0026-895X/99/020359-11$3.00/0

MOLECULAR PHARMACOLOGY, **56:**359–369 (1999).

Selective Killing of Cancer Cells Based on Loss of Heterozygosity and Normal Variation in the Human Genome: A New Paradigm for Anticancer Drug Therapy

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Received for publication March 4, 1999; accepted May 17, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

Most drugs for cancer therapy are targeted to relative differences in the biological characteristics of cancer cells and normal cells. The therapeutic index of such drugs is theoretically limited by the magnitude of such differences, and most anticancer drugs have considerable toxicity to normal cells. Here we describe a new approach for developing anticancer drugs. This approach, termed variagenic targeting, exploits the absolute difference in the genotype of normal cells and cancer cells arising from normal gene sequence variation in essential genes and loss of heterozygosity (LOH) occurring during oncogenesis. The technology involves identifying genes that are: 1) essential for cell survival; 2) are expressed as multiple alleles in the normal population because of the presence of one or more nucleotide polymorphisms; and 3) are frequently subject to LOH in several common cancers. An allele-specific drug inhibiting the essential gene remaining in cancer cells would be lethal to the malignant cell and would have minimal toxicity to the normal heterozygous cell that retains the drug-insensitive allele. With antisense oligonucleotides designed to target two alternative alleles of replication protein A, 70-kDa subunit (RPA70) we demonstrate in vitro selective killing of cancer cells that contain only the sensitive allele of the target gene without killing cells expressing the alternative RPA70 allele. Additionally, we identify several other candidate genes for variagenic targeting. This technology represents a new approach for the discovery of agents with high therapeutics indices for treating cancer and other proliferative disorders.

The fundamental challenge for cancer therapy is to identify specific differences between cancer and normal cells that are targets for chemotherapeutic drugs and will allow elimination of cancer cells with minimal toxicity to normal tissues. At least three classes of differences between cancer cells and normal cells are being investigated as targets for such therapeutic intervention. First, tumor-specific antigens have been identified and are being investigated as immunotherapeutic targets (Eynde and Boon, 1997). Second, tumor-specific oncogenes such as mutant Ha-*ras* (Monia et al., 1992; Schwab et al., 1994; Bennett et al., 1996) and *bcr-abl* rearrangements (Witte, 1993; Smetsers et al., 1997) are potential targets for therapeutic agents. Third, the loss of tumor-suppressor-gene function, which is an enabling step in oncogenesis, creates differences between cancer cells and normal cells that might be targeted by therapeutic agents. Therapies that are specifically toxic to p53-deficient cells, or cells deficient in other tumor suppressor genes, are currently under investigation (Bischoff et al., 1996; Heise et al., 1997). The therapeutic potential of these differences between cancer cells and normal cells is limited by the small number of targets that are truly tumor-specific and the fact that inhibition of many tumor-specific functions may not necessarily be cytotoxic to cancer cells. In this study, we describe a novel strategy for specific killing of cancer cells based on loss of heterozygosity (LOH) and normal genetic variation in genes that are essential for cell survival.

An early event in the clonal evolution of cancers is the loss of large chromosomal regions or even whole chromosomes (Lengauer et al., 1998). Presumably, these losses are driven, in part, by positive selection for cells in which LOH leads to the loss of tumor suppressor functions. In certain cancers, LOH can involve more than 20% of the total genome (Lengauer et al., 1998), and it is evident that thousands of genes are also lost from cancer cells because of LOH. Based on current estimates of the human gene number, this suggests

Financial support for the reported studies was provided by Isis Pharmaceuticals and Variagenics, Inc.

ABBREVIATIONS: LOH, loss of heterozygosity; RPA70, 70-kDa subunit of replication protein A; 20N-mer, 20-nucleotide, totally random phosphorothioate oligonucleotide; PCR, polymerase chain reaction; GAPDH, glyceraldehyde phosphate dehydrogenase.

that 15,000 to 20,000 genes that are not tumor suppressor genes are also reduced to hemizygosity in cancer cells by LOH. Among these genes are many that are essential for cell survival.

It is estimated that genetic variation occurs in approximately one nucleotide in 300 throughout the genome (Cooper et al., 1985). Because of the large number of polymorphisms or sequence variances found in the human genome, most individuals are heterozygous for one or more sequence variances in genes of normal tissues, including many genes that are essential for cell survival. LOH reduces many of these genes to hemizygosity in cancer cells, eliminating heterozygosity and creating a large number of absolute genetic differences between tumor and normal cells (Cavenee et al., 1991; Schwechheimer and Cavenee, 1993).

The approach described in this report, termed variagenic targeting, exploits the absolute genetic differences between cancer cells and normal cells that arise as a consequence of normal genetic variation and LOH. This strategy, shown schematically in Fig. 1, involves identifying gene targets that are: 1) known to be essential for cell survival or proliferation; 2) present in variant forms in the normal population; and 3) frequently subject to LOH in common cancers. Inhibitors are then identified that inactivate one or more variant forms of the target gene, but not the alternate forms that are present in the population. Inhibitors specific for the remaining allele expressed in the cancer cells, when administered to patients, would be selectively toxic to the cancer cells. Normal cells and tissues, which express both the sensitive and insensitive alleles, would escape significant toxicity. Because of the high frequency of many normal sequence variations and the high prevalence of LOH in common tumors, this technology could be generally applicable for the treatment of many important cancers.

The studies reported here test the feasibility of variagenic targeting as a paradigm for anticancer drug development. With antisense phosphorothioate oligodeoxynucleotides to target a high frequency sequence variance in the mRNA of the 70-kDa subunit of human replication protein A (RPA70), we demonstrate both variance-specific reduction of RPA70 mRNA levels and variance-specific inhibition of tumor cell growth in vitro. These data demonstrate the feasibility of this strategy for developing anticancer agents based on normal genetic variation in essential genes and LOH in cancer.

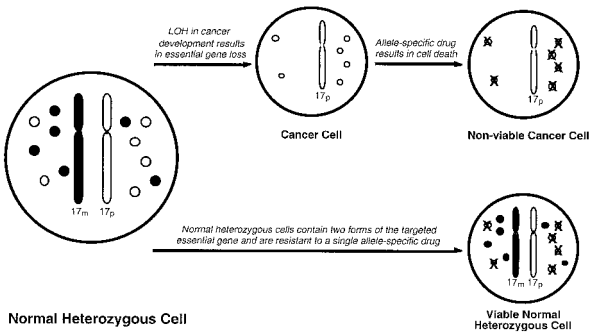


Fig. 1. The principle of variagenic targeting. Normal heterozygous cells have at least two variant forms of the targeted essential gene, maternally (M) and paternally (P) derived. LOH during malignant transformation deletes one form of the gene in the resultant cancer cell. Cancer cells, which have only one form of the essential gene remaining, will be sensitive to inhibitors of that allele. Normal heterozygous cells containing both forms of the gene will be unaffected. ●, maternal gene product; ○, paternal gene product.

Materials and Methods

Phosphorothioate Oligodeoxynucleotides. Phosphorothioate oligonucleotides, synthesized according to the method of Chiang (Chiang et al., 1991), were obtained from Isis Pharmaceuticals (Carlsbad, CA) or Synthetic Genetics (San Diego, CA) and were purified by reverse phase. The sequences of the oligonucleotides used in this study are available on request. As a control oligonucleotide for nonspecific phosphorothioate effects, a 20-nucleotide totally random phosphorothioate oligonucleotide (20N-mer) was synthesized by incorporating all four bases in equal proportion at each position in the oligonucleotide.

