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Use of antisense oligonucleotides to study homeobox gene function

Review Article

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Summary

Homeobox genes code for transcription factors known to be important in hematopoiesis and leukemogenesis. In order to understand the function of a homeobox gene in leukemia, we have used an antisense oligonucleotide technique to inhibit the expression of a homeobox gene, DLX7, which is expressed at high levels in some leukemia cell lines and patients. With careful design and use of the antisense oligonucleotide, we have found that the loss of DLX7 gene expression results in loss of expression of secondary genes, cell cycle arrest and apoptosis. These studies have led to an understanding of DLX7 gene function in leukemia cell growth and the identification of genes regulated by DLX7. These studies also raise the possibility that DLX7 antisense oligonucleotides may be useful in the treatment of patients with DLX7-positive leukemia.

I. Introduction

A. Homeobox genes as regulators of hematopoietic differentiation

Homeobox genes are a family of genes coding for transcription factors originally identified in the Drosophila as genes responsible for homeosis (McGinnis and Krumlauf, 1992; Scott et al., 1989), a phenomenon where Drosophila mutants exhibit abnormalities in pattern formation and cell fate decisions during embryogenesis. Homeobox genes have a strikingly conserved 60 amino-acid "homeodomain" encoding a DNA-binding domain.

We and many others have demonstrated that both lymphoid and non-lymphoid hematopoietic cells express homeobox genes (Baier et al., 1991; Crompton et al., 1992; Deguchi et al., 1993; Inamori et al., 1993; Kongsuwan et al., 1988; Petrini et al., 1992; Shen et al., 1989; Takeshita et al., 1993). It has therefore been attractive to speculate that homeobox genes may also play important role(s) in the regulation of hematopoiesis and pathogenesis of hematologic malignancies.

Several homeobox genes have been shown to have a function in normal hematopoiesis and in leukemia. Using over-expression strategies, several groups have shown that dysregulated expression of homeobox genes can result in increased cell proliferation (Sauvageau et al., 1995) and in some cases uncontrolled proliferation leading to leukemia. These studies have been instrumental in identifying some homeobox genes as leukemogenic genes in both mouse and human disease (Lawrence and Largman, 1992). The role of homeobox genes in leukemogenesis is corroborated by findings that some leukemia-associated chromosomal translocations affect homeobox genes.

Over-expression or ectopic expression of homeobox genes have been shown to cause leukemia. The murine acute monocytic leukemia line WEHI-3B has been shown to carry a retrovirus-like insertion near the Hox B8 gene, resulting in its over-expression (Blatt et al., 1988; Kongsuwan et al., 1989), while in BXH-2 mice, another homeobox gene is affected by retrovirus insertion (Moskow et al., 1995; Nakamura et al., 1996). The WEHI-3B cell line also has an IAP insertion upstream of the IL-3 gene, and co-infection of normal bone marrow with retrovirus vectors carrying the cloned IL-3 and Hox B8 genes resulted in myeloid leukemia (Perkins et al., 1990). There are also examples in humans of aberrant homeobox gene expression causing leukemia. In pre-B ALL with t(1;19) translocation a fusion protein is created between the transcription factor E2A gene and the homeobox gene PBX (Kamps et al., 1990; Nourse et al., 1990). In T-cell ALL with t(10;14) translocation, the Hox 11 gene is deregulated (Hatano et al., 1991; Lu et al., 1991). In myeloid leukemia with t(7;11) translocation, the HoxA9 gene is rearranged (Borrow et al., 1996; Nakamura et al., 1996). Rearranged homeobox genes have also been found in solid tumors such as rhabdomyosarcoma (Barr et al., 1993); ectopic expression of homeobox genes results in focus formation in the NIH 3T3 transformation assay (Maulbecker and Gruss, 1994). Thus, in addition to the well documented function in specifying body parts during embryogenesis, a major function of many human homeobox genes appears to be directed towards control of hematopoietic cell proliferation.

An alternative strategy to understand homeobox gene function has been to inhibit the expression of homeobox genes. The best experimental system to inhibit gene expression involves gene targeting to create engineered mutants in mice. Hoxa-9 mutant mice demonstrate decreases in the number of hematopoietic progenitor cells, although the peripheral blood counts are relatively normal (Lawrence et al., 1997). We have obtained similar findings in the Hoxc-8 knock-out mice (Shimamoto et al., 1998). Mice lacking genes known to control Hox genes, such as bmi-1 (vander Lugt et al., 1994) and mll (Yu et al., 1995) have more striking hematologic deficits. These knock-out mouse strategies indicate that some homeobox genes are important for hematopoiesis. However, these systems do not easily offer a detailed look on how the homeobox genes regulate hematopoiesis.

