A Novel Triplex-Forming Oligonucleotide Targeted to Human Cyclin D1 (*bcl*-1, Proto-Oncogene) Promoter Inhibits Transcription in HeLa Cells†

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ABSTRACT: The cyclin D1/*bcl-1* proto-oncogene is one of a series of genes encoding proteins which regulate the cell cycle and are involved in the multistep process of tumorigenesis. Translocation of the cyclin D1 proto-oncogene is a common event in B cell lymphoma, and cyclin D1 amplification occurs in breast, esophageal, hepatocellular, and head/neck carcinomas. The human cyclin D1 proto-oncogene promoter contains an 18-base pair purine-pyrimidine rich motif with three C·G interruptions. This motif is a potential target for purine·purine·pyrimidine triplex formation. We have designed a G-rich antiparallel triplex forming oligonucleotide (TFO) targeted to this region. Electrophoretic mobility shift analysis (EMSA) shows that this purine-pyrimidine rich motif is a binding site for the transcription factor Sp1 and that triplex formation by the target sequence prevents the binding of recombinant Sp1. The exact location of triplex formation was confirmed by DNase I footprinting. In an attempt to increase stability, we have used modified phosphorothioate oligonucleotides for cell culture experiments. Triplex formation by the cyclin D1 targeted phosphorothioate oligonucleotide occurs with a binding affinity approximately equal to that of phosphodiester oligonucleotides. This phosphorothioate modified TFO targeted to cyclin D1 also inhibits transcription of the cyclin D1 promoter in HeLa cells, as demonstrated by a decrease in luciferase expression from a stably integrated human cyclin D1 promoter driven luciferase construct. This suggests that triplex formation may represent a gene specific means of inhibiting cyclin D1 expression.

Oligonucleotides can form triple helical structures with polypurine tracts present in duplex DNA in a sequence specific manner. There are two widely known structural motifs by which these triple helical DNA form. Under acidic conditions, pyrimidine rich oligonucleotides bind in the major groove of DNA so that the triplex forming oligonucleotide is parallel to the purine strand of the duplex target. Acidic conditions are required for triplex formation by oligonucleotides containing cytosine since the cytosine residue must be protonated to form triplex. Sequence specificity is derived from the ability of thymine to recognize adenine·thymine base pairs (T*A·T base triplets) and cytosine (C^+) to recognize guanine·cytosine base pairs (C^+*G·C triplets) (*1*). Another class of triple helix was described by Cooney et al., who demonstrated that antiparallel purine rich oligonucleotides can specifically bind to purine tracts of double helical DNA in the major groove (*2*). Sequence specificity is based on the fact that guanine recognizes G·C base pairs (G*G·C) and adenine recognizes A·T base pairs (A*A·T). Although these purine rich oligonucleotides were originally thought to bind to DNA in a parallel orientation, Beal and Dervan showed that third strands bind to their target site in an antiparallel orientation with respect to the purine rich strand of duplex target (*3*).

Sequence specific intermolecular triple helix formation occurs in vitro, which raises the possibility of manipulating gene expression through triple helix formation. Maher et al. utilized triplex formation to inhibit restriction endonuclease cleavage at sites located in the vicinity of triplex forming regions (*4*). Triplex formation inhibits binding of the eukaryotic transcription factor Sp1 (*5*, *6*). Cooney et al. have shown that a homopurine oligonucleotide, which forms triple helix within a region of the c-*myc* P1 promoter, inhibits the in vitro transcription of the c-*myc* gene (*2*).

The cyclins are a major group of cell cycle regulatory proteins. They were first identified as proteins that accumulate during the cell cycle and are degraded rapidly in mitosis (*7*−*12*). The regulated synthesis and degradation of cyclin proteins appear to be critical for proper cell cycle control. Association of cyclins with cyclin-dependent kinases results in the subsequent activation of the complex and may activate specific targets whose phosphorylation is important to cell-cycle transition (*13*, *14*). There are five distinct classes of mammalian cyclins termed A−E, and the synthesis and function of these cyclins display cell cycle specificity. Cyclin D1 is encoded by the CCND1 gene located on chromosome 11q13 (*15*) which has also been known as the PRAD1 proto-oncogene and *BCL*1 proto-oncogene (*16*). Cyclin D1 is a nuclear protein whose expression is closely related to the cell cycle transition from G1 to S (*17*). Since loss of cell cycle control may contribute to tumor formation, it is interesting that cyclin D1 has been