Cell Culture. The human tumor cell lines Mia Paca II, T24, SW480, A549, and HeLa were obtained from the American Type Culture Collection (Manassas, VA) and cultured as recommended by the supplier. All media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS), 100 μg/ml penicillin-streptomycin (Life Technologies Inc., Grand Island, NY), and 2 mM L-glutamine (Life Technologies). All cell lines were grown under 5% CO_2/95% air in a humidified incubator at 37C.

LOH Studies. For LOH analysis, at least 180 breast, colon, ovarian, and nonsmall cell lung cancers were retrieved from archived pathological specimens at the Uppsala Pathology Institute (Uppsala, Sweden). All specimens were derived from individuals of Swedish decent. Analysis was performed as described here and in Sjogren et al. (1996). Tumor tissue was microdissected from normal tissue, and tumor DNA from informative patients (heterozygotes at nucleotide 1120 of RPA70) was amplified by polymerase chain reaction (PCR). Finally, a quantitative sequencing reaction with an Autoload and Alfexpress DNA Sequencer (Pharmacia Biotech, Uppsala, Sweden) was performed to determine the degree of LOH. Sequencing reactions were standardized with a set of mixed DNA solutions differing in allele proportions. Peak analysis was performed with a Fragment Manager (Pharmacia Biotech).

Genotyping of RPA70. PCR-single-strand conformation polymorphism was used to determine the extent of heterozygosity for each variance in the RPA70 gene. Total RNA was isolated from lymphoblast cell lines derived from a panel of 36 normal individuals. cDNA was synthesized and analyzed for variances with PCR-single-strand conformation polymorphism as described (Iwahana et al., 1992; Liu and Sommer, 1995). Changes in the DNA sequence were confirmed by sequencing. The panel used to determine the heterozygosity is described elsewhere (Stanton et al., in preparation).

Phosphorothioate Oligonucleotide Treatment of Cells. All antisense treatments were performed with phosphorothioate oligodeoxynucleotides. Cells were cultured in six-well plates to 60 to 80% confluency for use in oligonucleotide treatments. Cells were washed once with Opti-MEM (Life Technologies) prewarmed to 37°C. Transfections were carried out in 1 ml of Opti-MEM containing 3 μg of Lipofectin (Life Technologies) per ml of Opti-MEM per 100 nM added oligonucleotide. Opti-Mem containing the appropriate amount of Lipofectin was added to the cells followed by the addition of oligonucleotides from 1000× stocks (for dose-response studies, oligonucleotides were added from 20× stocks). Cells were incubated for 5 h at 37°C. After treatment, medium was removed and replaced with prewarmed replete media (Bennett et al., 1992; Monia et al., 1993).

In dose-response experiments, the total phosphorothioate oligonucleotide concentration was held constant at 400 nM by supplementing the tested oligonucleotide to 400 nM with the 20N-mer randomized oligonucleotide. This control oligonucleotide was synthesized by incorporation of all four bases in equal proportions at each position of the 20N-mer.

For screening of additional targets, Mia Paca II, SW480, A549, and T24 cells were transfected with 400 nM phosphorothioate anti-

sense oligonucleotide complementary to either allele of the variance (additional cell lines were included for some variances). Target mRNA levels were assessed by Northern blotting with target-specific, random-primed, [α-^{32}P]dCTP-labeled cDNA probes, as described below. cDNAs for probes were obtained by specific reverse transcription-PCR of cellular RNAs. All oligonucleotides were twenty bases with the variance at position 9 or 10.

Northern Blot Analysis. For determination of mRNA levels by Northern blot, total RNA was prepared from cells 24 h after oligonucleotide addition with a SDS-lysis method (Peppel and Baglioni, 1990). Northern analysis was performed as described (Brown and Mackey, 1987). To determine RPA70 mRNA expression, RNA blots were probed with a random-primed [α-^{32}P]dCTP-labeled cDNA probe corresponding to a 562-nucleotide sequence (1519–2081) from human RPA70 (Erdile et al., 1991). After transfer, membranes were prehybridized with Quik-Hyb solution (Stratagene, La Jolla, CA) for 1 h at 68°C and then hybridized 1 to 4 h with 12.5×10^6 cpm of cDNA probe and 2 mg of salmon sperm DNA carrier in a total of 10 ml of hybridization solution. After hybridization, membranes were washed twice at room temperature for 15 min in 2× SSC/0.1% SDS and then once at 60°C for 30 min in 0.1X SSC/0.1% SDS.

RNA blots were quantified by phosphorimaging on a Fuji FLA-2000 (Fuji Medical Systems, Stamford, CT). RPA70 mRNA levels are normalized to the level of RPA70 mRNA measured in cells treated with 400 nM control 20N-mer phosphorothioate oligonucleotide and expressed as a percentage of control-treated levels. Glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA levels were probed with a random-primed GAPDH cDNA generated by reverse transcription-PCR with human GAPDH primers (Stratagene, Inc.).

Assessment of Cell Survival. Cells were transfected either once (HeLa cells) or three consecutive times (Mia Paca II cells) with matched, mismatched, or nonallele-specific anti-RPA70 (ISIS 12790) oligonucleotides as described above. After the last transfection, the cells were allowed to recover for either 3 (HeLa cells) or 6 days (Mia Paca II cells). Recovery time periods were empirically determined (data not shown). The number of cells remaining attached to the tissue culture dish was quantified by sulforhodamine B staining (FluoReporter Colorimetric Cell Protein Assay Kit; Molecular Probes Inc., Eugene, OR).

For experiments where the cell number was measured directly by hemocytometer, cells were plated in six-well dishes 24 h before the experiment and transfected at approximately 50–70% confluency with various phosphorothioate oligonucleotides at 400 nM, as described above. After a single transfection, the cells were allowed to recover 72 h. After 72 h, the cells were washed and trypsinized, and the cell number was determined by hemocytometer. For each experiment, treatments were performed in triplicate wells. The number of cells corresponding to each well was determined twice.

Statistical Analysis of Data. Statistical analysis of mRNA levels and cell survival data (Figs. 4 and 5) was performed with the BMDP Statistical Package, Version 7.0 (BMDP Statistical Software, Inc., Los Angeles, CA). Data were subjected to ANOVA, and the results were expressed in terms of *F*-values, *t*-values, and significance. For cell survival data, analyses included repeated measurements with 3 between factors (drug, concentration, and position). For mRNA levels only, the 3 between factors were considered. Global comparisons were performed with *F*-tests and pairwise comparisons with *t*-tests. In each case, because there was no interaction with position, results were pooled and analyzed with two between factors (drug and concentration).

Results

Identification of RPA70 As a Candidate Target Gene for Variagenic Targeting. RPA70 is the 70-kDa subunit of a heterotrimeric protein complex, replication protein A, which was initially identified as a factor essential for simian virus 40 DNA replication in vitro (Wobbe et al., 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988). RPA homologues are structurally and functionally conserved in eukaryotes (Erdile et al., 1991; O'Donnell et al., 1993; Philipova et al., 1996), and a similar single-strand binding protein (SSB) exists in prokaryotes (Philipova et al., 1996). Human RPA70, the largest subunit of RPA, is encoded by a single gene locus and is required for multiple processes in DNA metabolism (Kenny et al., 1989; Karpel, 1990; Kornberg and Baker, 1992). Each of the three subunits of RPA has been shown to be essential for DNA replication, homologous recombination, and nucleotide excision repair in vitro (He et al., 1995), and disruption of any of the three subunits in yeast is lethal (Brill and Stillman, 1991).

Analysis of 36 unrelated individuals by single-strand conformation polymorphism and sequencing revealed that RPA70 mRNA contains five high-frequency polymorphisms or variances with heterozygosity frequency ranging from 25 to 42% (Fig. 2A). One of the five variances, at nucleotide 1120, codes for an alternative amino acid at position 351 of RPA70 (threonine to alanine).