In this article, we review our experience with the use of antisense oligonucleotides as a way to selectively inhibit the expression of a single homeobox gene in order to understand its function in hematologic cells (Shimamoto et al., 1998; Takeshita et al., 1993). The use of antisense oligonucleotides has been summarized in two recent reviews (Crooke, 1998; Gewirtz et al., 1998).

II. The DLX homeobox gene family

We have focused on a member of the DLX gene family, the founding member being the distal-less gene in the Drosophila fruitfly (Cohen et al., 1989). There are 6 members of this homeobox gene family, DLX1, DLX2, DLX3, DLX5, DLX6 and DLX7. These genes exist as closely located pairs in the chromosome, located at 2q32, 7q22 and 17q23, with the gene transcription units pointing toward each other. Therefore, the 3' end of one gene is also the 3' end of the other gene (Nakamura et al., 1996; Weiss et al., 1994) (see **Figure 1**).

At least four of the six DLX genes, DLX 1,2,3 and 7, are expressed in many leukemia cell lines. Since the known location of normal DLX gene expression is in the forebrain and the craniofacial structures, and since normal hematopoietic cells do not express or express only very low levels of DLX genes, expression of DLX genes in leukemia is likely to reflect either gene expression in progenitor cells or ectopic expression in malignant cells. Mutation in the DLX3 gene, a 4-bp deletion in the coding region of the DLX3 protein, has been identified on the tricho-dento-osseous syndrome, an autosomal dominant disorder characterized by kinky curly hair, enamel hypoplasia, and increased thickness and density of cranial bones (Price et al., 1998).

The DLX7 gene was chosen for our study for two reasons. First, it is expressed at high levels in many leukemia cell lines of an erythroid phenotype and in about 20% of leukemia cells from patients with acute myelogenous leukemia, but is expressed at very low levels in the normal bone marrow. The normal sites of expression of this gene are the central nervous system and craniofacial structures during development (Weiss et al., 1994).

A. DLX7 gene cloning and structure

DLX7 is expressed by leukemia cell lines of an erythroid phenotype at a high level (**Figure 2**, panel **C**). The major transcript is 2 kB, with a minor species of about 7 kB seen only in K562 erythroleukemia cells, which probably represents an alternative transcript, a phenomenon commonly seen in all DLX genes and other homeobox genes. Using the more sensitive RT-PCR, we have found that the gene is also expressed at a barely detectable level in normal bone marrow cells and in peripheral blood cells (**Figure 2**, panel **B**) and at a readily detectable level in about 30% of leukemia cell lines. High levels are seen in erythroleukemia cell line K562, as well as in TF1 cells treated with erythropoietin and in the human erythroleukemia cell line, HEL. Treatment of HEL cells with hemin increases DLX7 mRNA within 24 hr of hemin addition to a level equal to K562 (**Figure 2**, panel

