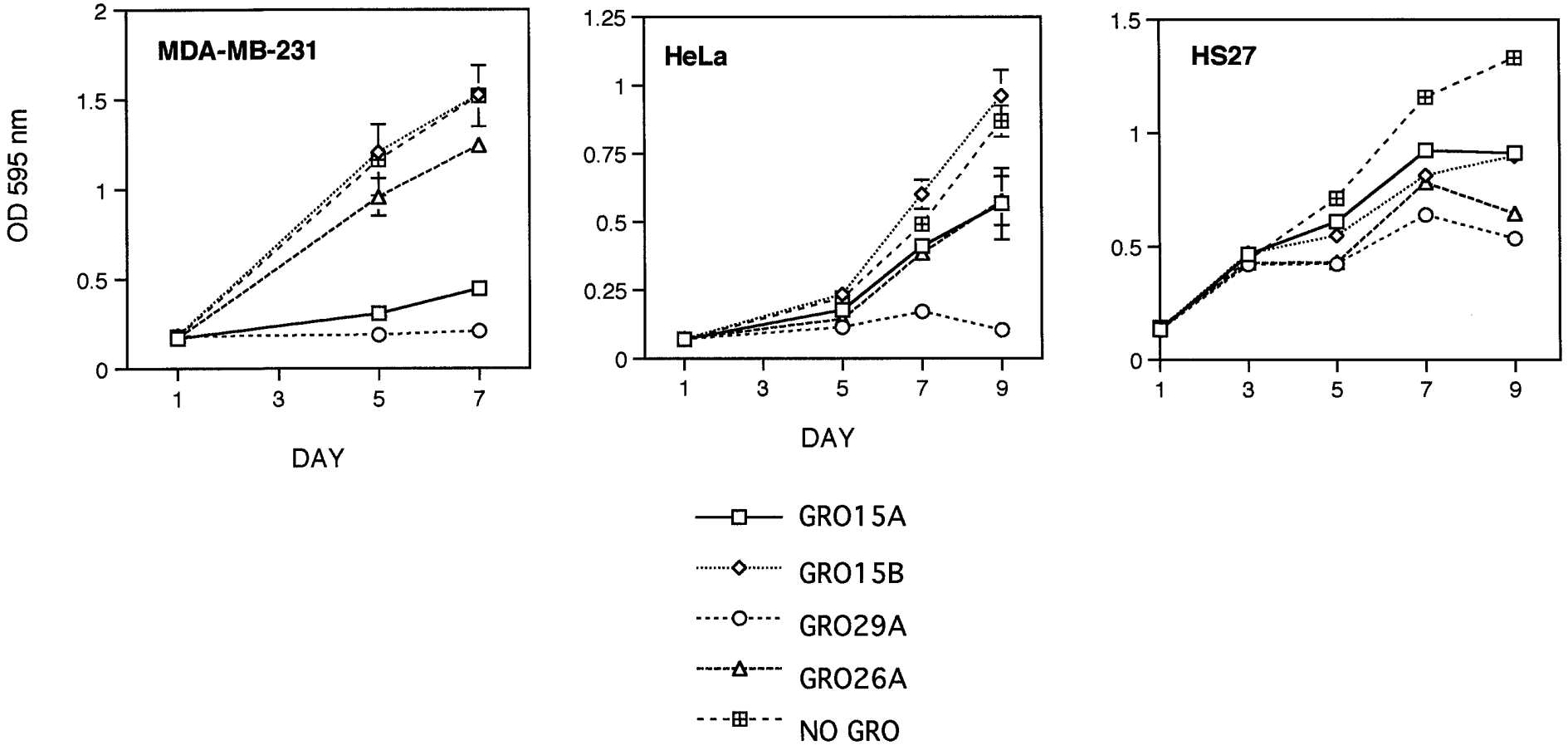


Figure 3: GROs bind to a specific nuclear protein that also binds to a telomere oligonucleotide

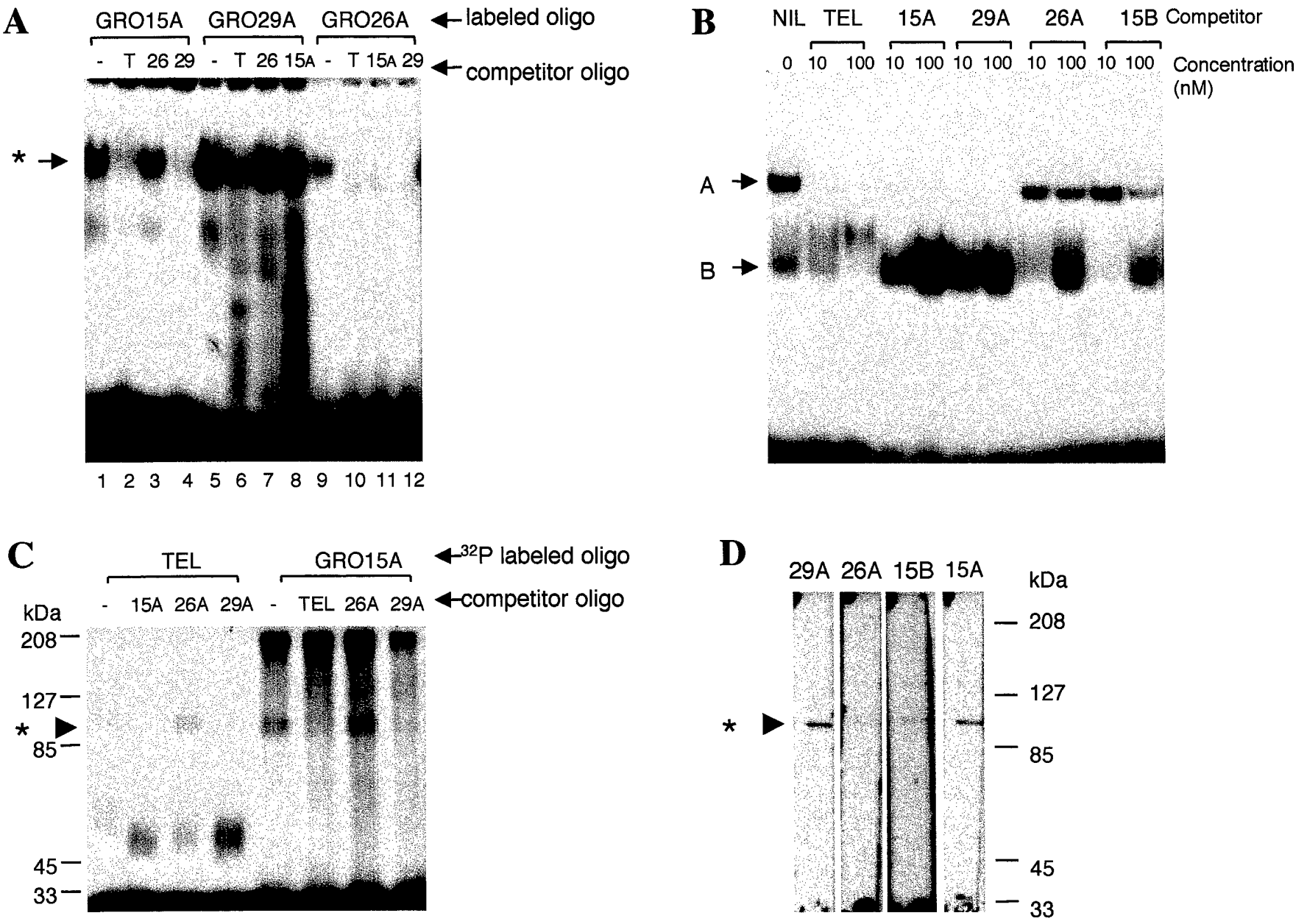
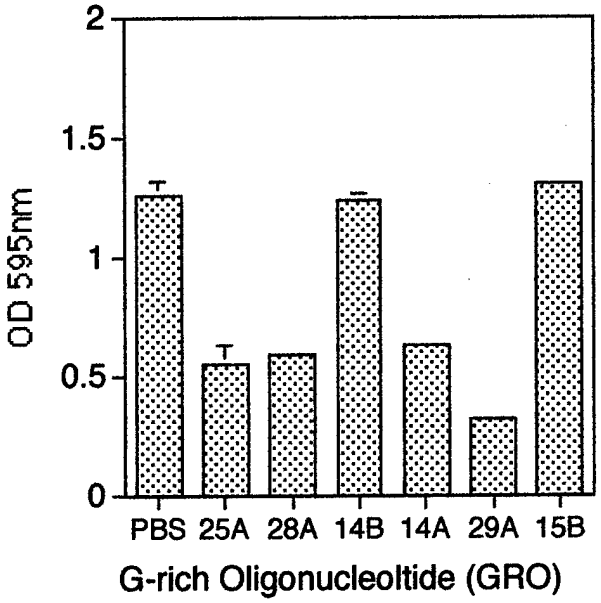


Figure 4: Correlation between GRO antiproliferative activity and protein binding

A



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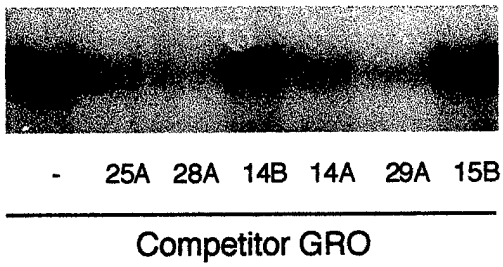


