# Inhibition of *DLX-7* homeobox gene causes decreased expression of *GATA-1* and c-*myc* genes and apoptosis

(divergent homeobox/TUNEL method/antisense oligonucleotide)

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ABSTRACT        The *DLX* gene family is a family of divergent homeobox genes which are related to the *Drosophila* distal-less (*Dll*) gene and has been reported to be expressed primarily in the forebrain and craniofacial structures. We have previously identified a new member of this family, *DLX-7*. We now report that this gene is expressed in normal hematopoietic cells and leukemia cell lines with erythroid characteristics. We used an antisense oligonucleotide targeted against the translation start site of *DLX-7* mRNA to inhibit its expression in a human erythroleukemia cell line K562, which expresses *DLX-7* at a high level. The antisense oligonucleotide efficiently reduced the *DLX-7* mRNA, while control oligonucleotides, including a mutant oligonucleotide identical to the antisense sequence except for four nucleotide mismatches, had no effect on *DLX-7* mRNA level. Inhibition of *DLX-7* expression decreased the plating efficiency by ≈70% compared with control. The antisense treatment caused apoptosis, as shown by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick end labeling (TUNEL) method. Downregulation of *DLX-7* expression by antisense treatment was associated with a reduction in *GATA-1* and c-*myc* mRNA levels. Thus, we conclude that *DLX-7* is expressed in hematopoietic cells and that the inhibition of its expression results in the decreased levels of *GATA-1* and c-*myc* genes, with an accompanying induction of apoptosis.

The homeobox gene family encodes for a group of transcription factors characterized by a highly conserved 60-amino acid DNA binding motif (1, 2). The homeobox genes, located outside the four *HOX* clusters, are designated "divergent" homeobox genes. Some divergent homeobox genes have implicated in leukemogenesis. In acute lymphoblastic leukemia with t(1;19) translocation, a fusion protein created between the transcription factor *E2A* gene and a homeobox gene *PBX* (3, 4). In common childhood T cell acute leukemia with t(10;14) translocation, *Hox-11* is transcriptionally activated (5, 6). *TRX*, also called *MLL*, located at 11q23 is rearranged in some biphenotypic leukemias (7).

*DLX* genes constitute a subfamily of divergent homeobox genes related to the *Drosophila* distal-less (*Dll*) gene (8–10). Six members in this family are found in the mouse and human genome, designated *DLX-1*, *-2*, *-3*, *-5*, *-6*, and *-7* (9–14). These genes are expressed in the forebrain, craniofacial, and limb structures (12–19). We have recently described the identification of *DLX-7*, a new member of this family and have mapped it to human chromosome 17q23 near *DLX-3* (14).

Antisense oligonucleotides have been employed as a powerful tool to modify gene expression (20, 21). This approach to

the study of specific gene function has been successfully used to study c-*myb* (22), c-*myc* (23) and some homeobox genes such as *HOXC6* (24) and *HOXB7* (25) in hematopoietic progenitor assays.

In this report, we demonstrate that *DLX-7* is expressed in normal bone marrow (BM) cells and at a particularly high levels in cell lines with the erythroid phenotype. Inhibition of *DLX-7* gene expression by an antisense oligonucleotide directed against *DLX-7* in K562 cells reduced the plating efficiency and induced apoptosis. The antisense treatment was accompanied by a reduction in *GATA-1* and c-*myc* mRNA levels. These results suggest that the function of the *DLX-7* gene may be to regulate events important in hematopoietic cell survival and/or proliferation.

## MATERIALS AND METHODS

**Cells and Cell Lines.** K562 and HEL cells were obtained from the American Type Culture Collection and maintained in RPMI 1640 culture medium supplemented with 10% fetal calf serum. For antisense experiments, K562 cells were grown in QBSF-58 serum free medium (Quality Biological, Gaithersburg, MD). Differentiation experiments using chemical inducers were performed by culturing cells in the presence of $1 \times 10^{-7}$ M phorbol 12-*O*-tetradecanoyl phorbol-13-acetate (Sigma) (26, 27) or $4 \times 10^{-6}$ M hemin (Sigma) (28, 29). Normal BM and peripheral blood (PB) samples were obtained from healthy volunteers or as discarded materials after consent. The mononuclear cells were purified by the Ficoll/Hypaque density gradient method from heparinized PB and BM aspirates.