The RPA70 gene has been mapped to chromosome 17p13.3 in close proximity to the tumor suppressor gene p53 at position 17p13.1. This segment of the genome is affected by LOH in many common epithelial cancers (Ozawa et al., 1993; Umbricht et al., 1993; Rodriguez et al., 1994). LOH at the RPA70 locus was determined for 189 paired normal and cancer tissues from patients selected for constitutional RPA70 heterozygosity. Our studies showed LOH for RPA70 is 44% for colon cancer, 58% for ovarian cancer, 19.5% for breast cancer, and 27% for nonsmall cell lung carcinoma.

Inhibition of Cell Survival with Antisense Oligonucleotides against RPA70. Antisense oligonucleotides were used to demonstrate that inhibition of RPA70 leads to inhibition of cell survival, and that RPA70 is indeed an essential gene in human cells. To identify antisense oligonucleotides that inhibit expression of RPA70 in human cells, a series of 14 phosphorothioate 20-mer deoxyoligonucleotides, targeting different segments of RPA70 mRNA, were synthesized (Fig. 2A). Three oligonucleotides, ISIS 12781, ISIS 12786, and ISIS 12791, targeted segments of RPA70 mRNA containing variances and were designed so that the polymorphic nucleotide was opposite position 10 (ISIS 12786) or position 11 (ISIS 12781 and ISIS 12791) of the oligonucleotide.

To assess the ability of these oligonucleotides to inhibit RPA70 expression, A549 cells were treated with oligonucleotide at 400 nM in the presence of Lipofectin, and the level of RPA70 mRNA was measured by Northern blot analysis (Fig. 2B). As reported with other genes, not all oligonucleotides against the RPA70 sequence produced mRNA suppression (Monia et al., 1996b). Three oligonucleotides did not suppress mRNA levels below 50%. The other 11 oligonucleotides reduced RPA70 mRNA levels by 50 to 94% (Fig. 2B). The most potent oligonucleotide, ISIS 12790, reduced RPA70 mRNA levels by 94%. This oligonucleotide targets a nonvariant region located in the 3′-UTR of RPA70 mRNA (Fig. 2A) and is not variance-specific. The most potent oligonucleotide targeting a variance was ISIS 12781, which resulted in ~75% reduction in RPA70 mRNA levels.

To further assess the activity of ISIS 12790, this oligonucleotide was transfected into four different human tumor cell lines, and both RPA70 mRNA levels and cell survival were

measured. As a control, experiments were also performed with a 20-mer with identical nucleotide composition but containing seven mismatches relative to the target (ISIS 13706). Treatment of T24, A549, Mia Paca II, and SW480 cells with 400 nM ISIS 12790 resulted in an 80% or greater reduction in RPA70 mRNA levels compared with cells treated with the control (Fig. 3A). Other, unrelated, control oligonucleotides had no effect on RPA70 mRNA levels (data not shown). As an additional control, levels of GAPDH mRNA were determined for HeLa and Mia Paca II cells and were found not to be significantly affected by treatment with oligonucleotide (Fig. 4).

To determine the effects of ISIS 12790 on tumor cell survival, T24, A549, Mia Paca II, and SW480 cells were treated with a single dose of either ISIS 12790 or ISIS 13706, and surviving cells were counted 72 h later (Fig. 3B). As an additional control, T24 cells were also treated with an antisense oligonucleotide, ISIS 2503, which has been shown to reduce Ha-*ras* mRNA expression and suppress proliferation in these cells (Bennett et al., 1996). Treatment with ISIS