Figure 5: GRO-binding protein is nucleolin

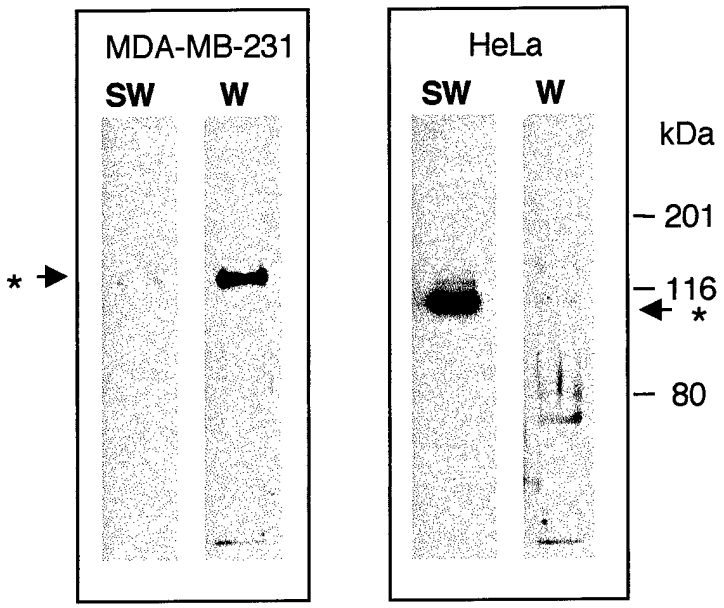
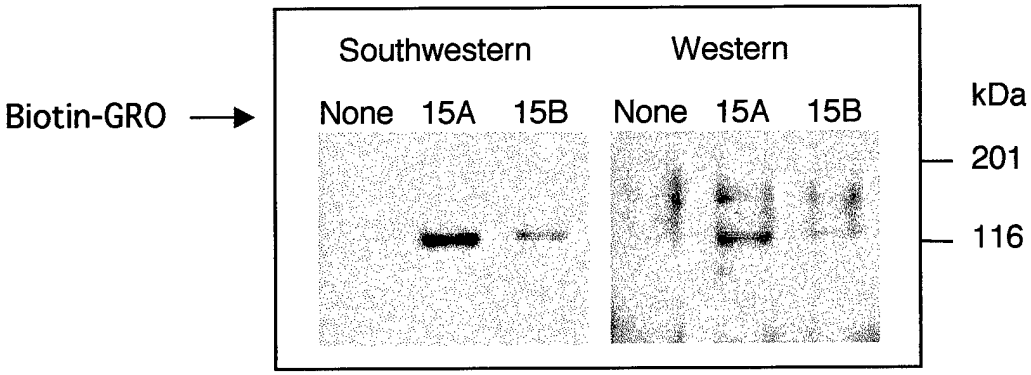


Figure 6: Capture of GRO-binding protein/nucleolin from treated cells



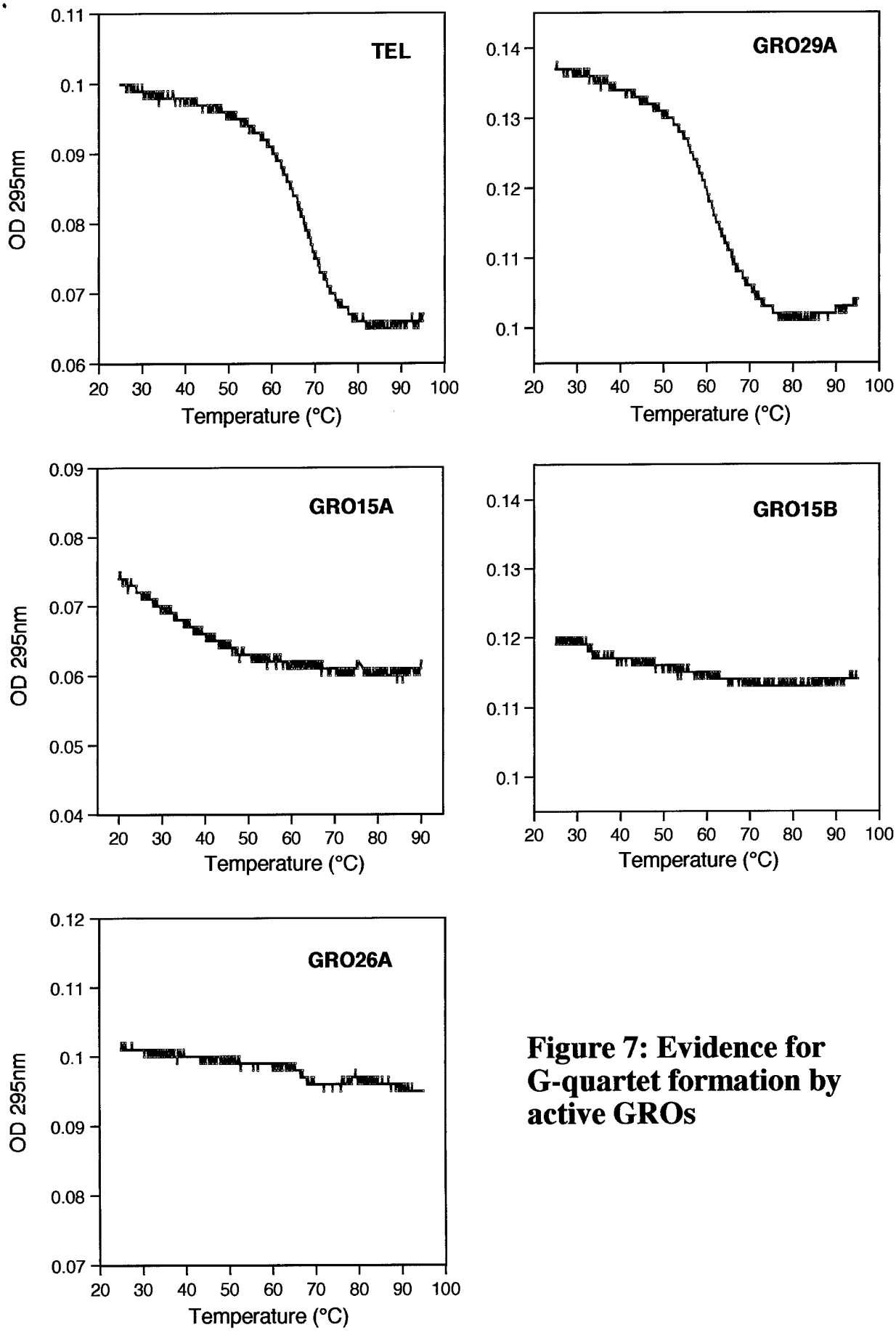


Figure 7: Evidence for G-quartet formation by active GROs

Figure 8: Relative cellular uptake of GROs

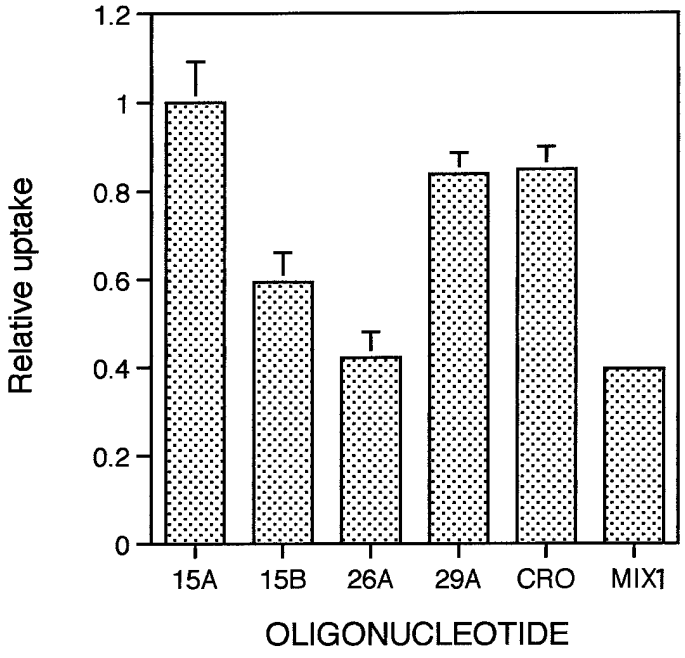
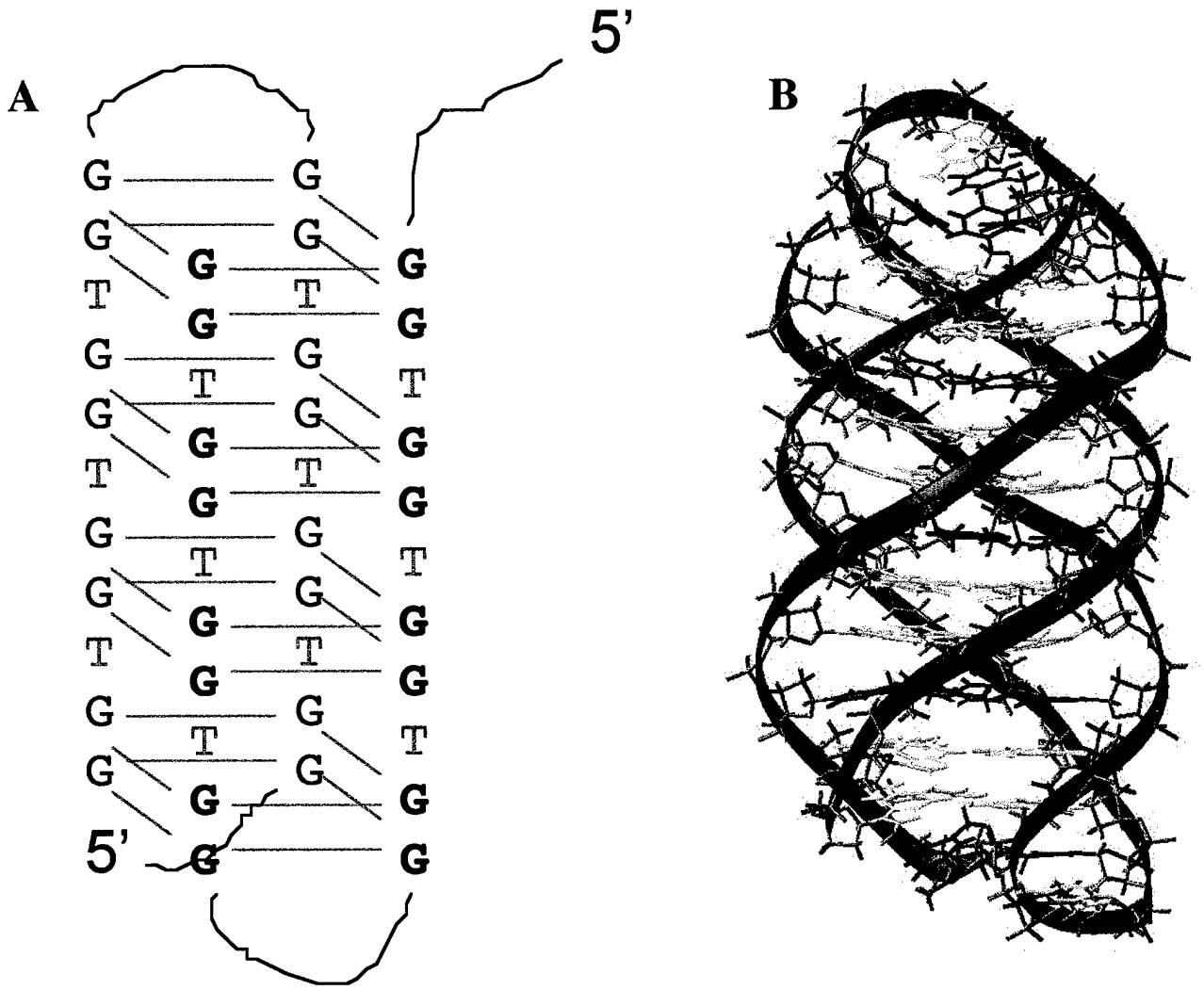