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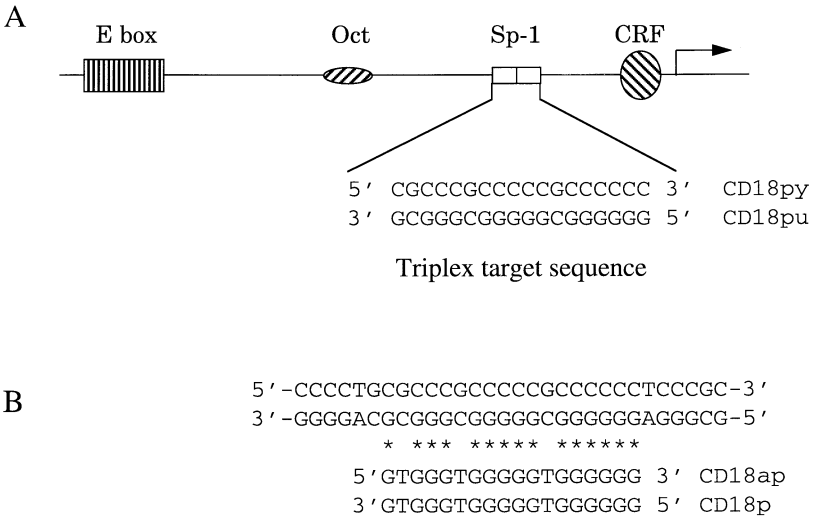

FIGURE 1: (A) Map of the human cyclin-D1 promoter region showing the 18-bp purine/pyrimidine rich motif and triplex target sequence relative to the transcription start site P2 (−116 to −99). (B) Oligonucleotide sequences and their alignment with the duplex target. The p and ap nomenclature indicate the parallel or antiparallel orientation of the oligonucleotide relative to the purine rich target strand, respectively.

found to be overexpressed in a wide variety of human cancers including esophageal, breast, and head/neck carcinomas (*18*, *19*). In centrocytic B cell lymphoma, cyclin D1 is translocated at the BCL-1 break point and brought under the control of the immunoglobulin heavy chain enhancer (*18*, *19*). Cyclin D1 expression is activated in certain head/neck, esophageal, breast, and hepatocellular carcinomas as a result of chromosomal amplification at the 11q13 region (*20*−*23*). Cyclin D1 is also overexpressed in some parathyroid adenomas by a chromosomal inversion, resulting in the control of the cyclin D1 expression by the parathyroid hormone gene promoter (*16*)

To develop specific transcriptional inhibitors of the human cyclin D1 proto-oncogene, we have characterized triplex formation by a purine rich oligonucleotide targeted to the 18-bp[1] pur·pyr rich motif in the promoter region of cyclin D1. We have also examined the effect of intermolecular triplex formation by the cyclin D1 target sequence on binding of recombinant Sp1. Even though the target site is not completely homopurine/homopyrimidine, these sequences can form triplex. Finally, the effect of triplex formation on the transcriptional activity of the cyclin D1 promoter was examined in HeLa cells which were stably transfected with a vector in which the cyclin D1 promoter drives the luciferase gene.

MATERIALS AND METHODS

Oligonucleotides and Enzymes. Four unmodified phosphodiester 18-mer oligonucleotides (sequences shown in Figure 1) were synthesized on a Milligen Cyclone Plus DNA synthesizer using standard phosphoramidite chemistry. All oligonucleotides were purified by OPEC. Phosphorothioate oligonucleotides were purchased from Oligo Etc. The structural integrity and purity of each oligonucleotide was verified by 5′ ^{32}P labeling using [γ-^{32}P]ATP and T4 polynucleotide kinase (PNK) followed by electrophoresis on a polyacrylamide gel. The concentration of oligonucleotides was determined by measurements of UV absorption at 260 nm using the following molar extinction coefficients for each base: 15400 (A), 7300 (C), 11700 (G), and 8800 cm^{-1} M^{-1} (T). DNase I was purchased from Pharmacia, and the restriction endonucleases, T4 ligase and T4 polynucleotide kinase, were purchased from GIBCO BRL.

Electrophoretic Mobility Shift Analysis (EMSA). For the oligonucleotide titration experiments the synthetic pyrimidine rich strand of the 18-base cyclin D1 target was 5′ labeled with [γ-^{32}P]dATP and T4 PNK and annealed to its oligonucleotide complement. The oligonucleotides were then incubated with the labeled 18-bp target in either a buffer consisting of 90 mM Tris, 90 mM borate (pH 7.4), and 10 mM $MgCl_2$ or protein binding buffer for 24 h at 37 °C. To stabilize triplexes, both the running buffer and the polyacrylamide gel contained 90 mM Tris, 90 mM borate (pH 7.4), and 10 mM $MgCl_2$. A 16% polyacrylamide gel was used. For gel shift experiments with recombinant Sp1, an 18-bp end-labeled duplex target was incubated with oligonucleotides in 25 mM Hepes, 12.5 mM $MgCl_2$, 70 mM KCl, 1 μM $ZnSO_4$, 1 mM DTT, 0.1% NP-40, and 10% glycerol (v/v) for 24 h at 37 °C. Cooling of the samples on ice was followed by the addition of recombinant Sp1 and 1 μg of poly(dI-dC). The mixture was then allowed to bind for 30 min on ice. Samples were analyzed by electophoresis on 5% native polyacrylamide gels at 150 V in 90 mM Tris borate (pH 7.4) and 2 mM EDTA, followed by autoradiography.