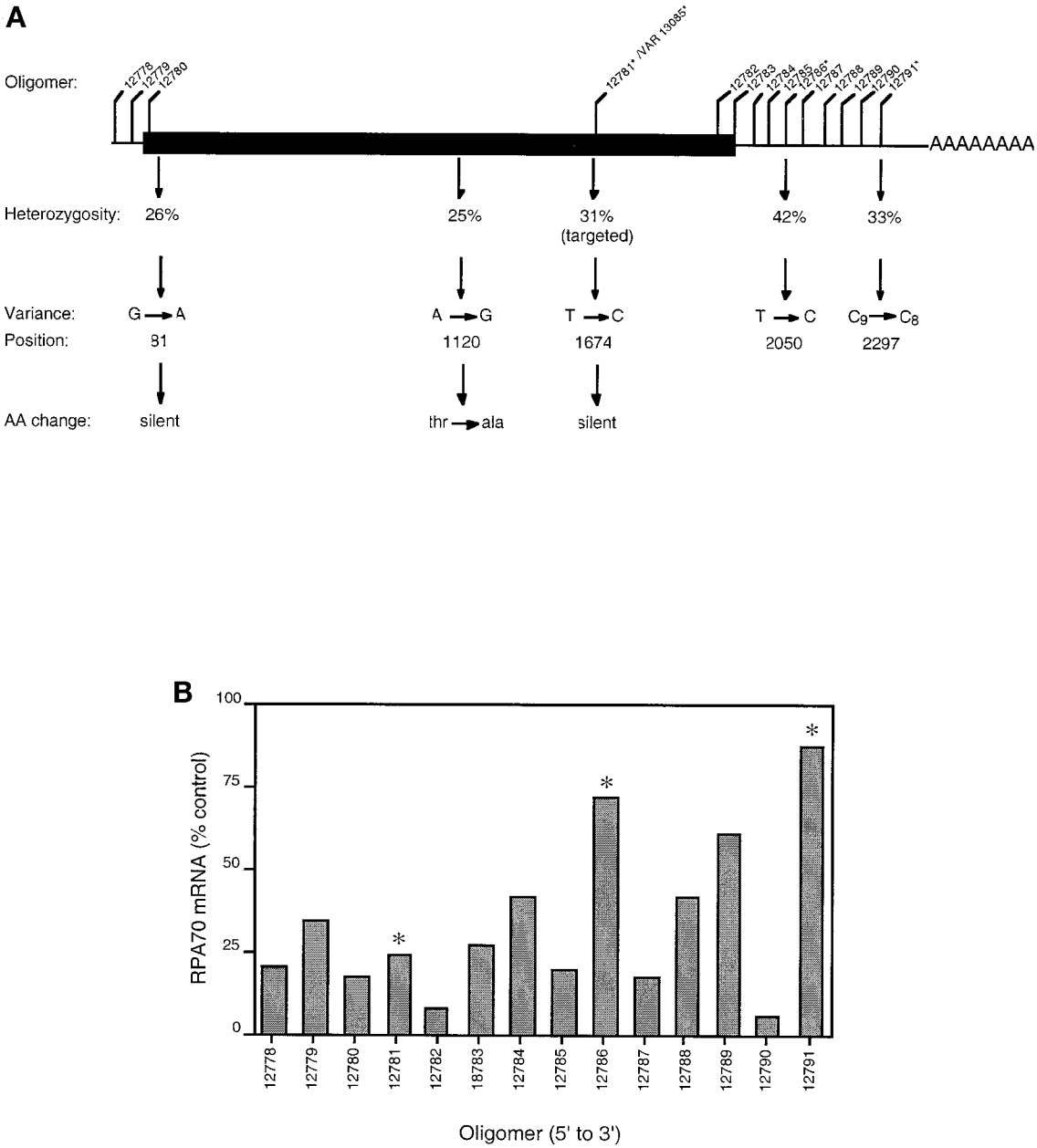


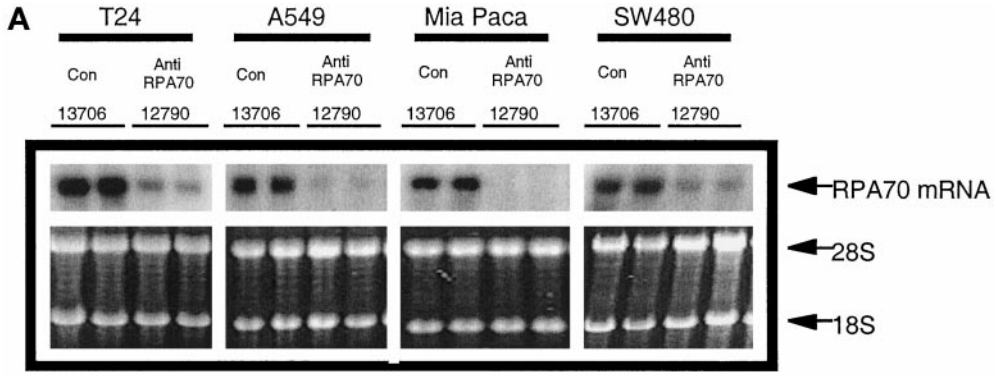
Fig. 2. Selection of anti-RPA70 antisense oligonucleotides for allele-specific targeting. A, schematic of RPA70 mRNA depicting the relative hybridization position of 15 antisense oligonucleotides that were tested for their ability to decrease RPA70 mRNA expression in tissue culture. Arrows indicate the variances contained in the RPA70 mRNA that occur at a heterozygosity rate of 25% or greater in the normal population. The location of the variances as well as the base and amino acid change are listed relative to the published cDNA sequence of the RPA70 gene (Erdile et al., 1991). B, RPA70 mRNA levels after antisense oligonucleotide treatment. A549 cells were treated for 5 h with 400 nM oligonucleotides, as described in *Materials and Methods*. RPA70 mRNA levels were measured 24 h later by Northern blot analysis. RPA70 mRNA levels were quantified and normalized to G3PDH as described (Monia et al., 1996b). * Oligomers ISIS 12781, ISIS 12786, and ISIS 12791 that target regions of the mRNA that contain variances. Sequences of oligonucleotides are available on request.

12790 was associated with decreased cell number in all four tumor cell lines (Fig. 3B). The decrease in cell number observed in T24 cells was 79%, comparable with that achieved with ISIS 2503. The decrease observed in the other three cell lines was A549, 98% decrease; Mia Paca II, 85% decrease; and SW480, 81% decrease. These data demonstrate that inhibition of RPA70 with antisense oligonucleotides is associated with a decrease in cell survival, confirming that this gene is essential for survival of the four tumor lines examined.

Identification of Variance-Specific Inhibitors of RPA70. Two phosphorothioate oligodeoxynucleotides, designed to target the variant sequences at position 1674 of the RPA70 mRNA were synthesized. ISIS 12781 was complementary to the T variance at position 1674. VAR 13085 was complementary to the C at position 1674, but was reduced in length relative to ISIS 12781 by the removal of two nucleotides from the 5′ end of the oligonucleotide. Shortening the length of the oligonucleotide enhanced oligonucleotide discrimination between the two variant alleles (data not shown).

Several cell lines were genotyped for the variance at position 1674 to identify cell lines expressing only one variant form of the gene. Mia Paca II cells were found to express only the C allele of RPA70, whereas A549 and HeLa cells expressed only the T allele.

Treatment of HeLa cells with ISIS 12781, which matches the target gene in these cells, resulted in a statistically significant dose-dependent inhibition of RPA70 mRNA expression over a concentration range between 50 and 400 nM ($p < .001$; Fig. 4, A and B). The IC_{50} for inhibiting expression of RPA70 was between 50 and 100 nM. In contrast, treatment of these cells with VAR 13085, which contains a single mismatch to the target in these cells, had only a small effect on the level of mRNA, even at 400 nM (Fig. 4, A and B). As a positive control, cells were treated with ISIS 12790. This oligonucleotide targets a different site within the gene and is not variance-specific. Treatment of the cells with ISIS 12790 resulted in a dose-dependent decrease of RPA70 mRNA levels with maximum suppression of mRNA levels occurring at 200 nM and an IC_{50} of less than 50 nM. None of the oligo-



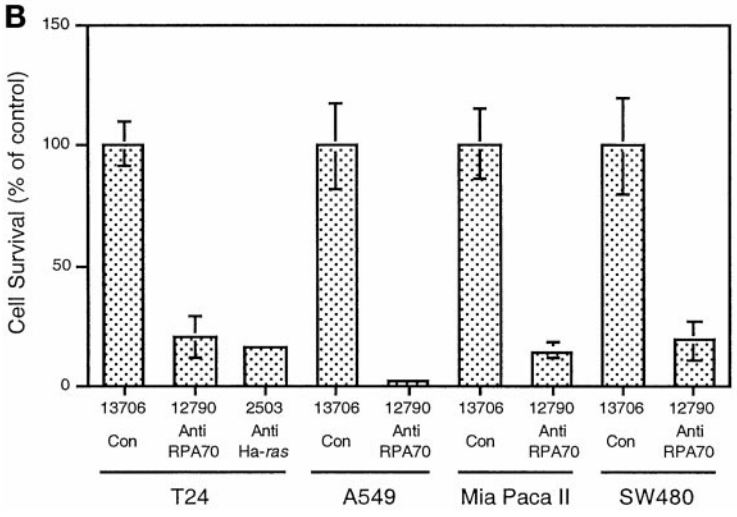


Fig. 3. Inhibition of cell growth and RPA70 mRNA expression by ISIS 12790. A, T24, A549, Mia Paca II, and SW480 cells were treated with 400 nM ISIS 12790 (Anti-RPA70), an all-phosphorothioate antisense oligonucleotide targeting a nonvariant region of RPA70, or a 7-mismatch control analog of ISIS 12790, ISIS 13706 (Con). Top, total RNA was prepared 24 h later and analyzed for RPA70 mRNA levels by RNA blot. Bottom, the EtBr-stained RNA before transfer. RPA70 mRNA was analyzed in duplicate. For all RNA blots, transfer efficiency was monitored by visualization with UV light. Treatment of cells with ISIS 13706 had no effect on RPA70 mRNA levels. B, the indicated cell lines were treated with 400 nM Anti-RPA70 (ISIS 12790) or the control antisense oligonucleotide ISIS 13706. After removal of the oligonucleotide, cells were allowed to recover for 3 days and the cell number was determined by hemocytometer. As a positive control, T24 cells were also treated with ISIS 2503 (Anti-Ha-*ras*). This oligonucleotide targets the Ha-*ras* gene in T24 cells and has previously been shown to have strong antiproliferative effects on these cells in vitro and in vivo (Bennett et al., 1996).