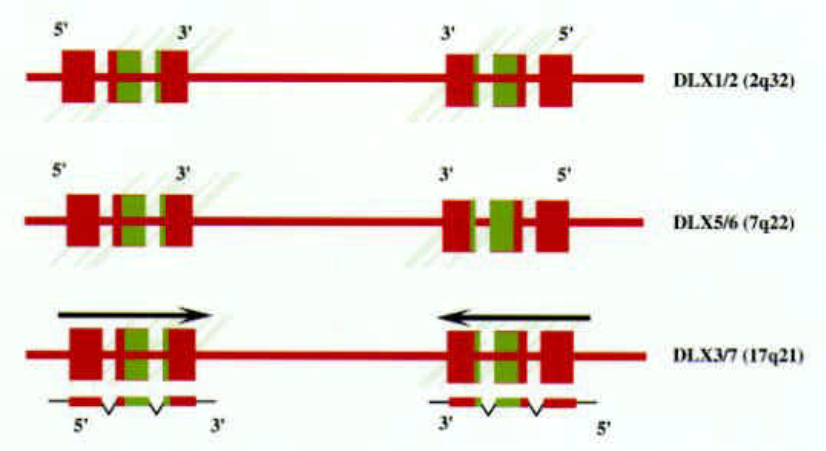
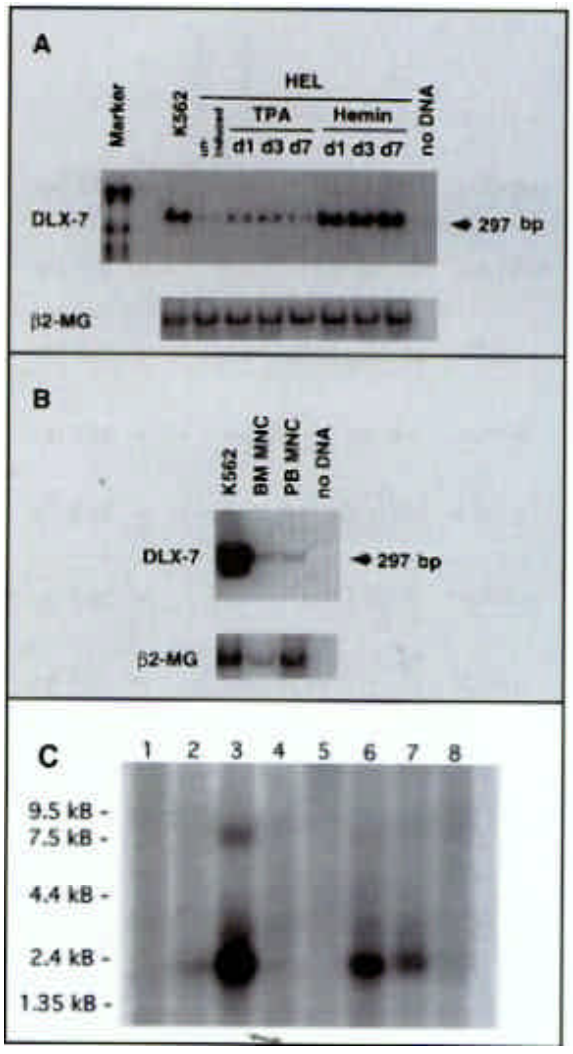


Figure 1. Genomic organization of DLX genes. The diagram depicts the genomic organization of DLX genes (not drawn to scale). The red boxes indicate coding regions, with the homeodomain highlighted in green. Three pairs of DLX genes are located on human chromosomes 2, 7 and 17 (Nakamura et al., 1996). The transcription units are separated by an intergenic distance of 5-10 kilobases and point toward each other. In addition to the transcript which gives rise to the DLX homeoprotein, there is substantial evidence indicating that many if not all DLX genes give rise to non-coding transcripts and antisense transcripts of unknown function (Ding et al., 1997; Liu et al., 1997; McGuinness et al., 1996; Ryoo et al., 1997).

Figure 2. Expression of DLX7 gene in various cancer cells, with a particular emphasis on leukemia cells (adapted from Shimamoto et al., 1997).
Panel A. RT-PCR indicating expression in K562 human erythroleukemia and HEL human erythroleukemia cell lines, showing high expression in K562 and high expression in hemin treated HEL cells. Hemin treatment induces an "erythroid" phenotype in HEL cells (Martin and Papayannopoulou, 1982).
Panel B. RT-PCR showing low expression of DLX7 in normal hematopoietic cells, bone marrow and peripheral blood, compared to K562 cells.
Panel C. A northern blot showing expression in human cancer cell lines. Lane 1, HL60 human myeloblastic leukemia cell line; lane 2, HeLa cervical carcinoma; lane 3, K562 erythroleukemia cell line from chronic myelogenous leukemia; lane 4, MOLT-4 T-lymphoblastoid cell line; lane 5, Raji B-lymphoblastoid Burkitt cell line; lane 6, SW480 human colon adenocarcinoma; lane 7, A549 human lung adenocarcinoma; lane 8, G361 human melanoma