Figure 9: Molecular modeling studies of GRO29A



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Antiproliferative Activity of G-rich Oligonucleotides Correlates with Protein Binding*

(Received for publication, March 16, 1999, and in revised form, June 17, 1999)

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Oligonucleotides have been extensively studied as antisense or antigene agents that can potentially modulate the expression of specific genes. These strategies rely on sequence-specific hybridization of the oligonucleotide to mRNA or genomic DNA. Recently, it has become clear that oligonucleotides often have biological activities that cannot be attributed to their sequence-specific interactions with nucleic acids. Here we describe a series of guanosine-rich phosphodiester oligodeoxynucleotides that strongly inhibit proliferation in a number of human tumor cell lines. The presence of G-quartets in the active oligonucleotides is demonstrated using an UV melting technique. We show that G-rich oligonucleotides bind to a specific cellular protein and that the biological activity of the oligonucleotides correlates with binding to this protein. The G-rich oligonucleotide-binding protein was detected in both nuclear and cytoplasmic extracts and in proteins derived from the plasma membrane of cells. We present strong evidence that this protein is nucleolin, a multifunctional phosphoprotein whose levels are related to the rate of cell proliferation. Our results indicate that binding of G-rich oligonucleotides to nucleolin may be responsible for their non-sequence-specific effects. Furthermore, these oligonucleotides represent a new class of potentially therapeutic agents with a novel mechanism of action.

Oligonucleotides have the potential to recognize unique sequences of DNA or RNA with a remarkable degree of specificity. For this reason they have been considered as promising candidates to realize gene-specific therapies for the treatment of malignant, viral, and inflammatory diseases. Two major strategies of oligonucleotide-mediated therapeutic intervention have been developed, namely the antisense and antigene approaches. The antisense strategy aims to down-regulate expression of a specific gene by hybridization of the oligonucleotide to the specific mRNA, resulting in inhibition of translation (1–4). The antigene strategy proposes to inhibit transcription of a target gene by means of triple helix formation between the oligonucleotide and specific sequences in the double-stranded genomic DNA (5). Clinical trials based on the antisense approach are now showing that oligonucleotides can be administered in a clinically relevant way and have few toxic side effects (1, 4).

Whereas both the antisense and antigene strategies have met with some success, it has become clear in recent years that the interactions of oligonucleotides with the components of a living organism go far beyond sequence-specific hybridization with the target nucleic acid. Recent studies and reexamination of early antisense data have suggested that some of the observed biological effects of antisense oligonucleotides cannot be due entirely to Watson-Crick hybridization with the target mRNA. In some cases, the expected biological effect (*e.g.* inhibition of cell growth or apoptosis) was achieved, but this was not accompanied by a down-regulation of the target protein and was thus unlikely to be a true antisense effect (6, 7). In many cases, it was demonstrated that other non-sequence-specific oligonucleotides could exert biological effects that equaled or exceeded the antisense sequence (8–10). Although there is currently a high awareness among antisense investigators of the importance of appropriate control oligonucleotides, and the necessity of demonstrating inhibition of target protein production (11), the mechanism of non-antisense effects is poorly understood.

In particular, phosphodiester and phosphorothioate oligodeoxynucleotides containing contiguous guanosines (G) have been repeatedly found to have non-antisense effects on the growth of cells in culture (9, 10, 12). There is evidence that this activity is related to the ability of these oligonucleotides to form stable structures involving intramolecular or intermolecular G-quartets (9, 10). These are square planar arrangements of four hydrogen-bonded guanines that are stabilized by monovalent cations. Such structures are thought to play an important role *in vivo,* and putative quartet-forming sequences have been identified in telomeric DNA (13), immunoglobulin switch region sequences (14), human immunodeficiency virus, type I, RNA (15), the fragile X repeat sequences (16), and the retinoblastoma gene (17).

It has been suggested that non-antisense effects may be due to sequestration of intracellular or surface proteins by the oligonucleotide (18, 19). For G-rich oligonucleotides that can form folded or G-quartet-containing structures, this binding is not mediated by recognition of the primary sequence of the oligonucleotides but rather of their unique three-dimensional shape. However, the protein targets of these oligonucleotides have not been well characterized.

Here we identify a G-rich oligonucleotide-binding protein, and we show that the ability of G-rich oligonucleotides to bind to this protein is correlated with their propensity to form G-quartets, and with their ability to inhibit the growth of tumor cells.

EXPERIMENTAL PROCEDURES

Oligonucleotides—3′-Modified oligonucleotides were purchased from Oligos Etc. (Wilsonville, OR) or synthesized at the University of Alabama at Birmingham using 3′-C3-amine CPG columns from Glen Research (Sterling, VA). Unmodified oligonucleotides were purchased from Life Technologies, Inc. Oligonucleotides were resuspended in water, precipitated with butan-1-ol, washed with 70% ethanol, dried, and

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resuspended in sterile water or phosphate-buffered saline (PBS).[1] They were then sterilized by filtration through a 0.2-μm filter. Each oligonucleotide was checked for integrity by 5′-radiolabeling followed by polyacrylamide gel electrophoresis. The results reported in this paper were reproducible and independent of the source of synthetic oligonucleotides.

Cell Growth Assays—Cells were plated at low density (10^2 to 10^3 cells per well, depending on cell line) in the appropriate serum-supplemented medium in 96-well plates. The following day (day 1) oligonucleotide (or water as control) was added to the culture medium to give a final concentration of 15 μM. On days 2–4 further oligonucleotide equivalent to half the initial dose was added. Cells were assayed using the MTT assay (20) on days 1, 3, 5, 7, and 9 after plating. The culture medium was not changed throughout the duration of the experiment (which was the time required for untreated cells to grow to confluence). Experiments were performed in triplicate, and bars represent the standard error of the data. For the experiments shown in Fig. 5, MDA-MB-231 breast cancer cells (5×10^2 cells per well) were plated in a 96-well plate. After 24 h, a single dose of oligonucleotide (or equal volume of PBS as a control) was added to the culture medium to a final concentration of 10 μM. Viable cells were assessed using the MTT assay 7 or 9 days (as indicated in the figure legend) after plating. For the experiment using 3′-unmodified oligonucleotides (Fig. 5*D*), serum-supplemented medium was replaced by serum-free medium containing oligonucleotide (or serum-free medium alone in control wells). After incubation at 37 °C for 4 h, fetal calf serum (Life Technologies, Inc.) was added to the medium to give 10% v/v. Heparin used in these experiments was USP grade sodium salt derived from porcine intestine, purchased from Apothecon (Bristol-Myers Squibb Co). Working solutions were diluted from the stock (1000 units/ml) in sterile PBS.

Detection of G-quartets by UV Spectroscopy—Oligonucleotides were resuspended in Tm buffer (20 mM Tris·HCl, pH 8.0, 140 mM KCl, 2.5 mM $MgCl_2$) at a concentration such that $A_{260} = 0.6$ (molar concentrations ranged from 2.0 to 3.9 μM). Samples were annealed by boiling for 5 min and allowing to cool slowly to room temperature and overnight incubation at 4 °C. Thermal denaturation/renaturation experiments were carried out using an Amersham Pharmacia Biotech Ultrospec 2000 instrument equipped with a Peltier effect heated cuvette holder and temperature controller (Amersham Pharmacia Biotech). Absorbance at 295 nm was monitored over a temperature range of 25–95 or 20–90 °C at a heating/cooling rate of 0.5 °C/min.