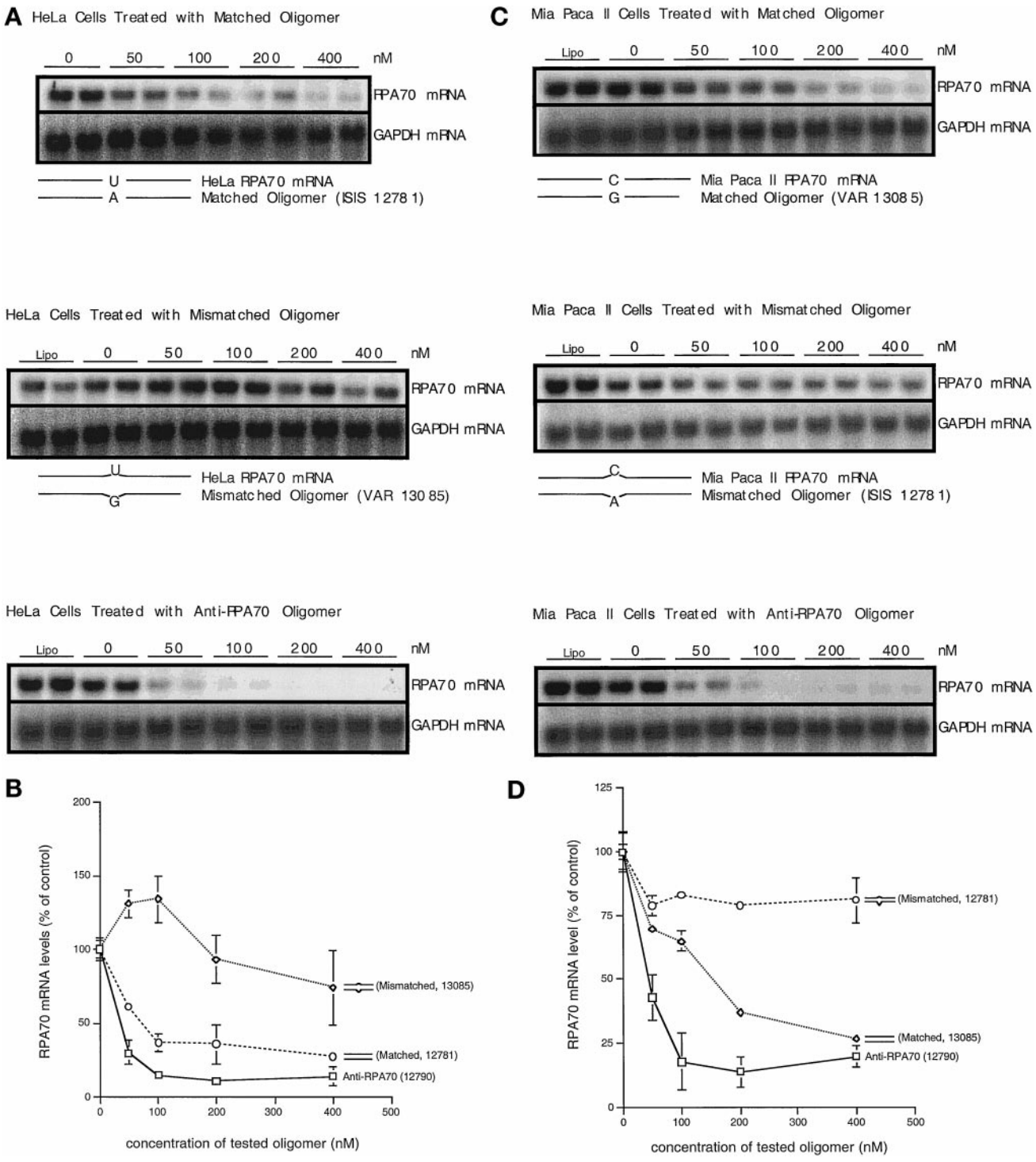


Fig. 4. Variance-specific inhibition of RPA70 mRNA expression in HeLa and Mia Paca II cells. Two cell lines, each expressing only one of the two variances identified at position 1674, were treated with increasing concentrations (50–400 nM) of perfectly matched, one-base mismatched, or Anti-RPA70 (ISIS 12790) antisense oligonucleotides. Total RNA was isolated 24 h after oligonucleotide treatment and the level of RPA70 mRNA expression was determined by RNA blot. To control for nonspecific phosphorothioate effects, each dose of oligonucleotide was supplemented to 400 nM with a randomized control oligonucleotide (see *Materials and Methods*). Where indicated, the effect of cationic liposomes alone (Lipo) on RPA70 mRNA was also tested. A, HeLa cells were treated with matched (ISIS 12781 [5′-TAGCTTCAGCAGACTCCTGG-3′]), one-nucleotide mismatched (VAR 13085; 5′-GCTTCAGCGGACTCCTGG-3′), or Anti-RPA70 (ISIS 12790; 5′TGGTCTGCAGTTAGGGTCAG-3′) antisense oligonucleotides. B, quantification of RPA70 mRNA levels from 4A. RPA70 mRNA levels were quantified on a Fuji FLA-2000 and graphed as a percentage of the control levels. Control levels were determined from cells treated with 400 nM control 20N-mer (lanes marked 0 nM; see *Materials and Methods*). 12790 and 12781 curves differ significantly from 13085 curve by ANOVA ($p < .001$). Each point represents duplicate analysis with error bars indicating ± S.D. C, Mia Paca II cells were treated with matched (VAR 13085), singly mismatched (ISIS 12781), or Anti-RPA70 (ISIS 12790) antisense oligonucleotides. D, quantification of RPA70 mRNA levels in 4C. 12790 and 13085 curves differ significantly from 12781 curve by ANOVA ($p = .03$ and .002, respectively). Each point represents duplicate samples with error bars indicating ± S.D.

nucleotides inhibited the level of GAPDH mRNA significantly in HeLa cells (Fig. 4A).

Treatment of Mia Paca II cells with increasing concentrations of VAR 13085, which matches the target gene in these cells, resulted in a statistically significant dose-dependent decrease in the level of RPA70 mRNA compared with mismatched oligonucleotide ($p = .002$; Fig. 4, C and D). This effect was not observed when VAR 13085 was applied to HeLa cells. Treatment of Mia Paca II cells with ISIS 12781, which contains a single mismatch from the target in these cells, had little effect on the level of RPA70 mRNA. ISIS 12790 resulted in a dose-dependent decrease in RPA70 mRNA levels quantitatively similar to the effect observed with this oligonucleotide in HeLa cells. None of the oligonucleotides inhibited the level of GAPDH mRNA significantly in Mia Paca II cells (Fig. 4C).

Variance-Specific Suppression of Cell Survival. To determine the effect of variance-specific antisense oligonucleotides on cell survival, cells were treated with oligonucleotides and cell survival measured by Sulforhodamine B staining. Treatment of HeLa cells with increasing concentrations of the matched antisense oligonucleotide, ISIS 12781, resulted in a statistically significant dose-dependent decrease in cell survival compared with mismatched oligonucleotide ($p < .001$), with an IC_{50} between 100 and 200 nM (Fig. 5A). At the maximum concentration of ISIS 12781, 400 nM, there was an 84% reduction of surviving cells. Treatment with VAR 13085, which contains a single base mismatch relative to the allele expressed in HeLa cells, resulted in little change in cell survival. After treatment with 400 nM oligonucleotide, the amount of cells remaining with VAR 13085 was 3.1-fold higher than with ISIS 12781. Treatment of cells with the nonallele specific anti-RPA70 oligonucleotide, ISIS 12790, caused a dose-dependent reduction in the number of surviving cells, with a 90% reduction in the number of surviving cells at 400 nM. The IC_{50} for the decrease was less than 100 nM. The IC_{50} values for inhibition of HeLa cell survival correlated with the IC_{50} values for RPA70 mRNA suppression by both ISIS 12781 and ISIS 12790 oligonucleotides.

Treatment of Mia Paca II cells with the matched antisense oligonucleotide, VAR 13085, resulted in a dose-dependent decrease in cell survival, with an IC_{50} between 100 and 200 nM (Fig. 5B) and a 90% reduction in the number of surviving cells at 400 nM. Inhibition of cell survival was seen also with 100 and 200 nM the mismatched oligonucleotide ISIS 12781. However, at 400 nM, there was a statistically significant difference in survival, with less survival seen in cells treated with the matched oligonucleotide, VAR 13085, than the mismatched oligonucleotide, ISIS 12781 ($p = .02$). At 400 nM, there was a 4.6-fold difference in cell survival between matched and mismatched oligonucleotides. As with HeLa cells, treatment of the Mia Paca II cells with increasing concentrations of ISIS 12790 yielded a dose-dependent decrease in the number of surviving cells, with an IC_{50} of less than 100 nM. The IC_{50} for inhibition of Mia Paca II cell survival was similar to that for suppression of RPA70 mRNA for both VAR 13085 and ISIS 12790. The effect of antisense treatment on cell survival for both cell lines appears to be cytotoxic rather than cytostatic, as the absolute number of cells decreases for both cell lines when treated with matched antisense oligonucleotides. For each of the cell lines, the successful inhibition of RPA70 expression and cell survival by two distinct oligonucleotides targeting different sites in the mRNA strongly suggests that inhibition of cell survival is target-dependent and acts through an antisense mechanism.