Cyclin D1 Promoter Fragment Isolation. pBS CD-1 containing 1.5 kb of the human cyclin D1 promoter fragment (kindly provided by Dr. Martin Eilers) was digested with *Xho*I. DNA was extracted once with phenol/chloroform and twice with chloroform and precipitated with ethanol. Digested plasmid was labeled with [α-^{32}P]dATP using the Klenow fragment of DNA polymerase I and then digested with *Hin*fI. The resulting 344-bp promoter fragment was purified by 5% preparative native polyacrylamide gel electrophoresis.

DNase I Footprinting. Triplex formation was performed in a buffer containing 10 mM Tris (pH 7.4) and 10 mM $MgCl_2$ by incubating the ^{32}P-labeled cyclin D1 promoter

[1] Abbreviations: bp, base pair; TFOs, triplex forming oligonucleotides; EMSA, electrophoretic mobility shift analysis; CD-1, cyclin D1; PRAD, parathyroid adenoma; PNK, polynucleotide kinase.

fragment with oligonucleotides for 24 h at 37 °C. Samples were precooled on ice, and 1 μg of poly(dI-dC) was added. Next, 0.0002 unit of DNase I was added and the mixture then incubated for 1 min on ice, and the reactions were stopped by adding 10 mM EDTA in 90% formamide. Samples were purified by organic extraction and ethanol precipitation. After precipitation, samples were heated at 95 °C for 5 min, quick-chilled on ice, and analyzed by electrophoresis on an 8 M urea, 8% polyacrylamide sequencing gel at 42 W. Bands were visualized by autoradiography.

Plasmid Construction. pMAMneoluc (Clontech) was cut with *Nhe*I, and both ends were filled with the Klenow fragment of DNA polymerase I. pBSCD-1 containing a 1.5 kb (−1.2 to +0.3 relative to the transcription start site) fragment of the human cyclin D1 promoter was cut with *Eco*RI and *Bam*HI, and both ends were filled with the Klenow fragment of DNA polymerase I to make the ends blunt. Digested plasmid was run on 1% low melting point agarose gels, and the 1.5 kb CD-1 promoter fragment was purified using Quiax DNA binding bead. pCDNA3 was cut with *Nru*I and *Eco*RV to obtain the CMV immediate early promoter. The 1.5 kb CD-1 fragment or 740-bp CMV early promoter fragment was cloned into the pMAMneoluc vector by blunt-end ligation, respectively. The resulting plasmids are designated as pCDLUCneo or pCMVLUCneo.

Cell Line. The HeLa human cervical carcinoma cell line was obtained from the American Type Culture Collection. It was grown in cell culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Cells were cultured at 37 °C with 85% humidity and 5% CO_2.

DOTAP/DOPE Preparation. A liposome vehicle consisting of a 1:1 (wt/wt) mixture of the cationic lipid DOTAP [1,2-dioleoyloxy-3-(trimethylammonium)propane] and the neutral lipid DOPE (dioleoylphosphatidylethanolamine) (Avanti Polar Lipids) has been shown in our laboratory to display transfection properties similar to Lipofectin, but with less toxicity (unpublished observations). DOTAP/DOPE liposomes were prepared by mixing 0.5 mg of DOTAP and 0.5 mg of DOPE and evaporating the chloroform solvent. Following the addition of 500 μL of cyclohexane, the mixture was placed on dry ice and lyophilized. One milliliter of sterile water was added to the powdered lipids, and the solution was vortexed every 5 min for 30 min.

Stable Transfection. To establish the HeLa neo^r cell line, cells were seeded at 30% confluency in a 100 mm dish and allowed to grow overnight. Immediately prior to transfection, cells were washed three times with sterile PBS. To transfect a 100 mm dish, 24 μg of DOTAP/DOPE (1 μg/μL) was mixed with 6 μg of plasmid DNA (1 μg/μL) and incubated for 15 min. Following a 15 min incubation, the liposome/DNA complexes were mixed with 270 μL of serum free media and added to a dish with 5 mL of serum free media. The plates were incubated for 4 h at 37 °C, and 5 mL of DMEM containing 20% FCS was added. Two days after transfection, the culture medium was replaced with DMEM containing 10% fetal calf serum and 1 mg of the neomycin analogue G418 (GIBCO BRL)/mL. The transfectants were replenished with fresh selection medium every 2−3 days. Resistant colonies were pooled into a 96 well plate after 2 weeks of selection and propagated in the presence of 1 mg of G418/mL.