Oligonucleotide Uptake—MDA-MB-231 cells were seeded in 24- well plates at a density of 5×10^5 cells/well. After 24 h, oligonucleotide (5 nmol of unlabeled oligonucleotide and 5×10^6 cpm (approximately 1 pmol) of 5′-^{32}P-labeled oligonucleotide) was added directly to the culture medium to give a final concentration of 10 μM. Cells were incubated at 37 °C for 10 or 26 h and were then washed 3 times with PBS. Cells were removed from the plate by trypsinization, washed, and collected in 100 μl of PBS. A 50-μl aliquot was counted by scintillation counting to assess cell-associated radioactivity. To ensure that the washing procedures were sufficient to remove all excess oligonucleotide, the final PBS wash was counted and found to be very low compared with the cell-associated radioactivity. The remaining 50-μl aliquots were boiled for 5 min and placed on ice. An equal volume of phenol/chloroform was added, and the oligonucleotides were extracted in the aqueous phase, precipitated with *n*-butyl alcohol, and analyzed by denaturing polyacrylamide gel electrophoresis on a 15% gel.

Electrophoretic Mobility Shift Assays (EMSAs)—Oligonucleotides were 5′-labeled with ^{32}P using T4 kinase. Labeled oligonucleotide (final concentration 1 nM, approximately 50,000 cpm) was preincubated for 30 min at 37 °C, either alone or in the presence of unlabeled competitor oligonucleotide. Nuclear extracts were added, and the sample was incubated a further 30 min at 37 °C. Both the preincubation and binding reaction were carried out in Buffer A (20 mM Tris·HCl, pH 7.4, 140 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 8% v/v glycerol). Electrophoresis was carried out using 5% polyacrylamide gels in TBE buffer (90 mM Tris borate, 2 mM EDTA).

UV Cross-linking—For the UV cross-linking experiments, samples were incubated as described above (EMSA). They were then placed on ice and irradiated at 5 cm from the source using the "autocross-link" function of a Stratagene UV Stratalinker. Following irradiation, samples were electrophoresed under denaturing conditions on a 8% polyacrylamide-SDS gel using a standard Tris glycine buffer and visualized by autoradiography.

Southwestern Blotting—Nuclear extracts were electrophoresed on an 8% polyacrylamide-SDS gel and transferred to polyvinylidene difluoride (PVDF) membrane by electroblotting using a Tris glycine/methanol (10% v/v) buffer. Immobilized proteins were denatured and renatured by washing for 30 min at 4 °C with 6 M guanidine HCl followed by washes in 1:1, 1:2, and 1:4 dilutions of 6 M guanidine in HEPES binding buffer (25 mM HEPES, pH 7.9, 4 mM KCl, 3 mM $MgCl_2$). The membrane was blocked by washing 1 h in a 5% solution of non-fat dried milk (NDM) in binding buffer. Hybridization with labeled oligonucleotide ($1–4 \times 10^6$ cpm) took place for 2 h at 4 °C in HEPES binding buffer supplemented with 0.25% NDM, 0.05% Nonidet P-40, 400 μg/ml salmon sperm, DNA, and 100 μg/ml of an unrelated, mixed sequence 35-mer oligodeoxynucleotide (5′-TCGAGAAAAACTCTCCTCTCCTTCCTTCC-TCTCCA-3′). Membranes were washed in binding buffer and visualized by autoradiography.

Western Blotting—Western blotting was carried out at room temperature in PBS buffer containing Tween 20 at 0.1% v/v (for polyclonal antibody) or 0.05% (monoclonal antibody). PVDF membranes were blocked with PBS/Tween 20 containing 5% NDM for 1 h, washed, and incubated for 1 h with a 1:1000 dilution of nucleolin antiserum or 1 μg/ml nucleolin monoclonal antibody (MBL Ltd., Japan) in PBS/Tween 20. The membranes were washed 3 times for 5 min with PBS/Tween 20 and incubated for 1 h with secondary antibody diluted in PBS/Tween 20 (1:1000 anti-rabbit IgG-horseradish peroxidase or 1:2000 anti-mouse IgG-horseradish peroxidase). After washing as above the blot was visualized using ECL reagent (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Capture of Biotinylated Oligonucleotide-Protein Complexes—MDA-MB-231 cells were grown to 50% confluence in 90-mm dishes. The cells were treated by addition of 5′-biotinylated oligonucleotide at a final concentration of 5 μM. After incubation for 2 h at 37 °C cells were washed extensively with PBS and lysed by addition of 1 ml of lysis buffer (50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 0.02% (w/v) sodium azide, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.5 mM dithiothreitol, 1 μg/ml aprotinin) followed by incubation at −20 °C for 10 min. Genomic DNA was sheared by repeated injection of the lysate through a fine gauge needle. Lysate was added to streptavidin-coated magnetic beads (MagneSphere, Promega Inc.) and incubated 10 min at room temperature. Beads were captured, and unbound sample was removed. Beads were then washed twice with 1 ml of lysis buffer and again with 1 ml of Buffer A. Finally proteins were eluted by addition of 50 μl of loading buffer (containing 1% SDS and 5% 2-mercaptoethanol) and incubation for 15 min at 65 °C.

Preparation of Nuclear, Cytoplasmic, and Membrane Protein Extracts—HeLa nuclear extracts used in EMSAs and Southwestern blotting were purchased from Promega Inc. (bandshift grade). Nuclear and cytoplasmic extracts were prepared from MDA-MB-231 cells using the protocol described in Ausubel *et al.* (21) Plasma membrane proteins were prepared from MDA-MB-231 cells using a method previously described (22, 23).

India Ink Staining—The membrane was incubated for 15 min at room temperature in PBS/Tween 20 containing 3 drops of Higgins India Ink 4415 and washed with distilled water.

RESULTS

Growth Inhibitory Effects of G-rich Oligonucleotides—We tested the effects of four G-rich phosphodiester oligonucleotides (GROs) on the growth of tumor cells in culture. These oligonucleotides consisted entirely of deoxyguanosine and thymidine and contained runs of at least two contiguous guanosines. For increased stability to serum nucleases, oligonucleotides were modified at the 3′-terminus with a propyl amino group. We have observed previously that this modification protects oligonucleotides from degradation in serum-containing medium for at least 24 h.

Fig. 1 shows the results of MTT assays for determining relative numbers of viable cells in treated cell lines derived from prostate (DU145), breast (MDA-MB-231, MCF-7), or cervical (HeLa) carcinomas. The sequences of the oligonucleotides used in this and later experiments are shown in Table I. Two oligonucleotides, GRO29A and GRO15A, consistently inhibited proliferation in all of the cell lines tested. For three of the cell lines, GRO29A had a more potent inhibitory effect than

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[1] The abbreviations used are: PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; GRO, guanosine-rich oligonucleotide; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NDM, nonfat dried milk; PVDF, polyvinylidene difluoride; CRO, C-rich oligonucleotide.

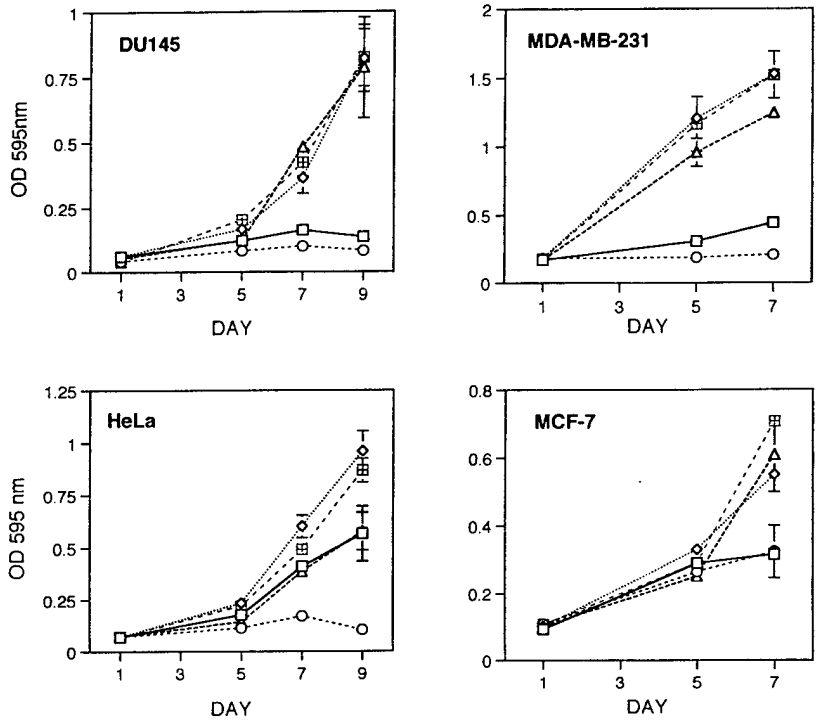

FIG. 1. **MTT assays showing the growth of tumor cells treated with 3′-protected G-rich oligonucleotides or water as a control.** The cell type used is shown in the *top left corner* of each curve. The $A_{595\ nm}$ value is proportional to the number of viable cells in the sample. □, GRO15A; ◇, GRO15B; ○, GRO29A; △, GRO26A; ⊞, water.