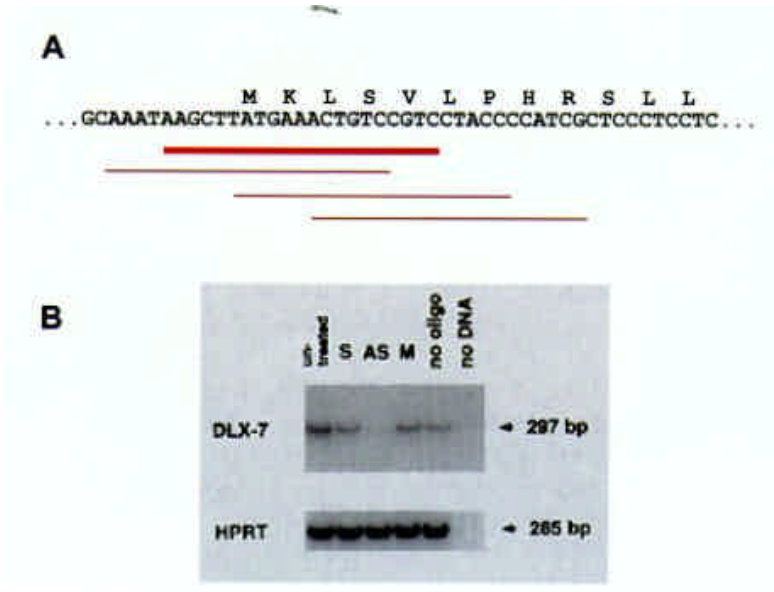


Figure 3. Design of antisense oligonucleotides directed against DLX7 gene (adapted from Shimamoto et al., 1997). **Panel A**. Location and sequence of oligonucleotides assayed for their ability to interfere with the expression of DLX7. The oligonucleotide exhibiting maximal effect is shown as a red line and has the sequence GACGGACAGTTTCATAAG. The thin line indicates oligonucleotides with minimal activity. **Panel B**. Effect of DLX7 antisense oligonucleotide on DLX7 mRNA level in K562 cells. RNA was extracted 3 hr after treatment with sense (lane S), antisense (lane AS), mutant (lane M) and mock treatment (lane no oligo), and analyzed by RT-PCR. Amplification of hypoxanthine phosphoribosyl transferase (HPRT) as a control is shown at the bottom. "Untreated" indicates cells not exposed to oligonucleotide. The oligonucleotide concentrations were 20 μM.

B. Possible function of DLX7 gene in hematopoietic cells

There is also a preliminary report that DLX7 is the protein which binds to BP1, a binding site located in the 5' upstream region of the beta globin gene that represses adult beta globin gene (Berg et al., 1998). Erythroleukemia cell lines, such as K562, TF1 and hemin treated HEL, express the fetal globin genes but not the adult globin genes. We have overexpressed DLX7 in the IL-3 dependent lymphoid cell line Ba/F3 and found that DLX7 relieves IL3 dependence and induces ICAM gene expression (Shimamoto et al., 1998), suggesting roles for both cell proliferation and differentiation. Additional roles have been suggested through the use of antisense oligonucleotides, as described below.

III. Use of antisense oligonucleotides to inhibit gene expression

The use of antisense oligonucleotides to inhibit gene expression has been reviewed by others. We have used the method as originally developed by Gewirtz and co-workers to inhibit gene expression in leukemia cells (Gewirtz and Calabretta, 1988; Szczylik et al., 1991). We have several randomly selected sequences near the translation start site. The oligonucleotides are generally 18 mers, with GC content of about 50% (**Figure 3** panel **A**).

For our purposes, it is particularly pertinent to note that questions have been raised about the specificity of biological effects seen with antisense treatment. Most of the reports of sequence-independent effects of oligonucleotides concern the phosphorothioate oligonucleotides, in which the sulfur entity is thought to result in a variety of biological effects (Abraham et al., 1997; Castier et al., 1998; Schobitz et al., 1997; Too, 1998; Wojcik et al., 1996; Yamaguchi et al., 1997). We have used phosphodiester oligonucleotides in all studies described here because it is cheaper and because the studies are based on cell lines where nuclease activity can be minimized. However, sequence independent effects have also been reported for phosphodiester oligonucleotides (Kabisch et al., 1994; Stull et al., 1993; Wu-Pong et al., 1994).

A. Inhibition of the target mRNA either at the protein level or at the mRNA level.

We have performed the following studies to demonstrate sequence specificity of the oligonucleotides we

have used. Antisense oligonucleotides are designed to bind to the target mRNA and cause either RNaseH-mediated degradation of the mRNA or block mRNA translation. However, in practice, many antisense oligonucleotides designed on the basis of the known mRNA sequence are ineffective (**Figure 3**, panel **A**). The reason for this is unclear but has been attributed to the complex secondary structure of the mRNA molecule which renders the mRNA inaccessible to the oligonucleotide.