Treating Stably Transfected Cells with Oligonucleotides. HeLa cells were grown up to 50% confluency in a 150 mm flask, washed twice with Hank's, and trypsinized. The cells were counted, and 10^6 cells were electroporated in 400 μL of PBS containing 40 μL of 100 μM oligonucleotides at 250 V. After electroporation, DMEM with 10% fetal calf serum was added to the cells, and the cells were plated into a 24 well plate. The plates were incubated for 24 h at 37 °C. Each experiment was performed in triplicate.

Luciferase Assays. Each well was washed three times with cold PBS, and 50 μL of lysis buffer (Luciferase Assay System, Promega) was added to each well of a 24 well plate. After incubation for 15 min at 37 °C, the lysate and cell debris were collected. A volume of 20 μL of the lysate was added to 50 μL of luciferase assay substrate (Promega) in a clear polystyrene 12 × 75 mm tube, which was immediately placed in a luminometer (Analytical Luminescence Laboratory model 2010), and light production was measured for 10 s. Some lysate samples were stored at −70 °C, and luciferase activity could be measured up to 7 days later without loss of signal. To normalize the amount of protein in each sample, total protein concentration was measured by reading absorbance at 620 nm with a micro titer plate reader (Molecular Dynamics).

RESULTS

Oligonucleotide Design. The human cyclin D1 promoter contains an 18-bp purine-pyrimidine rich sequence located at −116 to −99 from the transcription start site (Figure 1A). This region is a binding site for the transcription factor, Sp1. The sequence of this region is not strictly homopurine/homopyrimidine, but contains three C·G interruptions. The cyclin D1 targeted triplex forming oligonucleotide, CD18ap, was designed using guanine to recognize GC (G*GC triplets) and thymine aligned with the CG (T*CG triplets) base pairs in this target sequence. This base sequence was based on data obtained in our laboratory with a very similar target sequence in the human *Ha-Ras* promoter (*6*).

Triplex Formation by the Cyclin D1 Promoter. Triplex formation was demonstrated by gel mobility shift analysis and DNase I footprinting. Because of its decreased charge density, triplex DNA migrates more slowly than duplex

DNA in gel mobility shift analysis. As shown in Figure 2A, CD18ap shifted the duplex target (D) to a distinct, more slowly migrating band (T), indicating the formation of triplex DNA at 1.0 μM concentration (100-fold molar excess relative to target). On the other hand, control oligonucleotide of identical sequence but in a parallel orientation with respect to the purine rich strand of target did not show any triplex formation, even at 1000-fold molar excess.

Since the TFO is targeted to the Sp1 transcription factor binding site of the cyclin D1 promoter, we determined that triplex forming oligonucleotide can block the binding of recombinant Sp1 to cyclin D1 promoter. Recombinant Sp1 does not bind to the target sequence in 1× TBM as well as it does in a buffer containing 25 mM Hepes, 12.5 mM $MgCl_2$, 70 mM KCl, 1 μM $ZnSO_4$, 1 mM DTT, 0.1% NP-40, and 10% glycerol (v/v). Therefore, we could not use 1× TBM for Sp1 mobility shift analysis. To directly characterize the effect of triplex formation on Sp1 binding, we determined that triplex formation is feasible under the above Sp1 binding