Multiple controls were used to support the interpretation

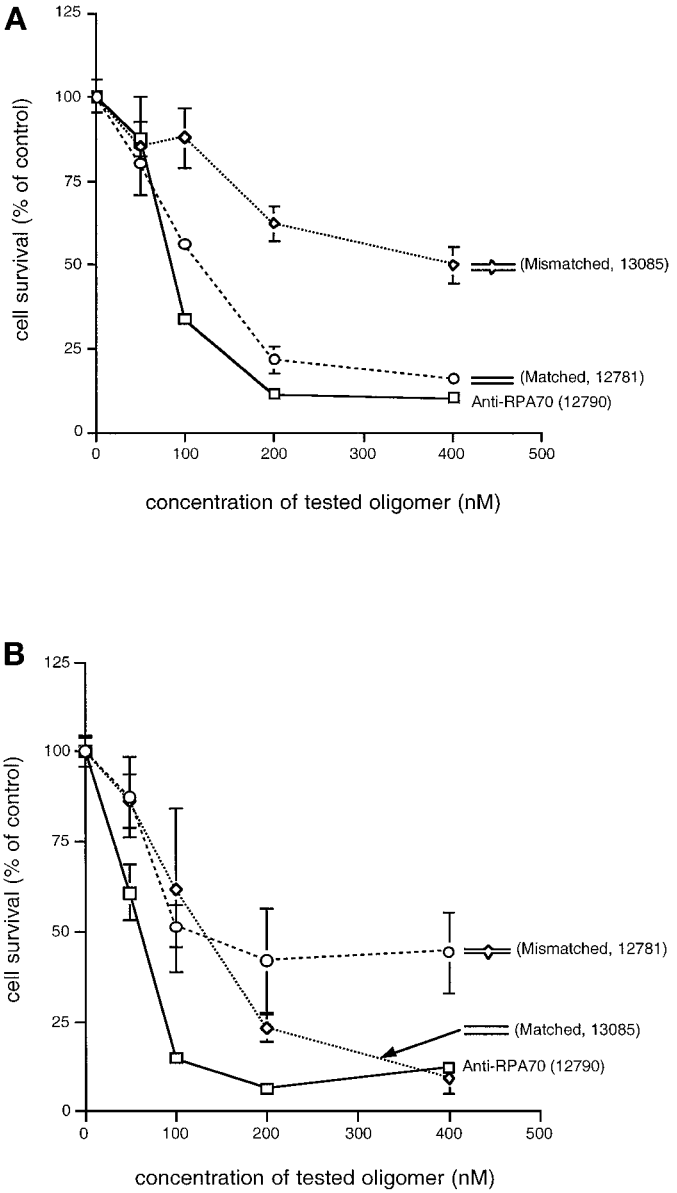


Fig. 5. Effect of various antisense oligonucleotides targeting RPA70 on HeLa and Mia Paca II cell survival. HeLa cells (A) or Mia Paca II cells (B) were treated either once (HeLa cells) or three successive times (Mia Paca II cells) with the indicated concentrations of matched and mismatched antisense oligonucleotides. As a positive control, both cell lines were also treated with ISIS 12790 (Anti-RPA70), which targets a nonvariant region of RPA70 mRNA. After the final treatment, the cells were allowed to recover either 3 (HeLa cells) or 6 days (Mia Paca II cells) and the number of surviving cells quantified by sulforhodamine B staining (see *Materials and Methods*). For both A and B, the amount of total phosphorothioate oligonucleotide was held constant at 400 nM by supplementing each dose to 400 nM with a randomized 20N-mer control phosphorothioate oligonucleotide. The number of transfections and recovery times were selected empirically and appear to be related to cell growth rates and sensitivity of cells to multiple transfections with cationic lipids. Survival is normalized to control treatments with 400 nM 20N-mer and is reported as a percentage of control. A, 12790 and 12781 curves differ significantly from 13085 curve by ANOVA ($p < .001$). B, 12790 and 13085 values at 400 nM differ significantly from 12781 value at 400 nM by ANOVA ($p = .03$ and $p = .02$, respectively). Error bars represent ± S.E. of the mean ($n = 3$).

that the inhibition of mRNA levels and cell survival reflect a specific antisense effect. First, of the 14 antisense oligonucleotides complimentary to the RPA70 mRNA, not all were effective inhibitors of RPA70 expression. This result is similar to what is commonly observed in scanning mRNA for antisense target sites. Second, in each cell line tested with the variance-specific oligonucleotides, the antisense perfectly matching the target gene produced greater inhibition than the control differing by a single base. A unique feature of these studies is the fact that the negative-control, mismatched oligonucleotide in each cell line is also shown to reduce RPA70 mRNA expression and to have antiproliferative effects when administered to cells that express the alternative matching allele of RPA70 (Figs. 4 and 5). Third, in each cell line, a positive control oligonucleotide, ISIS 12790, targeted to a different region of RPA70 mRNA, suppressed RPA70 gene expression and cell survival in a quantitatively analogous manner. Fourth, a variety of negative controls including transfection with a random 20N-mer oligonucleotide, transfection with antisense oligomers targeting unrelated genes, and oligonucleotides having the inverse sequence of either VAR 13085 or ISIS 12781 had no effect on RPA70 mRNA levels (data not shown). Fifth, we demonstrate that VAR 13085, ISIS 12781, and ISIS 12790 do not significantly suppress levels of GAPDH mRNA. In addition to these controls, for dose-response experiments, the total concentration of phosphorothioate oligonucleotide was maintained at 400 nM by supplementing the lower concentrations of specific oligonucleotides to 400 nM with a random 20N-mer phosphorothioate oligonucleotide.

Identification of Variance-Specific Inhibitors for Other Genes. To determine whether an antisense approach to variagenic targeting would be suitable for other gene targets, a screen of 15 additional genes was performed. Each of these genes is located in a region of a chromosome exhibiting substantial LOH in one or more cancers, and each variance has a heterozygosity frequency above 20% (a total of 35). Table 1 lists the variances successfully targeted by antisense oligonucleotides and the cell lines in which mRNA suppression was observed. Target mRNA suppression was observed for 22 of the 35 tested variances (63%), covering 13 of the 15 genes. Strikingly, oligonucleotide-selective mRNA suppression was observed at 20 of the 22 sites. Only two of the targeted sites showed equivalent mRNA suppression with both matched and mismatched oligonucleotide. With the exception of eukaryotic initiation factor 5A (site 623, at which the oligonucleotide targeting the G allele was found to suppress mRNA regardless of cell genotype), the oligonucleotide-selective mRNA suppression seen at the 20 sites correlated perfectly with the genotype of the cells. Figure 6 shows representative blotting results for six of the genes targeted in this screen. The left side shows three targets for which cells of both genotypes were tested, revealing reciprocal patterns of mRNA suppression.

Discussion

This study demonstrates in vitro the feasibility of a new strategy for development of anticancer agents. This technology exploits normal sequence variances in essential genes and LOH occurring during oncogenesis to define cancer-specific gene targets for antiproliferative drugs. The two principal advantages of this technology for cancer therapy are that the targets for this approach are genes known to be essential to cell survival, increasing the likelihood that inhibitors of these genes will be cytotoxic in vivo; and that this approach targets an absolute genetic difference between normal and diseased tissue, potentially enabling a greater therapeutic index than current therapies that are targeted to relative differences in the biological characteristics of normal and cancer cells.