**Oligonucleotides Treatment.** Phosphodiester oligonucleotides were used (Oligos Etc., Wilsonville, OR). Tests were initially performed to assess the toxicity of the oligonucleotides in culture. No obvious toxic effects were observed in K562 cells up to a concentration of 80 $\mu$M. The oligonucleotide sequences used here were as follows: *DLX-7*-antisense, GACGGA-CAGTTTCATAAG; *DLX-7*-sense, CTTATGAAACTGTC-CGTC; mutant-antisense, GAC<u>T</u>GA<u>A</u>AGT<u>G</u>TCATAC<u>G</u> (underlined bases indicate differences compared with the antisense sequence).

For oligonucleotide treatment, K562 cells were treated with streptolysin *O* essentially according to Spiller and Tidd (30) with some modifications. Cells ($5 \times 10^5$) were washed in permeabilization buffer (137 mM NaCl/Pipes, pH 7.4/5.6 mM glucose/2.7 mM KCl/2.7 mM EGTA/1 mM Na-ATP/0.1% bovine serum albumin), and resuspended in 0.5 units/ml of streptolysin *O* (Sigma) in permeabilization buffer, preacti-

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vated with 5 mM dithiothreitol for 2 h, containing 20 $\mu$M oligonucleotide. After 20-min incubation at 37°C, 5 ml of QBSF-58 serum-free medium was added to stop the perme-abilization reaction.

**Clonogenic Assay.** The clonogenic assay for K562 cells were performed as described (31). Briefly, 500 cells were treated with oligonucleotides and plated in quadruplicate in a culture medium consisting of Iscove's medium supplemented with 30% fetal bovine serum, 1% bovine serum albumin, $10^{-4}$ M 2-mercaptoethanol, 2 mM L-glutamine, and 0.8% methylcel-lulose (Stem Cell Technologies, Vancouver, BC, Canada). The colonies (>50 cells) were scored after 7 days of incubation at 37°C in 5% $CO_2$.

**Reverse Transcriptase–PCR (RT-PCR).** Expression of *DLX-7* gene and other genes was determined by RT-PCR (32). Total RNA was extracted using the acid guanidium thiocyanate-phenol-chloroform method (33). For oligonucleotide treated cells, the RNA extraction was performed 3 h after termination of the oligonucleotide treatment, using 10 $\mu$g of glycogen as a carrier. One microgram of total RNA was reverse transcribed using 1 $\mu$g of oligo(dT) and 200 units of murine leukemia virus reverse transcriptase (BRL/GIBCO) under recommended conditions. One-tenth of this cDNA was used as the template in a PCR using the following upstream and downstream primers: *DLX-7* (14), CTCCAGCCTGCAGCT-GCA (base pairs 402–419 of the published sequence) and CCAAAGCTGTTGCCATAGCCAC (base pairs 698–677) (expected PCR product, 297 bp); *HPRT* (34), GGTGGAGAT-GATCTCTCAAC (base pairs 439–458) and TCCAGTT-TCACTAATGACAC (base pairs 723–704) (285 bp); *GATA-1* (35), CCATTGCTCAACTGTATGGAGGG (base pairs 235–257) and ACTATTGGGGACAGGGAGTGATG (base pairs 483–461) (249 bp); *GATA-2* (36), AGCGTCTCCAGCCT-CATCTTCCGCG (base pairs 745–769) and CGAGTCTT-GCTGCGCCTGCTT (base pairs 1035–1016 (291 bp); *SCL* (37), CTCGGCAGCGGGTTCTTT (base pairs 168–185) and AGCAGCTTGGCCAAGAAGTT (base pairs 457–438) (290 bp); c-*myc* (38), TCTCCGTCCTCGGATTCTCT (base pairs 649–668) and GTCTCAGGACTCTGACACTG (base pairs 1003–984) (355 bp); c-*myb* (39), ATTAGGTAATGAATTG-TAGCCAG (base pairs 2256–2278) and ACTTAGAGTAAT-GCTTTTACTGA (base pairs 2483–2461) (228 bp); $\gamma$-globin (40), AGATGCTGGAGGAGAAACCCTG (base pairs 119–140) and CGAAATGGATTGCCAAAACG (base pairs 411–392); c-*abl* (41), GCTGGACCCAGTGAAAATGACC (base pairs 530–551) and CAAGAAGCTGCCATTGATCCC (base pairs 814–794) (285 bp); *bcl-2* (42), TTCTTTGAGTTCGGT-GGGGTC (base pairs 1906–1926) and CTTCAGAGA-CAGCCAGGAGAAATC (base pairs 2112–2089) (207 bp); *bcl-x*L (43) CATGGCAGCAGTAAAGCAAGC (base pairs 380–400) and CTGCGATCCGACTCACCAATAC (base pairs 636–615). All primers used flank at least one intron.