TABLE I
Oligonucleotide sequences

3′-Aminoalkyl modified	
GRO15A,	5′-GTTGTTTGGGGTGGT
GRO15B,	5′-TTGGGGGGGGTGGGT
GRO26A,	5′-GGTTGGGGTGGGTGGGGTGGGTGGGG
GRO29A,	5′-TTTGGTGGTGGTGGTTGTGGTGGTGGTGG
GRO14A,	5′-GTTGTTTGGGGTGG
GRO14B,	5′-TTCCCCGGGGTGGG
GRO25A,	5′-GGTTGGGGTGGGTGGGGTGGGTGGG
GRO28A,	5′-TTTGGTGGTGGTGGTTGTGGTGGTGGTG
CRO,	5′-TTTCCTCCTCCTCCTTCTCCTCCTCCTCC
MIX1,	5′-GACTGTACCGAGGTGCAAGTACTCTAT
Unmodified oligonucleotides	
TEL,	5′-TTAGGGTTAGGGTTAGGGTTAGGG
29A-OH,	5′-TTTGGTGGTGGTGGTTGTGGTGGTGGTGG
MIX2,	5′-TCGACGTGGGCCCCG
MIX3,	5′-CTAGCGGGGCCCACG

GRO15A (for MCF-7 cells, the oligonucleotides had similar effects). The growth of cells treated with two other oligonucleotides, GRO15B and GRO26A, was similar to that of the control water-treated cells (GRO26A had a weak growth inhibitory effect in MDA-MB-231 and HeLa cells). Examination of the growth-inhibited cells suggested that these effects may be cytostatic rather than cytotoxic. No indication of cell death (nonadherent cells, cell debris) was observed when viewed using a phase contrast microscope (not shown).

G-quartet Formation by G-rich Oligonucleotides—To investigate the formation of G-quartet structures by the G-rich oligonucleotides, we used a UV melting technique described by Mergny *et al.* (24). This method relies on the fact that dissociation of G-quartets leads to a decrease in absorbance at 295 nm and is reported (24) to give a more reliable indication of intramolecular G-quartet formation than measurement at 260 nm. As a control for G-quartet formation, we used a single-stranded oligonucleotide, TEL. This oligonucleotide contains four repeats of the human telomere sequence 5′-TTAGGG and is known to form a G-quartet structure *in vitro* (25). Fig. 2 shows the annealing curve for this sequence. G-quartet formation is indicated by a clear transition with a melting temperature of 66 °C. The transition was reversible and a slight hysteresis was observed between heating and cooling curves (not shown) at 0.5 °C/min indicating a fairly slow transition. The most active oligonucleotide, GRO29A, showed a similar profile, clearly indicating the presence of G-quartets. The slightly less active oligonucleotide, GRO15A, showed a decrease in absorbance between 20 and 50 °C. This is suggestive of G-quartet formation, but a clear transition is not seen since the melting temperature is lower than for TEL or GRO29A. The curves for the two inactive oligonucleotides, GRO15B and GRO26A, showed no transitions characteristic of intramolecular G-quartet formation under these conditions.

Relative Uptake of Oligonucleotides—To determine if the antiproliferative activity of G-rich oligonucleotides could be explained by their differential uptake into cells, we assessed the cellular uptake of 5′-radiolabeled oligonucleotides. Although this method may underestimate absolute cellular uptake of oligonucleotide due to the action of phosphomonoesterase in removing the 5′-label, it can provide useful information when comparing relative uptake (26, 27). Fig. 3 shows the relative uptake of oligonucleotides into cells after 10 h, as measured by cell-associated radioactivity. The order of uptake (*i.e.* GRO15A > GRO29A ~ CRO > GRO15B > GRO26A > MIX1) was the same at 26 h. The presence of intact oligonucleotide inside cells was verified by polyacrylamide electrophoresis of cell lysates.

Although Fig. 3 shows that there were differences in the extent of oligonucleotide uptake depending on sequence, these did not correlate with antiproliferative activity. For example, an inactive oligonucleotide, CRO (see Fig. 5*C*), was taken up with similar efficiency to the most active oligonucleotide, GRO29A. Hence, the differential growth inhibitory properties of the oligonucleotides cannot be explained in terms of differences in cell uptake. We noted that relative uptake appeared to correlate well with the proportion (but not the number) of thymidines in the sequence, but the significance of this observation is not clear at present.

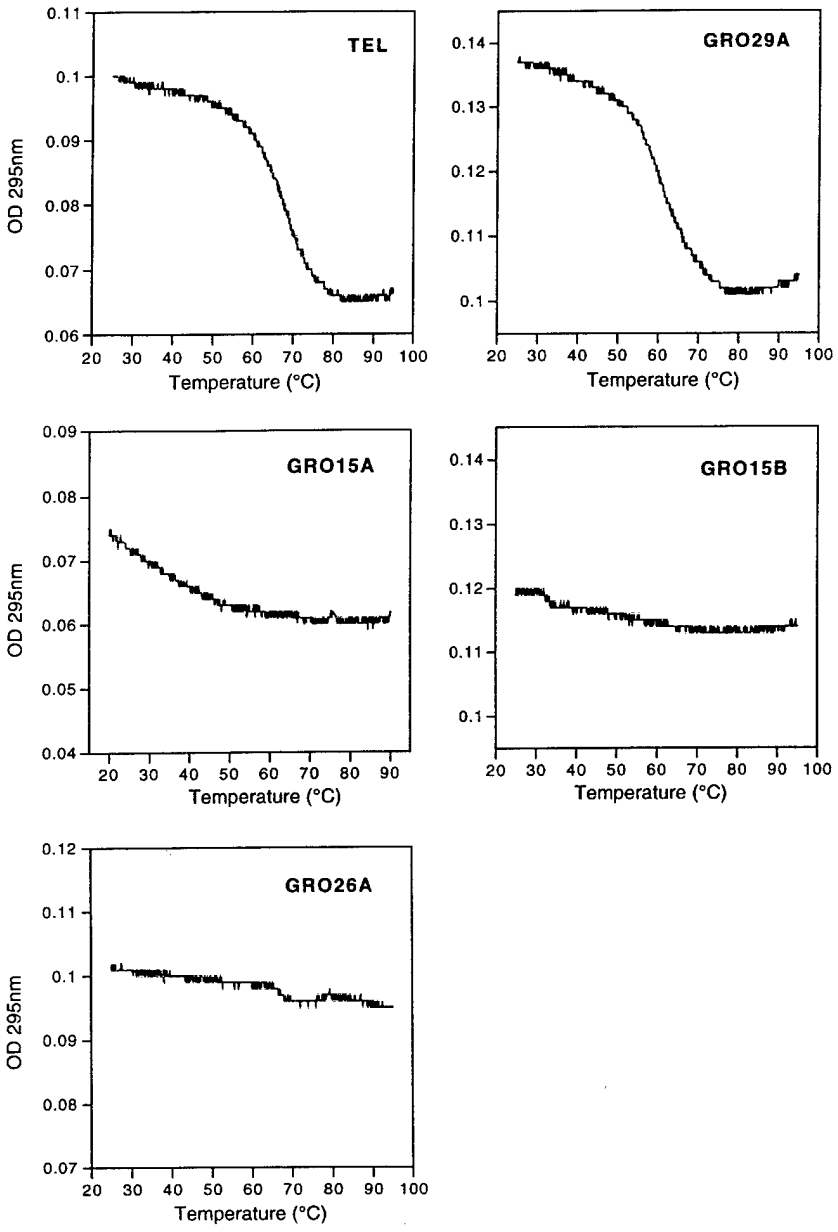

FIG. 2. **UV thermal renaturation curves to assess G-quartet formation by G-rich oligonucleotides.** The oligonucleotide name is shown in the *top right corner* of each graph. Experiments were carried out in Tm buffer (20 mM Tris·HCl, pH 8.0, 140 mM KCl, 2.5 mM $MgCl_2$).

Active G-rich Oligonucleotides Bind to a Specific Cellular Protein—To investigate further the mechanism of the growth inhibitory effects, we examined binding of the oligonucleotides to cellular proteins. 5′-Radiolabeled oligonucleotides were incubated with HeLa nuclear extracts, alone or in the presence of unlabeled competitor oligonucleotide, and examined by an electrophoretic mobility shift assay. The G-quartet forming telomere sequence oligonucleotide, TEL, was also included as a competitor in this experiment. Fig. 4*A* shows the formation of a stable protein-oligonucleotide complex (Fig. 4*A*, *). This band was intense when the labeled oligonucleotide was one of the growth inhibitory oligonucleotides, GRO15A or GRO29A (*lanes 1* and *5*), but the inactive oligonucleotide, GRO26A, formed only a weak complex (*lane 9*). This experiment also showed that the complex could be effectively competed by either unlabeled antiproliferative oligonucleotide or TEL but not by the inactive GRO26A.

To confirm further that the same protein is binding to TEL and to the growth inhibitory oligonucleotides, we carried out a similar experiment in which TEL was labeled. Labeled TEL formed two complexes with nuclear extracts in the absence of competitor oligonucleotides (*bands A* and *B*, Fig. 4*B*). The slower migrating TEL-protein complex (*band A*) was competed for by unlabeled growth inhibitory oligonucleotides (GRO15A and GRO29A) but not inactive oligonucleotides (GRO26A and GRO15B). The faster migrating complex (*band B*) was specific for TEL and was not competed for by G-rich oligonucleotides. Hence binding of competitor GROs was characterized by a decrease in the intensity of *band A* and an increase in the intensity of *band B* (due to release of labeled TEL from *band A* complex). This assay allowed comparison of the binding affinity of native GROs (without 5′-phosphorylation) and was used for assessment of protein binding in subsequent experiments. To ensure that competition was due to binding of the GRO to the protein component of complex A, and not a result of interaction between GRO and TEL oligonucleotide, we carried out a mobility shift on a 15% polyacrylamide gel. No shifted bands were observed when labeled TEL was incubated with GROs in the absence of protein (data not shown).