B. Lack of non-specific toxicity

In our experiments we have observed that some batches of oligonucleotide preparations obtained from many manufacturers and from our own institutional synthesizers give non-specific toxicity. The toxicity stems possibly from incomplete removal of many organic compounds used during the synthesis. Therefore, an initial concern is to demonstrate that any biological effect observed with an oligonucleotide is due to the oligonucleotide itself and not to impurities present in the oligonucleotide preparation. Several approaches are available for the purification of oligonucleotides, such as HPLC, gel electrophoresis, etc. We have also found that some manufacturers offer oligonucleotides which do not show non-specific toxicity, even at very high concentrations (>100 μM). These preparations do not appear to require additional specialized purification other than ethanol precipitation.

C. Demonstration of specificity by creating point mutations within the oligonucleotide sequence

In order to demonstrate specificity of any effect observed with an antisense oligonucleotide, we have introduced point mutations within the oligonucleotide sequence to demonstrate that the mutation abolishes the observed biological effect. Exactly how many mismatches can result in the loss of the antisense oligonucleotide is unknown. Therefore, we generally have introduced 3 or 4 mismatches in an 18-mer to create a "mutated" oligonucleotide.

The specificity of the DLX-7 antisense oligonucleotide is illustrated in **Figure 3**, panel **B** (Shimamoto et al., 1997). The specificity of mRNA inhibition by the antisense oligodeoxynucleotide used was demonstrated by measuring levels of DLX-7 mRNA in cells treated with sense, antisense, mutant (same sequence as the antisense except for 4 base changes), or no oligodeoxynucleotide. K562 erythroleukemia cells exposed to DLX7 antisense oligonucleotides showed a significant decrease in DLX-7 mRNA levels (**Figure 3**, panel **B**). In contrast, the negative control oligodeoxynucleotides, including the mutant oligo, had no effect (Shimamoto et al., 1997).

D. Are secondary changes seen with DLX7 antisense oligonucleotide treatment related to the binding of a DNA molecule with an mRNA, rather than specific inhibition of DLX7?

Some have argued that the mere existence of DNA-RNA hybrid per se in the cell causes physiological changes in cells independent of any gene inhibition, for example RNase H activation, and that secondary effects are unrelated to the biological effects of DLX7 gene inhibition.

Although we cannot exclude this possibility completely, we can present the following data which argue against this possibility. First, as we discuss below, we have observed secondary effects, such as down regulation of c-myc and GATA-1 genes, after DLX7 antisense treatment. In contrast an antisense oligonucleotide directed against MEIS1 homeobox gene does not cause secondary inhibition of c-myc or GATA-1, but instead inhibits a different set of oncogenes (JES unpublished).

Second, there are tissue-specific differences in response to DLX7 antisense oligonucleotide. For example, the human lung cancer cell line A549, expresses the DLX7 the gene and shows proper down regulation of DLX7 mRNA in response to the antisense oligonucleotide. However, A549 does not show the secondary gene changes (e.g. c-myc gene inhibition seen in K562 leukemic cells).

IV. Cellular effects of antisense oligonucleotide mediated inhibition of DLX7 gene

As reviewed above, there is good evidence suggesting that some homeobox genes may participate in leukemogenesis. In the mouse, it is possible to test such candidate leukemogenic homeobox genes using transgenic or retroviral systems. Usually after a latent period of weeks to months, leukemia develops in such mice. However, it is unclear whether such mouse leukemia accurately reflects events occurring in human leukemia because of the differences in the target cell that undergoes transformation. For example, genes isolated as giving rise to myeloid leukemia in humans have been noted to cause lymphoid leukemia in mice. In addition, the mechanisms leading to genetic dysregulation in leukemia are also different, namely retroviral gene activation in mice and chromosomal translocation in humans. There are also likely to be more potentially leukemogenic genes with no grossly apparent alterations in the gene structure. Thus, the antisense oligonucleotide approach is attractive in that genes can be inhibited in a perhaps more biologically meaningful setting.