In this study, we have used antisense phosphorothioate deoxyoligonucleotides to specifically suppress the expression of a gene, RPA70, that satisfies the criteria required for variagenic targeting. The data presented here demonstrate that RPA70 is indeed essential for cell survival, and are one of the first examples demonstrating that variance-specific differential cell killing, based on a single nucleotide difference in mRNA sequence, is achievable. These studies suggest that the strategy for developing anticancer agents based on normal genetic variation in essential genes and LOH in cancer is feasible.

Both ISIS 12781 and VAR 13085 effectively suppress RPA70 mRNA and inhibit cell survival in cells that express only the RPA70 mRNA with the exact complementary (matched) sequence. However, when administered to cells expressing the mismatched target, both oligonucleotides are less effective in suppressing RPA70 mRNA and in inhibiting cell survival. The discrimination is most apparent for VAR 13085, which inhibited mRNA and cell survival in Mia Paca II cells but exhibits little inhibition of mRNA or cell survival in HeLa cells. ISIS 12781 was most effective in inhibiting mRNA and cell survival in HeLa cells, but did exhibit toxicity to Mia Paca II cells disproportionately greater than the inhibition of mRNA observed in these cells. This result suggests that ISIS 12781 has some nonspecific toxicity not mediated by antisense inhibition of RPA70 mRNA. Nevertheless, despite this nonspecific toxicity, at 400 nM, there is also evident a statistically significant ($p = .02$) 4.6-fold variance-specific effect on cell survival that correlates with the specific effect on RPA70 expression. The observed specificity for different forms of the target gene, differing by only one nucleotide, is also consistent with previous reports with phosphorothioate oligonucleotides with single-base mismatches as controls in both in vitro and in vivo experiments. This specificity is also consistent with the ability of oligonucleotides to achieve specific inhibition of mutant forms of the Ha-*ras* protooncogene in vitro and in vivo (Monia et al., 1992, 1993; Duroux et al., 1995; Bennett et al., 1996).

The degree of inhibition observed with antisense inhibitors of RPA70 is comparable with that observed with other phosphorothioate oligonucleotides, including several products that have been shown to be effective antiproliferative agents in animal models and have moved successfully into clinical trials. These include ISIS 2503 (Phase I), an inhibitor of Ha-*ras* (Bennett et al., 1996), which was used as a positive control in Fig. 3B, ISIS 5132 (Phase II), an inhibitor of c-*raf* kinase (Monia et al., 1996a), ISIS 3521 (Phase II), an inhibitor of protein kinase C-α, and G-3139 (Phase II), an inhibitor of *Bcl-2*. Furthermore, positive clinical results have been reported for G3139 for the treatment of non-Hodgkin's lymphoma (Webb et al., 1997). All of these oligonucleotides have displayed very attractive safety profiles in the clinic, suggesting the possibility that they will exhibit a therapeutic index

that is attractive and, thus, quite unusual for anticancer agents. Therefore, we expect that oligonucleotides against RPA70 could be developed as effective antiproliferative agents in pharmacological models.

One of the potential limitations to any chemotherapeutic agent targeting essential cellular genes is drug toxicity caused by drug action on nondisease tissues. Because of the presence of an insensitive allele of the targeted gene in each normal cell, unwanted toxicity of these drugs to normal tissues will largely be limited by our ability to design highly selective variance-specific drugs. The present data are not sufficient to establish whether the degree of specificity exhibited by the phosphorothioate oligonucleotides will be sufficient to achieve killing of hemizygous cancer cells in vivo without toxic effects on heterozygous normal cells.

We do not expect the two variance-specific antisense oligonucleotides described here to be developed as pharmaceutical products; a greater specificity and efficacy is likely to be required to achieve specific killing of cancer cells in humans. Improvements may be achieved in several ways. First, phosphorothioates, with their relatively low affinity for RNA and corresponding requirement for relatively long sequences to achieve inhibition, may not be the ideal chemistry for achieving allele specificity. Advanced oligonucleotide chemistries and formulations that will modulate pharmacokinetics and increase the efficiency of delivery to tumor cells have been

TABLE 1

Allele-selective mRNA suppression of other genes

Sixteen genes were targeted for mRNA suppression. Each variance with a heterozygosity frequency above 20% (a total of 35) was targeted with oligonucleotide complementary to either allele of the variance. The effect of antisense oligonucleotides on mRNA levels was assessed by blotting. Listed in the table are variances successfully targeted by antisense oligonucleotides and the tumor cell lines in which mRNA suppression was observed. Of the 22 sites successfully targeted, 19 showed oligonucleotide-selective mRNA suppression that correlated perfectly with the genotype of each cell type. Genotyping of each cell line for each variance identified only two heterozygous lines, presumably due to frequent LOH in tumor lines. Data from these lines were excluded because of the difficulty of interpretation without knowledge of the ploidy of each allele.

Gene Targeted	Variance Targeted[a]	Cell Type Targeted	Genotype of Cell	Allele-Selectivity[b]
RPA 70	1674 T/C	Mia Paca	C	C > T
		HeLa	T	T > C
		SW480	T	T > C
		A549	T	T > C
		SW620	T	T > C
		Panc-1	T	T > C
Replication protein A, 32 kD[c]	40 G/A	Mia Paca II	G	G > A
		SW480	G	G = A
Ribonucleotide reductase	2410A/G	Mia Paca II	G	G > A
		HeLa	A	A > G
	2419A/G	Mia Paca II	A	A > G
Thymidylate synthase	1140C/T	Mia Paca II	C	C > T
		SW480	C	C > T
		A549	C	C > T
RNA polymerase II	1847C/T	SW480	C	C > T
	3059C/T	Mia Paca II	T	T > C
		SW480	C	C > T
	6466T/C	Mia Paca II	C	C > T
TATA associated factor 2H	554G/A	Mia Paca II	G	G > A
		SW480	G	G > A
		A549	G	G > A
Ribosomal protein S14	183G/A	SW480	G	G > A
Eukaryotic initiation factor 5A	623A/G	Mia Paca II	A	G > A
		SW480	G	G > A
		A549	A	G > A
		T24	G	G > A
		MCF7	A	G > A
		SKRC39	A	G > A
	1012C/T	Mia Paca II	C	C > T
		SW480	T	T > C
		T24	T	T > C
Alanyl tRNA synthetase	1013T/C	Mia Paca II	C	C > T
		SW480	T	T > C
Cysteinyl tRNA synthetase	1739C/T	T24	C	C > T
Glutamyl Prolyl tRNA synthetase	3247A/G	Mia Paca II	G	G > A
		A549	G	G > A
		MCF7	A	A > G
	4459G/A	A549	A	A > G
Threonyl tRNA synthetase	1608G/A	Mia Paca II	G	G > A
	1755G/A	T24	G	G > A
	2395T/C	SW480	T	T > C
NaK ATPase, alpha-1 subunit	2538T/C	Mia Paca II	T	T = C
		A549	T	T = C
		T24	T	T = C
	3375G/A	T24	G	G > A
Transferrin receptor	2387G/A	SW480	G	G = A

[a] Variances at which antisense oligonucleotides suppressed mRNA levels. The number and nucleotide listed refer to the position and variance identified for each cDNA, respectively. Position corresponds to numbering available from Genbank for each cDNA. The most abundant variance is listed first. Accession numbers for each gene are available on request.

[b] C>T indicates that the oligonucleotide matched to the "C" allele suppressed target mRNA better than the oligonucleotide matched to the "T" allele, etc.