To determine the approximate molecular weight of the pro-

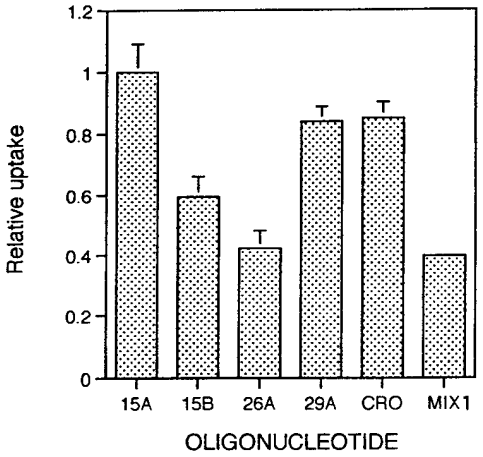

FIG. 3. **Relative uptake of G-rich oligonucleotide by MDA-MB-231 breast cancer cells.** A mixture of unlabeled and 5′-^{32}P-labeled oligonucleotide was added to the medium of cells to give a final concentration of 10 μM. After incubation for 10 h, cells were washed extensively to remove extracellular oligonucleotide, and uptake was assessed by counting cell-associated radioactivity.

tein involved in complex A, and to confirm direct binding of the protein to oligonucleotides, we carried out a UV cross-linking study. 5′-Labeled oligonucleotides and HeLa nuclear extracts were incubated alone or in the presence of unlabeled competitor oligonucleotides. The samples were then irradiated with UV light resulting in cross-link formation between protein residues and thymidines in the oligonucleotide. The protein was thus radiolabeled and could be detected on an SDS-polyacrylamide gel. Fig. 4*C* shows the results of this experiment. Both TEL and GRO15A cross-linked to a protein (Fig. 4*C*, *) which was competed for by antiproliferative oligonucleotides and TEL but not by inactive GRO26A. The most active oligonucleotide, GRO29A, also formed this approximately 100-kDa complex and another complex of higher molecular weight (not shown). Inactive GRO26A produced a barely visible band at ~100 kDa (not shown).

The molecular weight of the nuclear protein was more accurately determined by Southwestern blotting. HeLa nuclear extracts were electrophoresed on an 8% polyacrylamide-SDS gel and transferred to a PVDF membrane. The membrane was blocked and cut into strips. Each strip was incubated at 4 °C with a ^{32}P-labeled G-rich oligonucleotide in the presence of unrelated unlabeled double-stranded and single-stranded DNA to block nonspecific binding. Fig. 4*D* shows active oligonucleotides GRO15A and GRO29A hybridized to a single protein band at 106 kDa (the band was exactly adjacent to a 106-kDa molecular mass marker, not shown). Inactive oligonucleotides GRO15B and GRO26A hybridized only weakly to this protein. The data presented in Figs. 1 and 4 suggest a correlation between activity and protein binding, at least for the four oligonucleotides examined. These experiments also demonstrate that binding of GROs to p106 is highly specific, since only a single protein band is recognized with high affinity (see Fig. 4*D*). This was not simply a result of hybridization to an abundant protein, as India ink staining of immobilized nuclear extracts showed the presence of many other protein bands that were equally or more intense than the band at 106 kDa (data not shown).

Antiproliferative Activity of G-rich Oligonucleotides Correlates with Protein Binding—To confirm further the relationship between activity and binding to the 106-kDa protein, we synthesized four more G-rich oligonucleotides and compared their effects with active (GRO29A) and inactive (GRO15B) oligonucleotides. Fig. 5, *A* and *B*, shows that the growth inhibitory effect of the oligonucleotides correlated with their ability to compete for the TEL-binding protein. Three of the new oligonucleotides displayed a moderate antiproliferative activity but were not as potent as GRO29A. Oligonucleotide GRO14B showed no antiproliferative activity. Correspondingly, the moderately active oligonucleotides (GRO14A, GRO25A, and GRO28A) were able to compete with TEL for binding to the nuclear protein, although not as effectively as GRO29A. The non-inhibitory oligonucleotide, GRO14B, was unable to compete for protein binding.

Effects of Non-G-rich Oligonucleotides—To investigate the specificity of the antiproliferative effects, we examined the growth inhibitory effects of non-G-rich oligonucleotides and heparin, a polyanionic polysaccharide. Fig. 5*C* shows that at 10 μM concentration (equivalent to approximately 0.1 mg/ml for GRO29A), neither a 3′-modified C-rich oligonucleotide (CRO) nor a 3′-modified mixed base oligonucleotide (MIX1) were able to inhibit the growth of MDA-MB-231 breast cancer cells. This result showed that the growth inhibiting activity of GRO15A and GRO29A was not simply nonspecific effects resulting from the presence of 3′-modified oligonucleotide but rather relied on some unique feature of these sequences. Heparin also had no effect on cell growth when added to the culture medium at a concentration of 20 units/ml (approximately 0.12 mg/ml), further demonstrating that the antiproliferative effects of active oligonucleotides are not simply a result of their polyanionic character. To examine the antiproliferative properties of non-3′-protected oligonucleotides, we used a slightly modified treatment protocol in which oligonucleotides were added to cells in serum-free medium (see "Experimental Procedures"). Fig. 5*D* shows that similar effects could also be seen with unmodified oligonucleotides under these conditions. Both 29A-OH (a 3′-unmodified analog of GRO29A) and TEL inhibited the growth of cells, whereas two mixed sequence oligonucleotides had no growth inhibitory effects.

We also compared the protein binding properties of these non-G-rich oligonucleotides and heparin (not shown). As expected, the unlabeled growth inhibitory oligonucleotides GRO29A, 29A-OH, and TEL competed strongly for protein binding in the competitive electrophoretic mobility shift assay (using labeled TEL oligonucleotide and MDA-MB-231 nuclear extracts) at 10 nM concentration (approximately 0.1 μg/ml for GRO29A). In accord with its lesser antiproliferative activity, TEL competed slightly less effectively than 29A-OH or GRO29A. No competition was observed using 10 nM unlabeled CRO, MIX2, or MIX3 or in the presence of 0.02 units/ml heparin (approximately 0.12 μg/ml). However, the mixed sequence oligonucleotide, MIX1, was anomalous. Although this oligonucleotide had no effect on the growth of cells, it appeared to compete for protein binding in the competitive EMSA.

Evidence That G-rich Oligonucleotide-binding Protein Is Nucleolin—Two previous reports describe binding of the nucleolar protein, nucleolin, to the G-rich telomere sequence. Ishikawa *et al.* (28) identified a 50-kDa protein from HeLa extracts that bound to 5′-$(TTAGGG)_4$-3′. Microsequence determination suggested that this was a proteolytic fragment of nucleolin. Binding of the full-length, purified 106-kDa nucleolin protein was demonstrated independently by Dickinson and Kohwi-Shigematsu (29). Since our protein was of the correct molecular weight and also bound to 5′-$(TTAGGG)_4$-3′ (TEL), we tested the hypothesis that the G-rich oligonucleotide-binding protein was nucleolin. Nuclear extracts from HeLa cells (purchased from Promega) or MDA-MB-231 breast cancer cells (obtained in our laboratory by standard procedures) were electrophoresed and transferred to PVDF membrane. The immobilized proteins were probed for binding to ^{32}P-labeled GRO15A using the