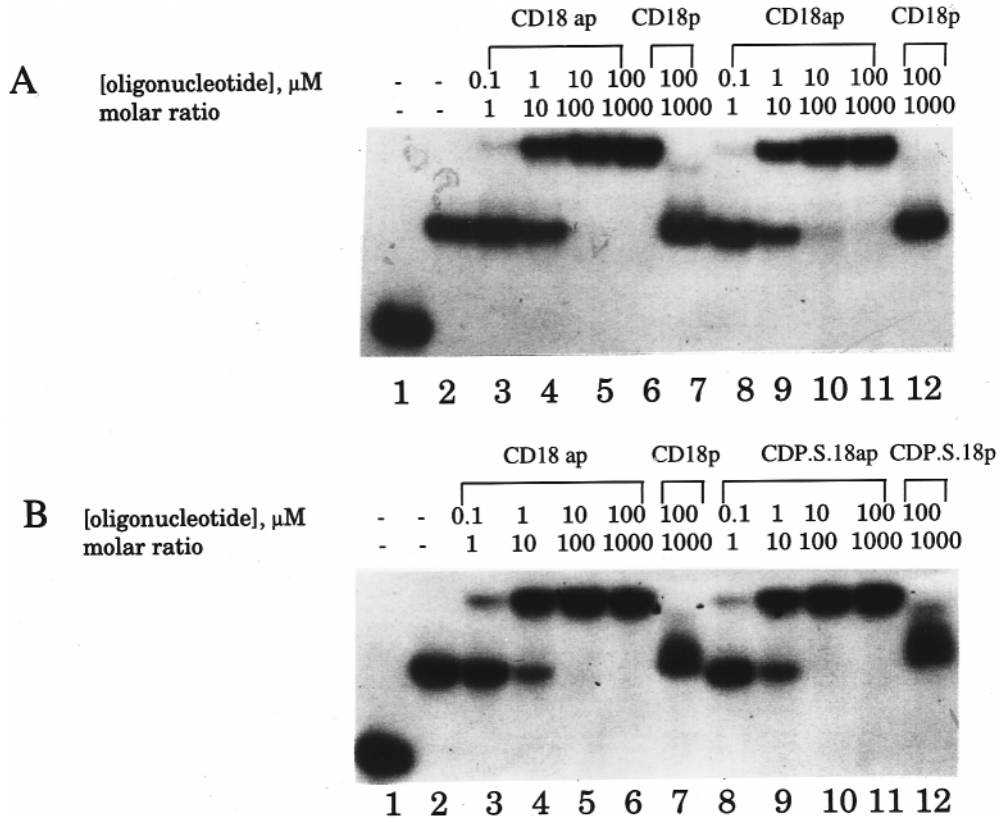

FIGURE 2: (A) EMSA of oligonucleotide directed triplex formation in the cyclin-D1 promoter target in 1× TBM or under protein binding condition. The concentration of triplex forming oligonucleotide or control oligonucleotide added to 10 nM ^{32}P-labeled 18-bp duplex and their molar ratio to the target duplex DNA are indicated above each lane. Lanes 1 and 2 are control DNA containing single strand 18-mer and 18-bp duplex, respectively. S = single strand DNA. D = duplex DNA. T = triplex DNA. The 18-bp target was incubated with oligonucleotide at the concentrations indicated either in 1× TBM (lanes 3−7) or under the protein binding conditions described in Materials and Methods (lanes 8−12). (B) Comparison of phosphodiester oligonucleotide with phosphorothioate oligonucleotide in the capability of triplex formation. Lanes 1 and 2 are control DNA containing single strand 18-mer and 18-bp duplex, respectively. S = single strand DNA. D = duplex DNA. T = triplex DNA. The 18-bp target was incubated with either phosphodiester oligonucleotide (lanes 3−7) or phosphorothioate oligonucleotide (lanes 8−12) at the concentrations indicated.

conditions by gel mobility shift. As shown in Figure 2A, lanes 7−11, triplex formation under the Sp1 binding conditions was comparable to that observed in 1× TBM.

DNase I footprinting experiments support the results obtained from gel mobility shifts and confirm that triplex formation occurs in a sequence specific manner only when the oligonucleotide third strand is oriented antiparallel relative to the purine rich strand of target duplex. Protection of the target sequence by the triplex forming oligonucleotide is concentration dependent in a manner consistent with the gel mobility shift analysis. Figure 3 shows that at oligonucleotide concentrations of 30 μM (3000-fold molar excess with respect to the 465-bp promoter fragment), the antiparallel CD18ap yields DNase protection patterns documenting complete protection of the 18-bp target sequence (lane 6). On the other hand, the control parallel oligonucleotide, CD18p, which does not form triplex at concentrations up to 30 μM, does not show any DNase protection (lane 1). These data further suggest that, under these conditions, triplex formation occurs exclusively with the third strand oriented antiparallel to the purine-rich strand of the cyclin D1 promoter target.

Binding of Phosphorothioate Oligonucleotide Relative to Phosphodiester Oligonucleotide. The comparison of triplex formation by unmodified phosphodiester oligonucleotides to that of phosphorothioate oligonucleotide is shown in Figure 2B. The extent of triplex formation with phosphorothioate oligonucleotides (lanes 3−6) is comparable to that with phosphodiester oligonucleotides at identical concentrations (lanes 8−11). The concentration-dependent shift of the cyclin D1 target duplex to triplex begins at 0.01 μM oligonucleotide, which corresponds to a one-to-one molar ratio of oligonucleotide to duplex target. At 1.0 μM (100-fold excess) concentration of either phosphodiester or phosphorothioate oligonucleotides, all labeled duplex target sequences are shifted to triplex (lanes 6 and 11). Again, there was no triplex formation with control oligonucleotides even at 10 μM (1000-fold excess), which indicates that the parallel oligonucleotide does not form triplex under these conditions (lanes 7 and 12). The only difference in triplex forming ability between CD18ap and CD18P.S.ap is the slight difference in the relative amount of duplex and triplex species in the presence of 0.01 μM solutions of each oligonucleotide.