The amplification conditions were denaturation at 94°C for 30 sec, annealing for 30 sec at 50°C for *DLX-7*, at 55°C for *HPRT*, c-*myb*, and $\gamma$-globin, at 58°C for *GATA-1*, at 60°C for *SCL*, c-*myc*, c-*abl*, *bcl-2* and *bcl-x*L, and at 63°C for *GATA-2*, and extension at 72°C for 1 min for 25 cycles for *DLX-7* and 20 cycles for other genes. Solution hybridization using 10% of the PCR product was performed in a thermal cycler with the oligonucleotide probe 5'-end labeled with $[\gamma$-$^{32}$P]ATP (44). The probes used were as follows: *DLX-7*, CTGGCAGCG-CAGCTCGGC (base pairs 478–495 of the published se-quence); *HPRT*, CTTGCTGGTGAAAAGGACC (base pairs 573–591); *GATA-1*, TGGATGGAAAAGGCAGCACC (base pairs 371–390); *GATA-2*, ACGACTACAGCAGCGGACTC (base pairs 948–967); *SCL*, TCGAGTGAAGAGGAGAC-CTT (base pairs 224–243); c-*myc*, GAGCCCCTGGTGCTC-CATGA )base pairs 706–725); c-*myb*, CTGGTATTTTA-AAGGATCC (base pairs 2331–2349); $\gamma$-globin, CAGCTTT-GGCAACCTGTCCTC (base pairs 184–205); c-*abl*,

CACAATGGGGAATGGTGTGAAG (base pairs 647–668); *bcl-2*, ACAACATCGCCCTGTGGATGAC (base pairs 1970–1991); *bcl-x*L, ATGGGGTAAACTGGGGTCGCATTG (base pairs 532–555). Each hybridization sample was run on an 8% polyacrylamide gel, and the gel was autoradiographed at room temperature for 4 h.

**Northern Blot Analysis.** Northern membrane with 2 $\mu$g of poly(A)$^+$ RNA per lane (CLONTECH) was hybridized for 24 h at 42°C to $[\alpha$-$^{32}$P]dCTP-labeled probe prepared by the random priming labeling method. After hybridization, the membrane was washed under standard conditions and auto-radiographed.

***In Situ* Detection of Apoptosis.** Cytospin preparations of K562 cells were prepared and fixed in 4% paraformaldehyde for 30 min, washed in PBS for 5 min, and incubated in 0.1% Triton X-100 for 2 min on ice and washed in PBS twice, after which the TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling) (45) assay was performed using the In Situ Cell Death Detection Kit (Boehringer Mannheim) according to the manufacturer's instructions.