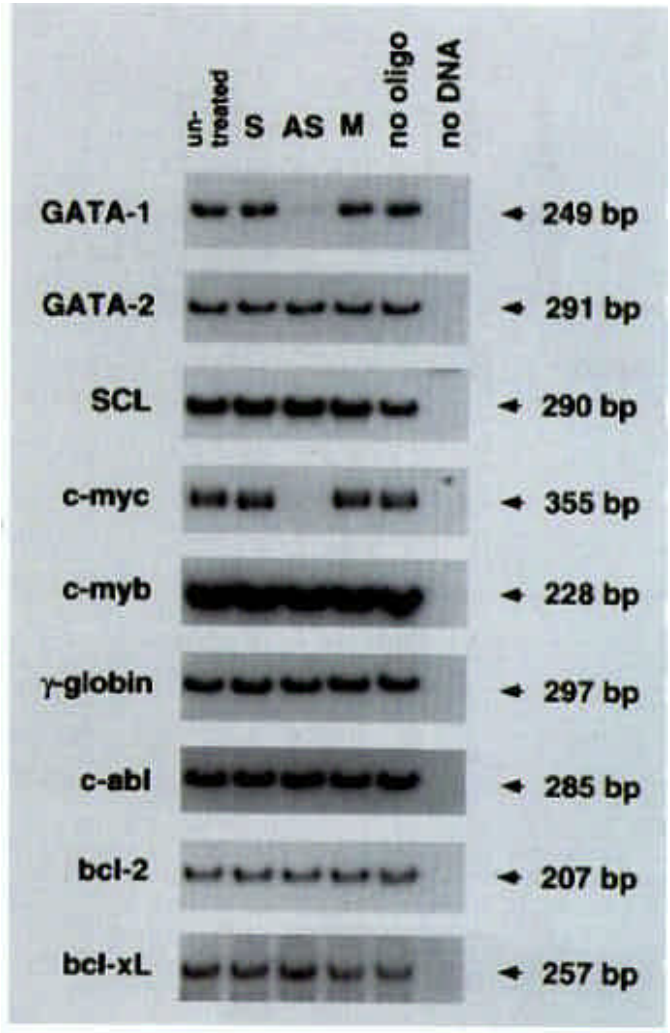


Figure 4. Analysis of gene expression in K562 cells treated with DLX7 antisense oligonucleotide (adapted from (Shimamoto et al., 1997). RNA was extracted 3 hr after oligonucleotide treatment and analyzed by RT-PCR for the genes indicated.

A. c-myc and GATA-1 genes are down regulated following antisense oligonucleotide treatment

Since DLX7 is a homeoprotein and thus likely to code for a transcription factor, we investigated the effects of inhibition of DLX7 gene expression on the expression of other genes. We selected several genes known to be important in hematopoiesis, cell proliferation or apoptosis. At 3 hours after DLX-7 antisense treatment, GATA-1, HOXC8 and c-myc mRNAs were decreased compared to control oligos (**Fig. 4**). GATA-1 and c-myc are well known regulators of hematopoiesis (Dubart et al., 1996; Mouthon et al., 1993; Weiss and Orkin, 1995). HOXC8 is a homeobox gene which also regulates hematopoiesis (Shimamoto et al., 1998); it is of note that the Drosophila versions of HOXC8 and DLX7 interact (Cohen et al., 1993; OHara et al., 1993; Panganiban et al., 1994). In contrast, GATA-2, SCL, c-myb, gamma-globin, c-abl, bcl-2 and bcl-xL mRNA were unaffected (Shimamoto et al., 1997). Preliminary data from nuclear run off studies indicate that the c-myc gene inhibition is mediated at the transcriptional level, with an associated alteration in the level of E2F activity, the transcription factor which regulates c-myc gene transcription. In contrast, the loss of GATA-1 gene expression is mediated at the mRNA level (data not shown).

B. Decreased colony forming efficiency after DLX-7 antisense treatment

We emphasize that the changes in gene expression seen after antisense oligonucleotide treatment described above occurred at 3 hours after the treatment. During this time, no changes in the cell viability, morphology, etc., are seen.

However, we note that both GATA-1 and c-myc have been reported to be involved in apoptosis. The role of c-myc in cell proliferation is well known (Dubart et al., 1996; Mouthon et al., 1993; Weiss and Orkin, 1995). In the case of GATA-1, GATA-1 deficient cells exhibit apoptosis at the normoblast stage and inhibition of GATA-1 in erythroleukemia cells causes apoptosis (Blobel and Orkin, 1996; Dubart et al., 1996; Weiss and Orkin, 1995). Thus, the loss of these proteins might be expected to inhibit cell proliferation and cause apoptosis.