Effect of Triplex Formation on Recombinant Sp1 Binding. The effect of oligonucleotide directed triplex formation on recombinant Sp1 binding by the cyclin D1 promoter was demonstrated by electrophoretic mobility shift analysis (Figure 4). Lane 1 shows the labeled 18-bp cyclin D1 probe alone (F), while lane 2 shows that the labeled 18-bp target is bound by Sp1 as evidenced by retardation of labeled duplex oligonucleotide following incubation with recombi-

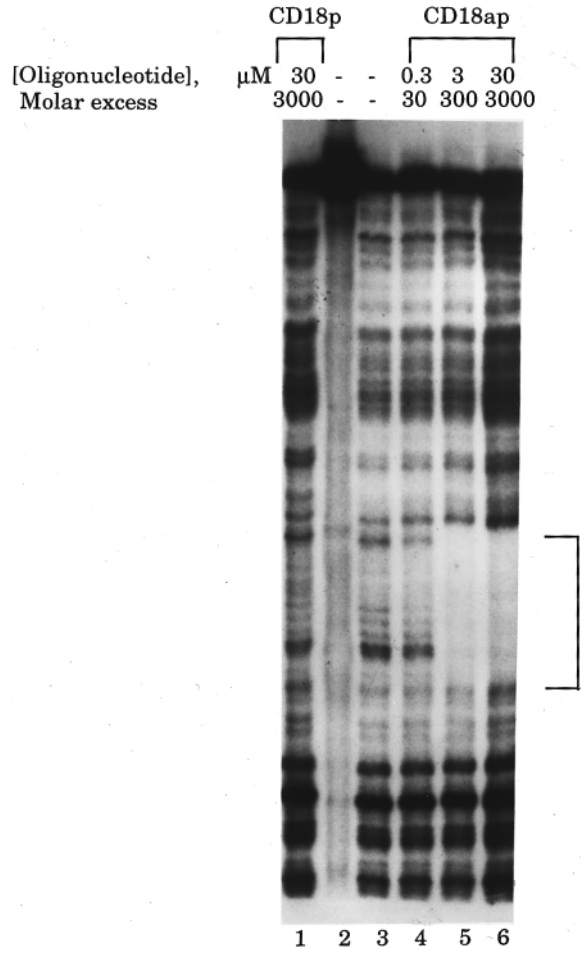

FIGURE 3: DNase I footprinting analysis demonstrating sequence specific binding of triplex forming oligonucleotide to cyclin-D1 promoter target. Oligonucleotides were incubated at the concentration indicated above each lane with 10 nM 344-bp ^{32}P-Klenow-labeled promoter fragment followed by limited DNase I digestion. Control digest (lane 4) was promoter fragments incubated in the absence of any oligonucleotide.

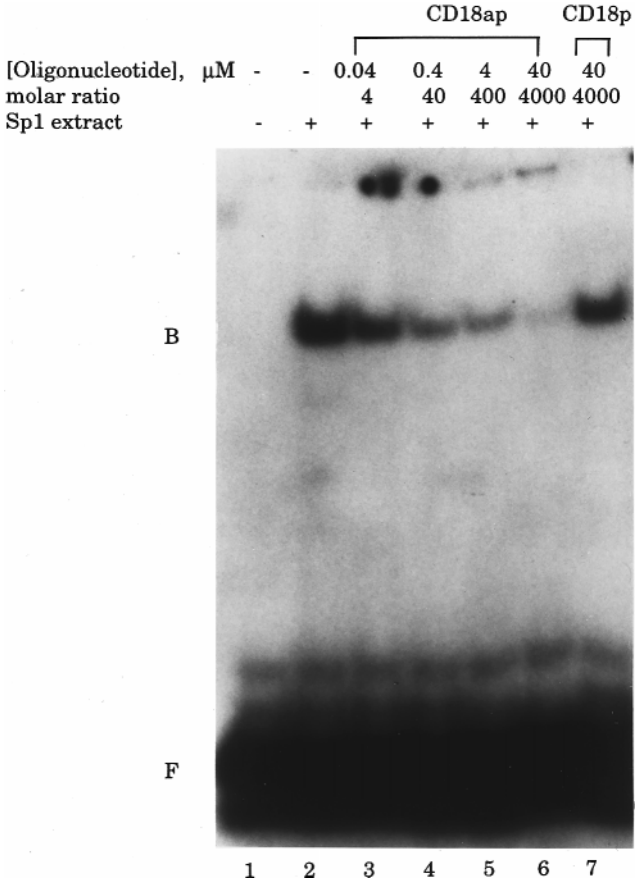

FIGURE 4: EMSA demonstrating recombinant Sp1 binding to the human cyclin-D1 duplex target and its inhibition by triplex forming oligonucleotide. ^{32}P-labeled 18-bp human cyclin D1 fragment was incubated with oligonucleotides as described, and then recombinant Sp1 was added. B represents protein DNA complexes, and F represents unbound DNA or free probe.