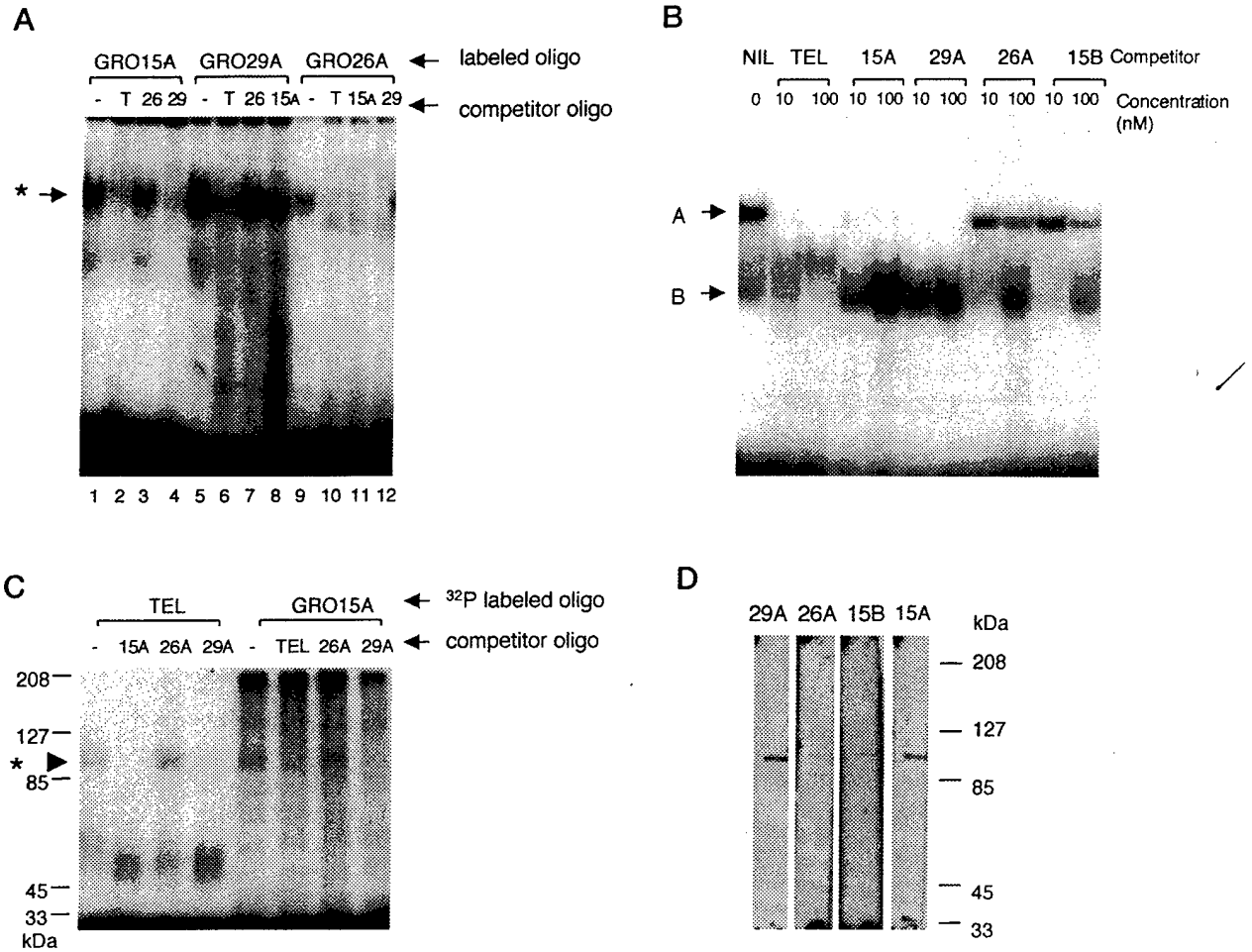

FIG. 4. *A*, EMSA showing binding of ^{32}P-labeled G-rich oligonucleotides to 5 μg of HeLa nuclear extracts and competition by unlabeled competitor oligonucleotides (100-fold molar excess over labeled oligonucleotide). Competitor oligonucleotides are abbreviated as *T* (TEL), *29* (GRO29A), *26* (GRO26A), and *15A* (GRO15A). *B*, EMSA showing complexes formed between ^{32}P-labeled TEL oligonucleotide (1 nM) and 5 μg of HeLa nuclear extracts and competition by unlabeled competitor G-rich oligonucleotides (10 or 100 nM). *C*, SDS-polyacrylamide gel showing complexes formed by UV cross-linking of labeled oligonucleotides and HeLa nuclear extracts incubated in the absence or presence of unlabeled competitor (100-fold molar excess). *D*, Southwestern blots of HeLa nuclear extracts probed with ^{32}P labeled G-rich oligonucleotides (2×10^6 cpm, approximately 0.75 nmol). The probe GRO is indicated at the *top* of each strip.

Southwestern procedure described and were visualized by overnight exposure to autoradiographic film. The same membrane was stripped of oligonucleotide by the denaturation/renaturation steps described (see "Southwestern Blotting" under "Experimental Procedures") and Western-blotted using nucleolin antiserum as primary antibody and a horseradish peroxidase-conjugated anti-rabbit secondary antibody. The blot was visualized by incubation with a chemiluminescence detection reagent followed by a 20-s exposure to autoradiographic film. The results are shown in Fig. 6*A*. Southwestern blots of nuclear extracts showed an intense band upon hybridization with radiolabeled GRO15A at 106 kDa (HeLa) or 116 kDa (MDA-MB-231). The Western blot of MDA-MB-231 nuclear proteins shows one intense band at 116 kDa and weaker bands at about 50 kDa. In HeLa extracts the nucleolin antibody recognizes multiple bands at approximately 50, 75, 106, and 120 kDa. Most importantly, in both cell lines the band that was recognized by GRO15A exactly corresponded to a band recognized when the membrane was stripped and Western-blotted with nucleolin antibody. Nucleolin is a protein that can be phosphorylated in cells by a number of kinases and is also highly susceptible to proteolysis (30–36). We believe that the difference in the molecular weight of proteins detected in these blots may arise from the different methods of preparation of the nuclear extract leading to differently phosphorylated or degraded forms of nucleolin being the predominant species. The difference in the intensities of the bands shown in the Southwestern blots in Fig. 6*A* may be due to the preferential binding of GRO15A to one form of nucleolin (apparently the 106-kDa species) over others.

To determine whether binding of the specific protein occurred within the cell, we used biotinylated G-rich oligonucleotides to treat MDA-MB-231 breast cancer cells. Streptavidin-coated magnetic beads were then used to capture oligonucleotide-protein complexes after lysing the cells cells with an immunoprecipitation-type buffer (see "Experimental Procedures"). This procedure was carried out for cells that were treated with either an active oligonucleotide (5′-Biotin-GRO15A) or an inactive oligonucleotide (5′-Biotin-GRO15B) and untreated cells as a control. Equal volumes of each sample were electrophoresed and transferred to a PVDF membrane. This was analyzed by india ink staining, Southwestern blotting with radiolabeled GRO15A, and Western blotting with a nucleolin monoclonal antibody. India ink staining of the membrane showed a major protein band at 116 kDa that was present in cells treated with biotinylated GRO15A but was absent in untreated cells and of a lower intensity in cells treated with inactive biotinylated GRO15B (data not shown). The Southwestern and Western blots (Fig. 6*B*) confirm that this captured protein binds to both GRO15A and a nucleolin antibody.

This experiment showed that a 116-kDa protein was specif-

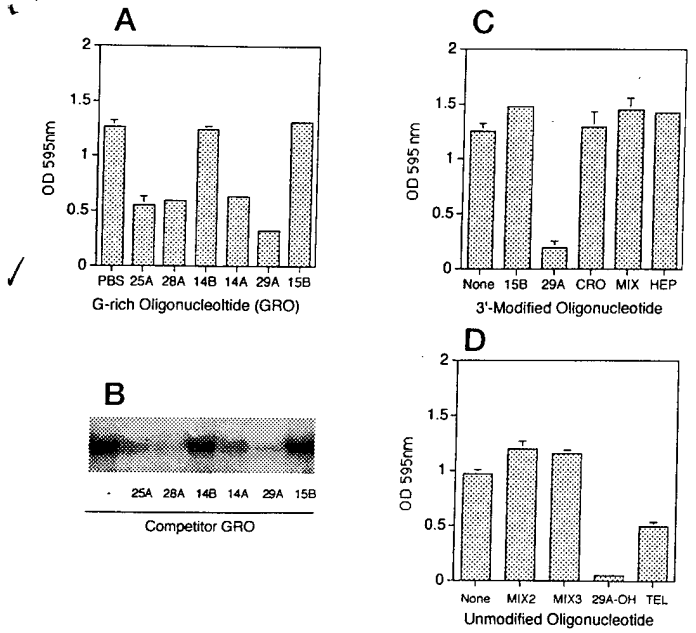

FIG. 5. *A*, MTT assay of MDA-MB-231 cells treated with a single 10 μM dose of G-rich oligonucleotide or PBS as a control. The assay was performed on day 9 (oligonucleotide added on day 1). *B*, EMSA showing complex formed by binding of 5 μg of MDA-MB-231 nuclear extracts to ^{32}P-labeled TEL oligonucleotide and competition by unlabeled G-rich oligonucleotides (10-fold molar excess). *C*, MTT assay of MDA-MB-231 cells treated with a single 10 μM dose of 3'-protected C-rich or mixed sequence oligonucleotide or with 20 units/ml heparin, in comparison with inactive (GRO15B) and active (GRO29A) G-rich oligonucleotides. The assay was performed on day 7. *D*, MTT assay of MDA-MB-231 cells treated with a single 10 μM dose of unmodified mixed sequence oligonucleotides, in comparison with an unmodified GRO29A analog (29A-OH) and TEL. To treat cells, culture medium was replaced by serum-free medium containing 10 μM oligonucleotide. After 4 h at 37 °C, fetal calf serum was added to give 10% v/v. The MTT assay was performed on day 7.