**Statistical Analysis.** Statistical tests were performed using the STATVIEW (Abacus Concepts, Berkeley, CA) software. The number of each colony or the percentage of positive cells was shown as the mean ± SD. Comparisons of groups were analyzed using the Student's *t* test or ANOVA test. Values of $P < 0.05$ were considered significant.

## RESULTS

***DLX-7* mRNA Expression in Normal and Leukemic Hema-topoietic Cells.** In a preliminary study, we used RT-PCR to survey the expression of *DLX-7* gene in 50 leukemia cell lines and found that K562 erythroleukemia cell line expresses *DLX-7* at a high level. The expression was confirmed by Northern blot analysis, which indicated a major transcript at 2.3 kb and a minor transcript at a 7.3 kb in K562 cells. Two additional nonhematopoietic cell lines, SW480 (colon adeno-carcinoma) and A549 (lung adenocarcinoma), also expressed the *DLX-7* gene (Fig. 1).

To determine whether *DLX-7* gene is associated with the erythroid phenotype, we also examined HEL cells, which can be induced to undergo erythroid maturation when cultured in hemin (29). As indicated in Fig. 2*A*, the hemin treatment resulted in an increase in the *DLX-7* mRNA to a level equivalent to K562 cells.

*DLX-7* is also expressed at detectable levels not only in BM cells but also PB mononuclear cells, indicating that the ex-pression of this gene is not specific to leukemia cells (Fig. 2*B*).

**Inhibition of *DLX-7* mRNA Expression Level by an Anti-sense Oligonucleotide.** Antisense oligonucleotides inhibit gene expression by binding to its complementary site on the mRNA, resulting in an RNase H-mediated mRNA degradation (46). We tested the activity of several antisense oligonucleotides and
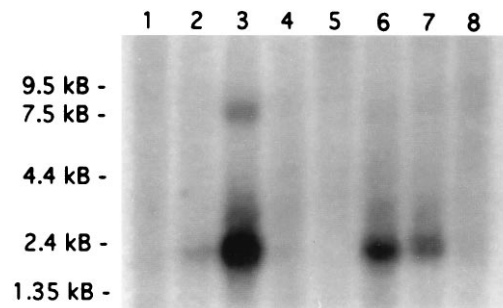


Fig. 1.   Northern blot analysis demonstrating *DLX-7* expression in leukemia cell lines and other cancer cell lines. Lanes: 1, HL-60; 2, HeLa; 3, K562; 4, MOLT-4; 5, Raji; 6, SW480; 7, A549; 8, G361.
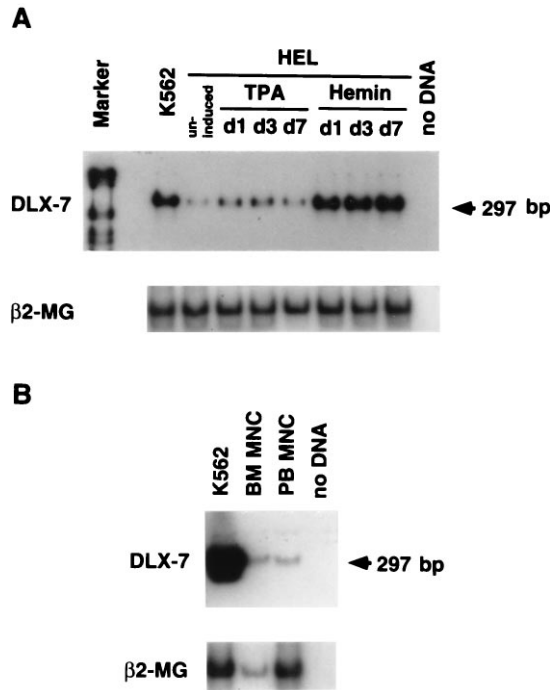
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*Proc. Natl. Acad. Sci. USA* 94 (1997)   3247