nant Sp1. There are some slowly migrating species which are not protein DNA complexes; instead, they represent DNA retained in the well. When the CD18ap TFO is incubated with the target, the pattern of migration is not changed by triplex formation (lane 3). However, incubation with 10 μM TFO completely inhibits the binding of Sp1 to the target. As demonstrated in lane 4 (0.1 μM), lane 5 (1 μM), and lane 6 (10 μM), this inhibition of Sp1 binding by triplex formation is concentration dependent. The control oligonucleotide, CD18p, of identical sequence but opposite orientation to the target, appears to have little if any effect on protein binding at 10 μM (4000-fold excess) (lane 7). Because a low percentage gel (5%) was used to analyze protein binding, the migration of the triplex DNA was indistinguishable from that of duplex and was not detectable in Figure 4. However, when a 16% TBM gel was used, it was possible to demonstrate triplex formation by CD18ap but not by CD18p (Figure 2A). These data suggest that significant inhibition of protein binding to the cyclin D1 target is a direct result of triplex formation.

Effect of Triplex Formation on Cyclin D1 Transcription. To test the effect of triplex formation on cyclin D1 transcription, we have created HeLa cell lines which were stably transfected with a luciferase reporter gene driven by the cyclin D1 promoter. We have used pMAMneoluc (Clontech) containing 1.5 kb of the CD-1 promoter region for stable transfection. Oligonucleotides were delivered by electroporation. After electroporation of HeLa cells with the triplex forming CD18P.S.ap at 10 μM, a significant decrease of luciferase activity (60% inhibition) was observed, but the nontriplex forming CD18P.S.p had little or no effect at the same concentration (Figure 5A). The effect of the cyclin D1 targeted triplex forming oligonucleotide on transcription from a promoter unrelated to that of cyclin D1 was examined by treating stably transfected HeLa cells expressing a luciferase reporter gene driven by the cytomegalovirus immediate early promoter with either TFO or control oligonucleotides. As shown in Figure 5B, at the same concentration which inhibits cyclin D1 transcription, the CD18P.S.ap TFO has little effect on transcription from the cytomegalovirus promoter under the same conditions. Both CD18P.S.ap and CD18P.S.p do not decrease luciferase expression from the CMV promoter. These results suggest that inhibition of cyclin D1 transcription by CD18P.S.ap is due to sequence specific triplex formation within the cyclin D1 promoter.

DISCUSSION

The data presented in this paper demonstrate that a triplex helix structure of the pur*pur·pyr motif can be formed by the cyclin D1 promoter, the third strand being antiparallel to the purine rich strand of the duplex. The ability of this

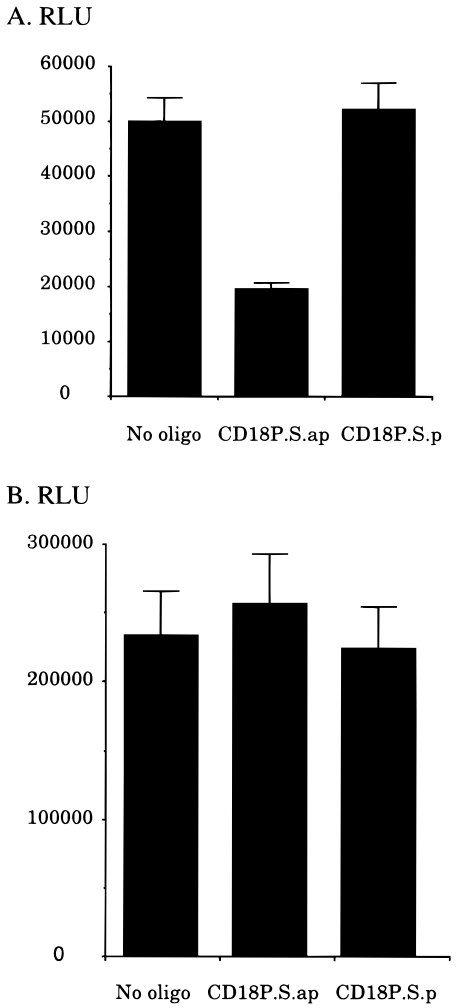

FIGURE 5: Luciferase assay demonstrating specific inhibition of cyclin D1 transcription. (A) HeLa cells stably transfected with luciferase driven by cyclin D1 promoter were electroporated with 10 μM phosphorothioate oligonucleotides. (B) HeLa cells stably transfected with luciferase gene driven by CMV immediate early promoter were treated with 10 μM oligonucleotide by electroporation. The luciferase activity is inhibited by triplex forming oligonucleotide, whereas the purine rich control oligonucleotide has no effect on luciferase activities. The vertical bar represents the standard error of the mean for the three samples.

oligonucleotide to inhibit expression of a reporter gene suggests that triplex can also be formed in intact cells.