[c] Note that RPA, 32 kDa subunit, was scored as showing oligonucleotide-selective mRNA suppression even though only one of the two cell lines in the table showed differential suppression.

used to achieve antisense inhibition with shorter sequences. This may allow greater variance-specific discrimination. Second, optimization of the length and position of the oligonucleotide sequence relative to the position of the variance may identify products with greater specificity. Third, various strategies for increasing the ability of antisense oligonucleotides to discriminate single-base mismatches with advanced chemistries or oligonucleotides with hybrid chemistries have been described. Finally, it is likely that there will be differences in the ability to achieve variance-specific inhibition of different sequence variances comprising distinct base changes and occurring in different sequence contexts or secondary or tertiary structures.

We have identified many genes that meet the three criteria enumerated above and are potential targets for drug discovery through variagenic targeting. We predict that several hundred genes will be identified that meet these criteria. Although the number of essential human genes is not known, studies of disrupted *Saccharomyces cerevisiae* genes suggest that over 25% are essential for growth (Hodges et al., 1998). Of these genes, ~500 have homologues in humans, and preliminary studies to date have identified at least one putative variance in ~300 of these genes (D. Steffen, R. M. Adams and V. P. S., unpublished data). Thirty genes that are known to be essential for cell survival have now been studied in detail, and we have identified variances with >20% heterozygosity and LOH at >40% frequency in at least one major cancer type in 16/30 genes. The large number of potential targets for variagenic targeting provides a spectrum of challenge and opportunity for drug development.

In our study of 16 genes containing 35 variances, only two of the genes were not affected by antisense oligonucleotides. The 14 remaining genes contained 22 variances that were targeted by antisense oligonucleotides (Table 1). We have achieved variance-specific suppression by antisense oligonucleotides at 86% (19/22) of these variances. We have also identified six variances in amino acid sequence that may be candidates for inhibition by small molecules, several of which occur in regions of the protein that may be involved in biological function. Lastly, we have identified at least one sequence variance in an extracellular domain of a protein that is a potential target for inhibition by monoclonal antibodies (F. Baas, A. ten Asbroek, D. E. H., and V. P. S., manuscript in preparation). Thus, we believe that a wide variety of different chemistries and therapeutic approaches may be used to achieve effective cancer therapy through variagenic targeting. The major challenge for this technology will be the development of pharmaceutical products capable of achieving variance-specific inhibition, based on a single nucleotide or amino acid change in the target. The in vivo specificity of such an inhibitor will depend on the kinetics of inhibition of sensitive and insensitive alleles of the target gene, the ploidy of the remaining chromosome after LOH, and on the relative bioavailability of the inhibitor to different normal and cancer tissues.

It should be noted that the therapeutic strategy described here will face many of the same obstacles that must be overcome by more conventional chemotherapeutics, namely, the selection of drug-resistant cancer cells. For variagenic targeting, the selection of resistant cancer cells could very well be attributable to mutation of the targeted variance. However, because LOH presumably will affect more than one polymorphic essential gene (more than one essential gene is located in the region of a chromosome that undergoes LOH) therapeutic strategies with multiple agents and targeting more than one essential gene (or more than one site in the

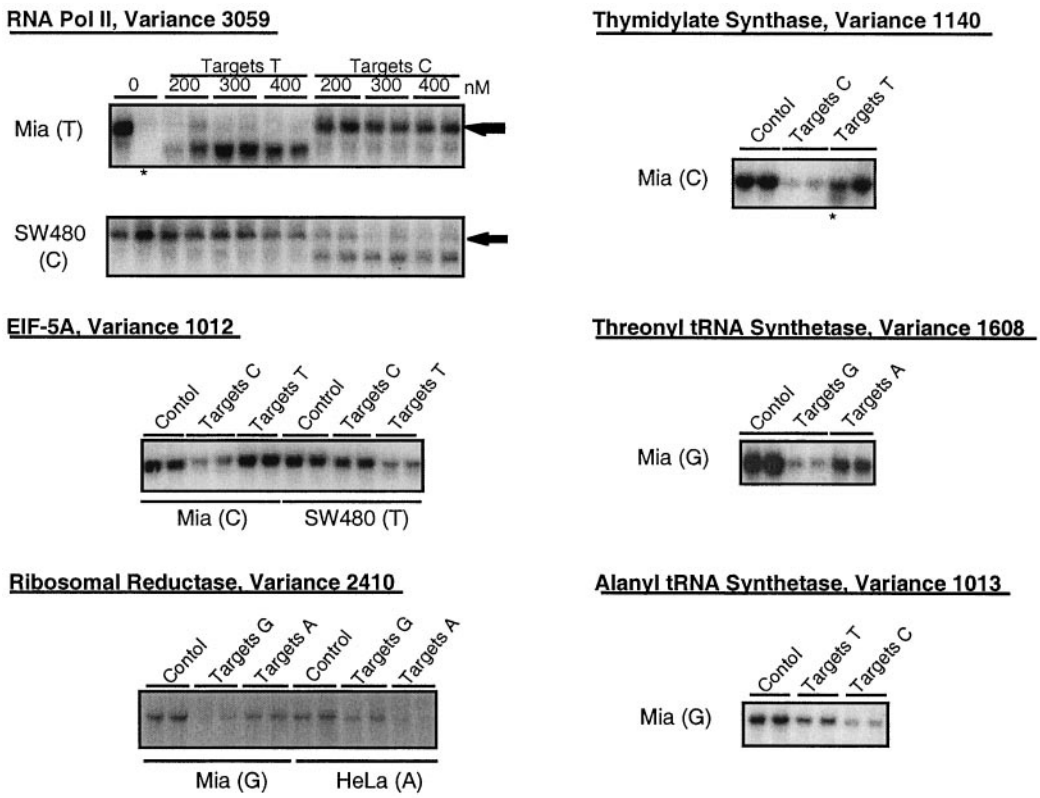


Fig. 6. RNA blotting showing variance-selective mRNA down-regulation. Tumor cell lines were transfected with control (ISIS 13706) or allele-selective phosphorothioate oligonucleotides, as described in the legend to Table 1. RNA blots are shown for RNA polymerase II (RNA Pol II), the eukaryotic initiation factor 5A (eIF-5A), ribosomal reductase, thymidylate synthase, and the threonyl and alanyl tRNA synthetases. The left side shows three targets for which cell lines of both genotypes were tested. Arrows indicate RNA polymerase II mRNA (the lower band presumably represents the product after cleavage). Total RNA concentration was measured by spectrophotometer and equal amounts loaded in each lane. Equivalency of loading was confirmed by EtBr staining of ribosomal RNA (not shown). *, RNA was found to be degraded.

same gene) should help prevent selection of drug-insensitive cancer cells.

Ultimately, we hope that variagenic targeting will allow development of a series of multiple agents with high therapeutic indices that could be used effectively alone or in combination for anticancer therapy. The use of these agents would be linked to a panel of diagnostic tests to identify patients who are heterozygous for the target genes, and pathological analysis to determine which forms of the target genes were retained in the tumor. This would allow cancer therapy, like antimicrobial therapy, to be selected based on foreknowledge of the sensitivity of the tumor to the prescribed therapy.

Acknowledgments

We thank our numerous colleagues at Variagenics, Inc. and Isis Pharmaceuticals for their involvement, assistance, and advice on this project. We would also like to thank Drs. Claude Hélène, Phillipe Gros, and Frank Baas for their thoughtful critiques of the manuscript, and Anneloor L. M. A. ten Asbroek for sharing unpublished single nucleotide polymorphism (SNPs) data. Dr. Housman is Novartis Professor of Biology at the Center for Cancer Research, Massachusetts Institute of Technology. LOH studies were performed through a contract with Eurona Medical AB (Uppsala Sweden).

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