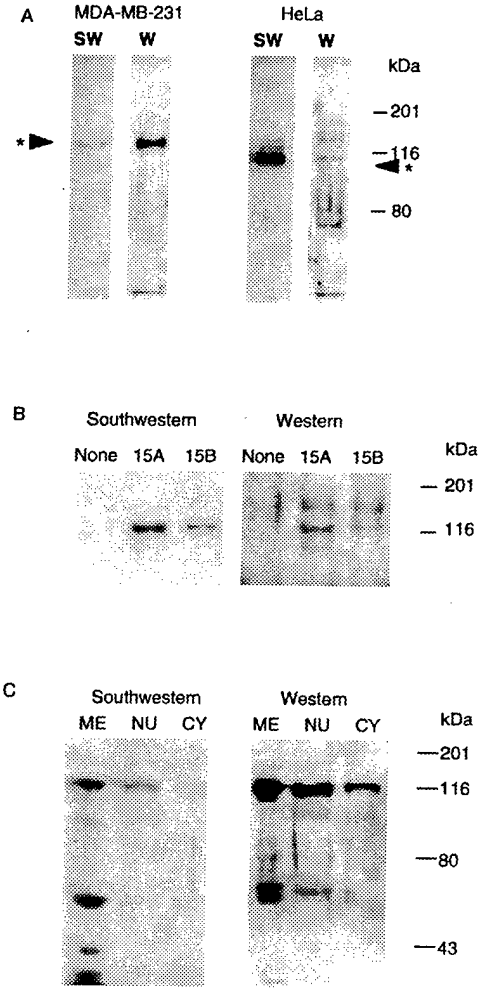

FIG. 6. *A*, Southwestern (*SW*) and Western (*W*) blots probed respectively with ^{32}P-labeled active G-rich oligonucleotide (*GRO15A*) or nucleolin antiserum. *Left panel* shows MDA-MB-231 nuclear extracts (5 μg/lane); *right panel* shows HeLa nuclear extracts (Promega Inc., 5 μg/lane). *B*, Southwestern and Western blots of proteins captured from the lysates of MDA-MB-231 cells which had been treated with no oligonucleotide (*none*), active G-rich oligonucleotide (*15A*), or less active G-rich oligonucleotide (*15B*). *C*, Southwestern and Western blots showing binding of GRO15A and nucleolin antibody to protein extracts (3 μg/lane) from MDA-MB-231 cells: nuclear extracts (*NU*), cytoplasmic extracts (*CY*) and membrane proteins (*ME*).

ically captured from cells treated with biotinylated GROs, that this protein was recognized also by a nucleolin antibody, and also that more of this protein was captured by active GRO15A than was captured by the less active GRO15B. Although we cannot absolutely exclude that the protein-oligonucleotide association took place during cell lysis or oligonucleotide capture, it is unlikely that the oligonucleotide would exist in a free, uncomplexed state inside the cell. We believe, therefore, that these results provide strong evidence for binding of oligonucleotide to the 116-kDa protein inside the cell (or possibly at the cell surface).

To determine the subcellular location of the G-rich oligonucleotide-binding protein, we carried out Southwestern and Western blotting experiments to compare nuclear extracts, cytoplasmic extracts, and proteins derived from the cell membrane (5 μg of extract per lane). Fig. 6*C* shows the results of these studies. The Southwestern blot shows a 116-kDa protein capable of binding labeled GRO15A is present in the nuclear extracts and, to a lesser extent, in the cytoplasmic fraction. The same band was present in plasma membrane extracts and hybridized strongly to GRO15A. Western blotting of the same membrane showed that a monoclonal antibody to nucleolin also recognized these bands at 116 kDa in each fraction. (A band at approximately 70 kDa was also recognized by both GRO15A and nucleolin antibody and may be a proteolytic fragment of nucleolin.) Since both the location and relative intensity of the bands recognized by GRO15A and nucleolin antibody are the same, these results provide further evidence that the protein that binds to antiproliferative G-rich oligonucleotides is nucleolin. The detection of GRO-binding protein in the plasma membrane extracts also suggests the possibility that binding to cell surface protein may be important in the mechanism of action of G-rich oligonucleotides.

DISCUSSION

Oligonucleotides are polyanionic species that are internalized in cells, probably by receptor-mediated endocytosis (37). They are likely to interact with many biomolecules within the cell and also in the extracellular membrane by virtue of both their charge and their shape, as well as sequence-specific interactions with nucleic acids. The proteins that bind to oligonucleotides and mediate non-antisense effects have not yet been unequivocally identified.

We have described G-rich oligonucleotides that have potent growth inhibitory effects that are unrelated to any expected antisense or antigene activity. Although we have not yet delineated the mechanism of these effects, we have demonstrated that the antiproliferative effects of these oligonucleotides are related to their ability to bind to a specific cellular protein. Because the GRO-binding protein is also recognized by anti-nucleolin antibodies, we conclude that this protein is either

nucleolin itself or a protein of a similar size that shares immunogenic similarities with nucleolin.

Nucleolin is an abundant multifunctional 110-kDa phosphoprotein, thought to be located predominantly in the nucleolus of proliferating cells (for reviews, see Refs. 38 and 39). It has been implicated in many aspects of ribosome biogenesis including the control of rDNA transcription, pre-ribosome packaging, and organization of nucleolar chromatin (38–40). Another emerging role for nucleolin is as a shuttle protein that transports viral and cellular proteins between the cytoplasm and nucleus/nucleolus of the cell (41–43). Nucleolin is also implicated, directly or indirectly, in other roles including nuclear matrix structure (44), cytokinesis, and nuclear division (45) and as an RNA and DNA helicase (46). Its multifunctional nature is reflected in its multidomain structure, consisting of a histone-like N terminus, a central domain containing RNA recognition motifs, and a glycine- and arginine-rich C terminus (47). Levels of nucleolin are known to relate to the rate of cellular proliferation (48, 49), being elevated in rapidly proliferating cells, such as malignant cells, and lower in more slowly dividing cells. For this reason, nucleolin may be an attractive therapeutic target for the treatment of malignant disease.

Although considered a predominantly nucleolar protein, our finding that nucleolin was present in the plasma membrane is consistent with several reports identifying cell surface nucleolin and suggesting its role as a cell surface receptor (50–53).

Previously, several mechanisms have been proposed to explain the non-sequence-specific effects of oligonucleotides. These include binding to cellular receptors (54, 55), modulation of cytokine or growth factor activity (56–60), inhibition of cell cycle progression (9), changes in cell adhesion (12), and binding to an uncharacterized 45-kDa protein (26).

In this present report, we have identified nucleolin (or a nucleolin-like protein) as a G-rich oligonucleotide-binding protein, and we have shown a strong correlation between binding to this protein and antiproliferative activity for a series of G-rich oligonucleotides. We believe that these findings strongly suggest a mechanistic role for nucleolin in non-antisense inhibition of cell growth by G-rich oligonucleotides. This belief has been strengthened by our recent immunofluorescence experiments that show significant differences in nucleolin levels between cells treated with GRO29A and untreated cells.[2]

The relationship between nucleolin binding and antiproliferative activity for other, non-G-rich, oligonucleotides has not yet been fully evaluated. One mixed sequence oligonucleotide (MIX1) was found to bind to nucleolin, although it had no growth inhibitory effect. Nucleolin contains RNA binding domains that can recognize specific sequences of RNA or single-stranded DNA (29, 61). It is possible that this particular oligonucleotide contains a sequence or structure that resembles such a recognition element.

In support of our findings that nucleolin binds selectively to G-rich oligonucleotides that form stable G-quartet structures, Maizels *et al.* (62, 63) have recently demonstrated binding of purified nucleolin to G-quartet forming DNA sequences from immunoglobulin switch regions and ribosomal DNA. It is likely that nucleolin has currently undefined functions *in vivo* that depend on recognition of G-quartet forming sequences in ribosomal DNA, switch region sequences, or telomeres.

It is our hypothesis that nucleolin contains a specific binding site that recognizes certain G-quartet structures and that binding at this site by a G-rich oligonucleotide inhibits one or more of the normal functions of nucleolin. The consequences of nucleolin inhibition on the growth of cells have not been well studied, but it is easy to envisage that inhibition of a protein whose functions include ribosome production, nuclear transport, and cell entry could have profound effects on the growth of cells.

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APPENDIX 4: ABSTRACTS

Presented by Paula Bates at the AACR 90th Annual Meeting, April 10-14 1999, Philadelphia:

Novel antiproliferative G-rich oligonucleotides that bind to a specific cellular protein.

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There have been several recent reports concerning the non-antisense effects of phosphodiester and phosphorothioate oligonucleotides, particularly those that are rich in guanosine and able to form G-quartet structures. The non-sequence specific effects of these oligonucleotides are thought to be related to protein recognition of their unique tertiary structure, but their exact mechanism of action is still unknown. We have discovered G-rich phosphodiester oligonucleotides that have potent inhibitory effects on the growth of several tumor cell lines in culture. Preliminary experiments suggest that active oligonucleotides can also inhibit the growth of breast cancer xenografts in a nude mouse model. In investigating the mechanism of these effects, we have observed a striking correlation between the antiproliferative activity of the G-rich oligonucleotides and binding to a specific cellular protein, using gel shift assays and southwestern blotting. Recently we have tentatively identified this protein and evidence suggests that levels of this protein are higher in malignant cells than in non-malignant fibroblasts. The same protein was found to bind to an oligonucleotide representing the G-rich strand of the human telomere, a sequence known to form G-quartets.

Presented by Paula Bates at the AACR 89th Annual Meeting, March 28 - April 1 1998, New Orleans:

Inhibition of prostate cancer cell growth by novel G-rich oligonucleotides.

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In recent years, a new class of potentially therapeutic oligonucleotides have emerged. These oligonucleotides are rich in guanosine and often exist in a conformation that involves the formation of intramolecular or intermolecular G-quartets. They exert potent biological effects that appear to be related to their unique tertiary structure, but the exact mechanism of their action remains largely unknown. We have discovered a G-rich oligonucleotide (GRO15A) which can completely inhibit the growth of DU145 prostate cancer cells in culture relative to untreated cells and cells treated with other G-rich oligonucleotides. Preliminary observations indicate that another oligonucleotide (GRO29A) may be even more potent. Native polyacrylamide gel electrophoresis of GRO15A and GRO29A has shown the existence of multimeric structures. Higher order structures are not observed with oligonucleotides which are not growth inhibitory. Electrophoretic mobility shift assays show the formation of two specific complexes when GRO15A is incubated with nuclear extracts. GRO29A competes for binding to these proteins, but other biologically inactive G-rich oligonucleotides do not.