**A**



**B**



FIG. 2.   (*A*) Expression of *DLX-7* gene in HEL cells treated with 12-*O*-tetradecanoyl phorbol-13-acetate (TPA; $1 \times 10^{-7}$ M) or hemin ($4 \times 10^{-6}$ M). RNA was reverse transcribed into cDNA, amplified by PCR using *DLX-7*-specific primers, and hybridized with a *DLX-7* gene-specific internal oligonucleotide probe. Hemin treatment caused a marked increase in *DLX-7* mRNA levels by day 1. (*B*) Expression of *DLX-7* gene in normal BM cells and PB mononuclear cells.

selected one that was the most effective in reducing *DLX-7* mRNA (Fig. 3*A*). The specificity of the antisense oligonucleotide effect was demonstrated by the lack of any effect of sense and mutant oligonucleotides (Fig. 3*B*). A negative control, *HPRT* gene, was not affected by antisense treatment.

**Decreased *DLX-7* Expression Results in Reduced Plating Efficiency.** To investigate whether *DLX-7* is required for K562 cell growth, we determined the plating efficiency of K562 cells treated with oligonucleotides. The *DLX-7* antisense treatment
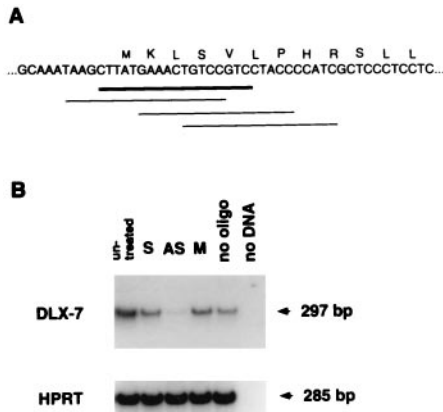
**A**



**B**



FIG. 3.   (*A*) Location and sequence of oligonucleotides assayed for their ability to interfere with the expression of *DLX-7*. The oligonucleotide exhibiting the maximal effect is shown as thick line. (*B*) Effect of *DLX-7* antisense oligonucleotide on *DLX-7* mRNA expression. RNA was extracted from K562 cells 3 h after treatment with sense (lane S), antisense (lane AS), mutant (lane M), and no (lane no) oligonucleotides and analyzed by RT-PCR. Amplification of hypoxanthine phosphoribosyltransferase (HPRT) as a control is shown at the bottom. The final concentration of antisense and negative control oligonucleotides used was 20 $\mu$M.

at 20 $\mu$M suppressed the plating efficiency by $\approx$60%, while negative control oligonucleotides had no effect (Fig. 4*A*). Furthermore, the inhibitory effect of the *DLX-7* antisense oligonucleotide was dose-dependent, ranging from 30% inhibition at 5 $\mu$M to 70% inhibition at 40 $\mu$M (Fig. 4*B*).

**Reduced *DLX-7* Expression Results in Apoptosis.** To determine whether the reduced plating efficiency is due to induction of apoptosis, K562 cells treated with antisense oligonucleotides were stained by the TUNEL method to detect *in situ* endonucleolytic cleavage characteristic of apoptosis. There was no detectable apoptosis at 3 h (data not shown), but after 24 h of exposure of oligonucleotides, significant apoptosis was detected in antisense-treated K562 cells compared with control cultures (Fig. 5). Thus, the reduced plating efficiency of K562 cells by *DLX-7* antisense treatment is likely to be due to induction of apoptosis.

**Analysis of Gene Regulation by *DLX-7*.** Because homeobox genes are likely to control the expression of other genes, we surveyed the expression of several genes known to be important in hematopoiesis, cell proliferation, or apoptosis. Three hours after the *DLX-7* antisense treatment, K562 cells had decreased levels of *GATA-1* and c-*myc* mRNA compared with various negative controls (Fig. 6). In contrast, *GATA-2*, *SCL*, c-*myb*, $\gamma$-globin, c-*abl*, *bcl-2*, and *bcl-x*$_L$ mRNA were unchanged (Fig. 6). We have not been able to locate any homology between the antisense oligonucleotide sequence and either *GATA-1* or c-*myc* cDNA sequence, indicating that the effect of the antisense oligonucleotide is likely to be secondary to the effect on *DLX-7* expression.