To examine this hypothesis, we tested the viability of K562 cells after antisense oligo treatment by determining the plating efficiency of K562 cells treated with oligonucleotides in methylcellulose, a viscous culture medium which allows cells to grow as a colony. After a 30 min oligonucleotide treatment, the cells were plated in methylcellulose and the colonies counted after 7 days. 20 μM of DLX-7 antisense treatment suppressed the colony forming efficiency of K562 cells, whereas sense and mutant oligodeoxynucleotides had no effect on the plating efficiency, indicating essentially no non-specific toxicity of the oligonucleotide treatment (**Fig. 5**, panel **A**) (Shimamoto et al., 1997). Furthermore, the inhibitory effect of DLX-7 antisense oligodeoxynucleotide was dose-dependent, ranging from 30% inhibition at 5 μM to 70% inhibition at 40 μM (**Fig. 5**, panel **B**) (Shimamoto et al., 1997).

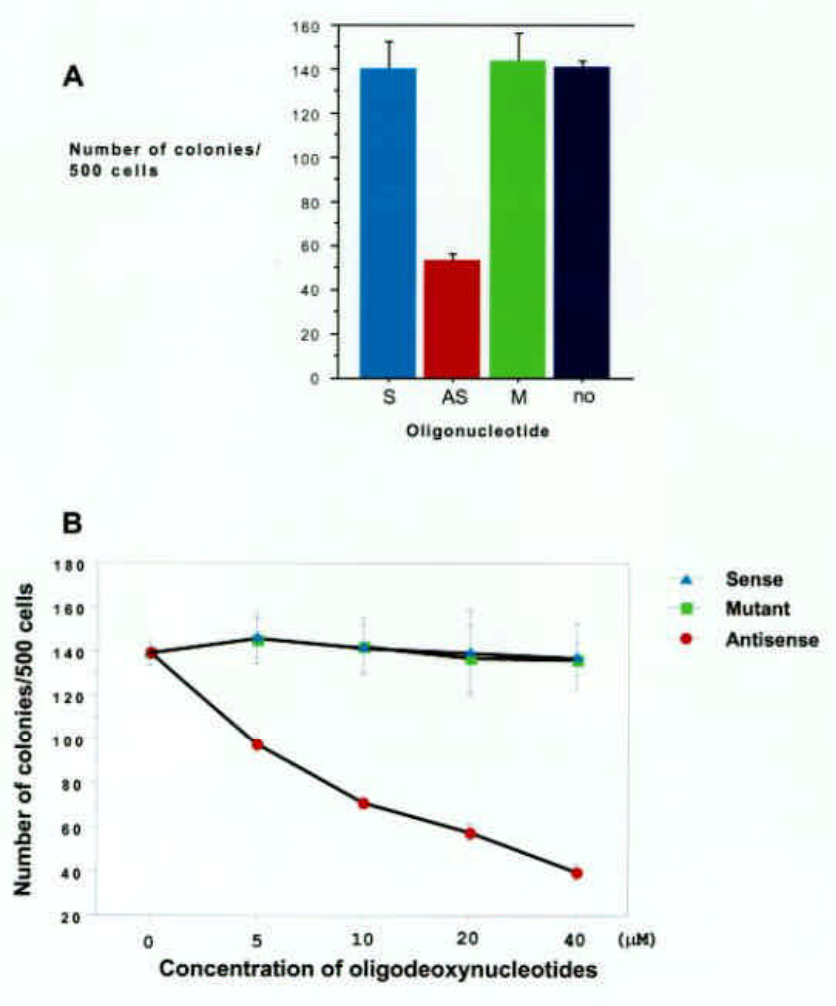


Figure 5. Effects of DLX7 antisense oligonucleotide treatment on K562 cell viability (adapted from (Shimamoto et al., 1997). **Panel A**. After exposure to the oligonucleotides, 500 cells were plated per well in quadruplicate and cultured for 7 days in a viscous methylcellulose-containing culture medium; after which the leukemia colonies were counted. The concentration of the antisense used was 20 μM. **Panel B**. Dose response curve of the oligonucleotides on the colony formation by K562 leukemia cells. Studies were carried out as in panel A, with the oligonucleotide concentration varied as indicated.

C. Apoptosis after antisense oligonucleotide treatment

To determine whether cells treated with antisense underwent apoptosis, K562 cells were stained by the TUNEL method, which detects in situ endonucleolytic cleavage characteristic of apoptosis. Apoptosis was assayed at 6 hr and at 12 hr after the oligonucleotide treatment. No apoptosis was seen at 6 hr. However, at 12 hr after the oligonucleotide treatment, apoptotic cells were observed (**Figure 6**) (Shimamoto et al., 1997). Additional studies indicate that the apoptosis is preceded by a block in the G1 to S progression in the cell cycle (data not shown), in agreement with the known site of action of c-myc and E2F.