## DISCUSSION

*Dlx* genes such as *Dlx-1*, *-2*, *-5*, and *-6* have previously been reported to be expressed primarily in the brain and craniofacial structures (9–19). We have found that *DLX-7* is expressed at
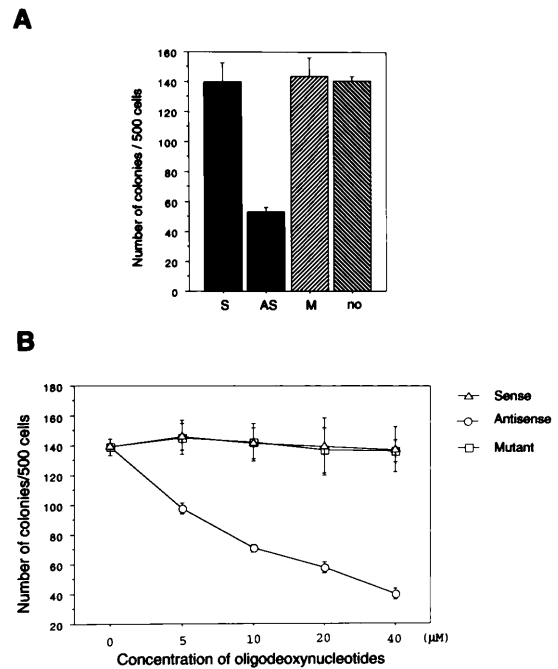
**A**



**B**



FIG. 4.   (*A*) Effect of *DLX-7* antisense treatment on the plating efficiency of K562 cells. After exposure to the oligonucleotides as indicated, 500 cells were plated per well in quadruplicate and cultured for 7 days in methylcellulose, after which colonies were counted. The concentration of the oligonucleotides was 20 $\mu$M. (*B*) Dose-response curve of oligonucleotides on the plating efficiency of K562 cells. Studies were carried out as in *A*, with the oligonucleotide concentration varied as indicated.
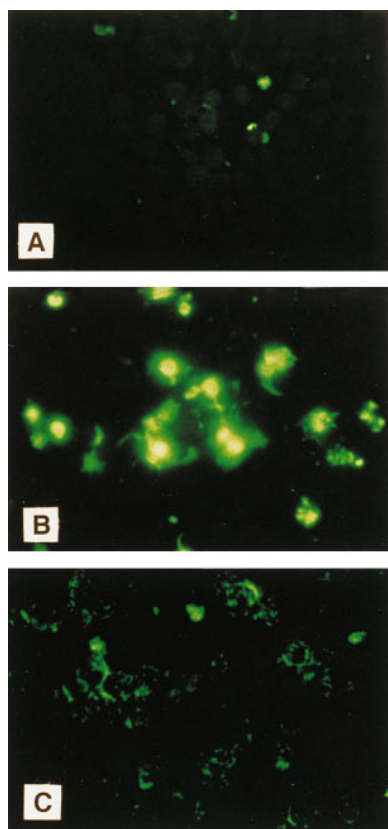
high levels in cell lines with erythroid characteristics such as K562 and HEL, as well as at low levels in normal BM cells. Hemin-induced erythroid differentiation of HEL cells resulted in an increase in *DLX-7* mRNA levels after 24 h of exposure to hemin. We have also found that other members of the *DLX* gene family are expressed in hematopoietic cells (K.T., unpublished data).

We took advantage of its expression in well studied leukemia cell lines to study the possible function of *DLX-7*. Several authors have overexpressed homeobox genes to investigate their role in hematopoiesis (29, 30, 47, 48). The inhibition of gene expression by antisense oligonucleotides has also shown to be useful in elucidating homeobox gene function (24, 25). In this study, we have used an antisense oligonucleotide directed against *DLX-7*.

The antisense oligonucleotide treatment reduced *GATA-1* and c-*myc* mRNAs at 3 h after the treatment, at a time when no apoptosis is detectable by the TUNEL assay. Apoptosis is seen at 12 h after antisense treatment and is accompanied by a reduction in the plating efficiency. The apoptosis might be mediated directly by *DLX-7* or through downstream genes. At least one homeobox gene has been shown to prevent apoptosis (49). Since induction of cell death is preceded by down regulation of c-*myc* and *GATA-1*, down-regulation of one or both of these genes may be sufficient to account for the observed apoptosis (50–52). c-*myc* is well known to be involved in cellular decisions regarding apoptosis or proliferation (53–55), and decreasing the c-*myc* expression is known to cause apoptosis in leukemia cells (51, 52). *GATA-1* has also recently been shown to control apoptosis. *GATA-1* deficient embryonic stem cells undergo apoptosis at the proerythroblast stage (52) and overexpression of *GATA-1* prevents estrogen-induced
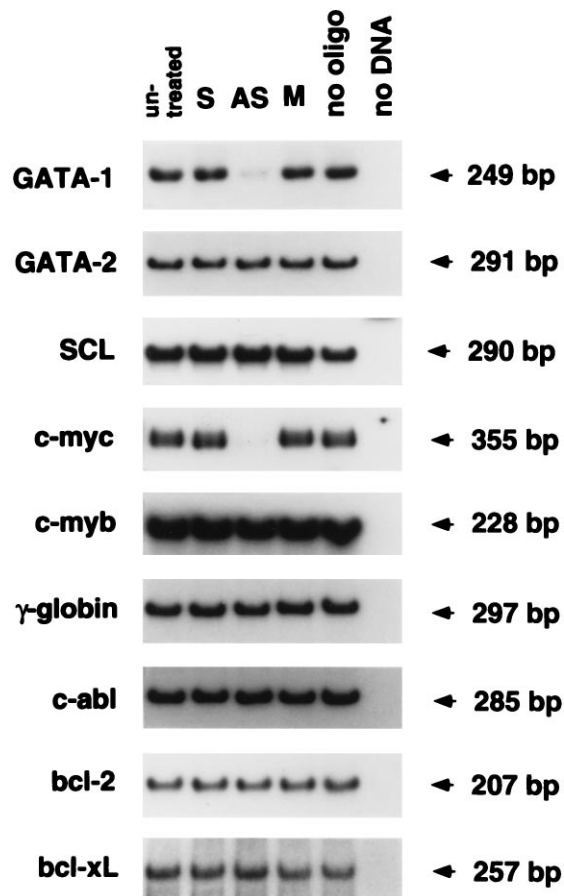
apoptosis (56). Therefore, apoptosis induced by *DLX-7* gene inhibition might be due to inhibition of *GATA-1* and/or c-*myc* gene expression, and the role of *DLX-7* gene might simply be to maintain expression of *GATA-1* and c-*myc*.

The relationship between the c-*myc* and *GATA-1* gene expression and *DLX-7* gene expression may either be direct or indirect. Homeobox-containing proteins are known to act at both transcriptional and posttranscriptional levels (57). Preliminary evidence suggests that the loss of *GATA-1* mRNA in antisense-treated cells is due to increased mRNA instability (T.S., unpublished data). On the other hand, c-*myc* mRNA has a short half-life (58), which is not altered in antisense oligonucleotide treated K562 cells (T.S., unpublished data), suggesting that the loss of c-*myc* mRNA occurs at the transcriptional level.

Because *DLX-7* is expressed at high levels in erythroleukemia cell lines, mediated by hemin in the case of HEL cells, and at low levels in the normal BM, it is tempting to speculate that the *DLX-7* gene may be involved in some aspect of erythropoiesis, possibly in the regulation of apoptosis that occurs during normal erythropoiesis (59). Further studies are needed to address the function of *DLX-7* and other *DLX* genes in normal hematopoiesis and to determine whether other *DLX* genes play a similar role in hematopoietic cells.

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