V. Concluding remarks and observations

In this review, we have outlined the uses of antisense oligonucleotide technology, primarily as a way to probe the function of the DLX7 gene. These data suggest that DLX7 homeobox gene controls directly or indirectly the expression of secondary genes such as c-myc and GATA-1, that the mechanism of control might be transcriptional or mRNA stability, and that the loss of DLX7 gene expression results in apoptosis.

However, as physicians, we also believe that the real value of antisense oligonucleotides may come in the form of better treatment for leukemia and other disorders. Currently, several antisense oligonucleotides directed against other genes are in clinical trials. These include phase II and III clinical trials for antisense oligonucleotides

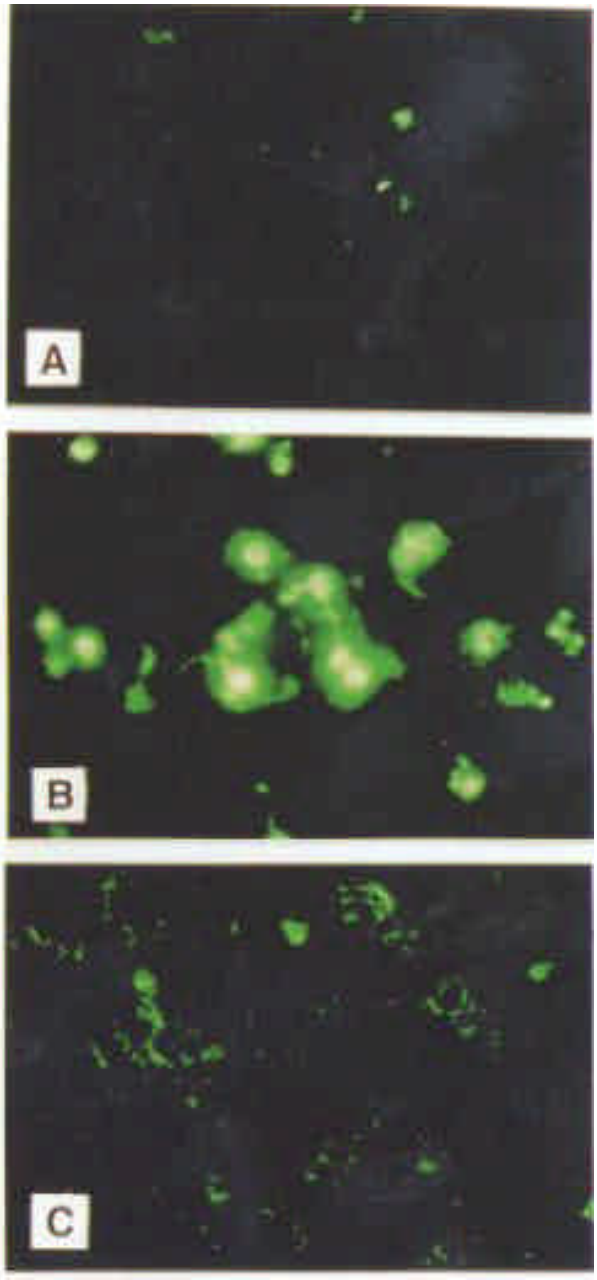


Figure 6. In situ analysis for DNA fragmentation by the TUNEL assay. At 12 hr after oligonucleotide treatment, cytocentrifugation preparations of K562 leukemia cells were stained by the TUNEL assay (adapted from Shimamoto et al., 1997). **Panel A**, no oligonucleotide; **panel B**, antisense oligonucleotide treatment; **panel C**, sense oligonucleotide treatment. Apoptotic cells were identified by labeling with fluorescent dUTP. No apoptosis was observed at 3 hr and 6 hr after oligonucleotide treatment (not shown).

directed against BCL2, c-myb, BCR-ABL, protein kinase A for a wide range of diseases, including hematologic malignancies, solid tumors, as well as immunologic diseases, such as ulcerative colitis, and infectious diseases. One antisense oligonucleotide has recently been approved by the United States Food and Drug Administration for the treatment of cytomegalovirus induced retinitis (Isis Co., 1998). Our intention is to continue to study the function of the DLX7 gene, with an emphasis on clinical applications.

Acknowledgments

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