There have also been several studies which looked at the functional consequences of triplex formation. McShan et al. demonstrated that a TFO targeted to the Sp1 binding sites in the long terminal repeats of human immunodeficiency virus inhibits viral transcription in infected cells (*24*). It has been demonstrated that TFOs targeted to the human *ki-Ras* or *Her-2*/*Neu* promoter inhibit the binding of a protein in HeLa nuclear extract (*25, 26*). We have shown that triplex within the human c-*myc* P2 promoter also inhibits binding of the important regulatory factor, MAZ, and blocks transcription of the *c-myc* in a cell free in vitro transcription system (*27*) in addition to *Ha-Ras* (*6*) and *Her-2*/*Neu* (*25*).

Cyclin D1 is a G1 cyclin and appears to be functional in the late part of the G1 phase of the cell cycle. Because loss of cell cycle control may contribute to tumor formation, it is intriguing to note that cyclin D1 has been found to be overexpressed in a wide variety of human cancers. It has been shown that ectopic expression of cyclin D1 leads to a shortening of the G1 phase of the cell cycle (*28*). Baldin et al. and Ewen et al. have shown that the neutralization of cyclin D1 by microinjected antibodies and antisense RNA arrests cells in G1 (*17, 29*). Weinstein et al. have expressed an antisense cyclin D1 RNA, either constitutively or inducibly, in the HCE−7 human esophageal carcinoma cell line in which cyclin D1 is amplified and expressed at high levels (*30*). They have demonstrated that the expression of antisense cyclin D1 RNA led to the decreased expression of cyclin D1 which has been closely related to a marked decrease of inhibition of cell proliferation. These findings suggest that cyclin D1 is an excellent target for inhibition by triplex formation.

A closer inspection of the human cyclin D1 promoter sequence revealed the presence of a number of potential transcription factor binding sites. These included a TRE at −935 which is composed of a sequence shown to represent a perfect Jun/Fos binding site in vitro as well as binding sites for Myc, E2F, OTF, Sp1, and CREB/ATF (*31*). Because of its strong polypyrimidine/polypurine nature, we have chosen the Sp1 binding site as the target for triplex formation. In an effort to provide sequence specific DNA binding agents which may inhibit cyclin D1 transcription, we have designed a triplex forming oligonucleotide targeted to this region of the cyclin D1 promoter. Beal and Dervan have previously shown that G*GC, A*AT and T*AT, triplets stabilize a triple helix to a greater extent than the other 13 natural triplets (*32*). They have also compared the relative cleaving ability of 15-mer oligonucleotides which differed in sequence at a single position which were equipped with a thymine−EDTA moiety at each 3′ end so that binding could be monitored by the affinity cleaving method. Their results indicated that while oligonucleotides containing adenosine, cytosine, or guanine opposite a single C·G base pair did not provide cleavage, thymidine substituted oligonucleotide gave weak but better cleavage than other oligonucleotides. Based on these facts, a potential triplex forming oligonucleotide targeted to the human cyclin D1 pur·pyr motif was designed in both parallel and antiparallel orientation with respect to the purine rich strand, containing guanine to recognize GC (G*GC triplets), adenine to recognize AT (A*AT triplets), and thymine to recognize CG (T*CG triplets) (Figure 1B). The parallel oligonucleotide was used as a control oligonucleotide after it was shown that it would not form triplex with the target sequence.

The oligonucleotide CD18ap which is oriented antiparallel to the purine rich strand of duplex was shown to bind in a sequence specific manner to the target sequence. A control oligonucleotide, CD18p, containing a sequence identical to CD18ap but in a parallel orientation does not form triplex. These results are consistent with those from previous studies by Beal and Dervan and Durland et al., which have shown that the third strand of pur/pyr*pur·pyr triplex binds in an antiparallel orientation with respect to the purine rich strand of the duplex target (*3, 33*). Giovannangeli et al. have also demonstrated that an oligonucleotide containing three bases (thymine, cytosine, and guanine) is capable of binding to the target in a parallel orientation with respect to the homopurine sequence of a homopurine/homopyrimidine target of human immunodeficiency virus (HIV) proviral DNA (*34*).

Maher et al. have shown that repression of transcription by triplex formation upstream of the TATA box occurs mainly at the level of transcription initiation (*35*). One possible explanation is that this triplex mediated repression of transcription results from the inhibition of formation of the proximal initiation complex assembly. This mechanism of inhibiting transcription could not explain repression at long distance, nor does it give an explanation for the repression by triplex-helix formation further upstream of the TATA box. They proposed an alternative model suggesting that oligonucleotide-directed triplex formation bends or stiffens the target duplex in a way that might antagonize promoter function.

We used modified oligonucleotides for our cell experiments, since unmodified phosphodiester oligonucleotides have a relatively short half-life due to intracellular nucleases or nucleases in the serum of tissue culture media. Modifications of the phosphodiester backbone have been shown to impair stability and allow for enhanced affinity and increased cellular uptake of oligonucleotides. In the phosphorothioate linkage, a nonbridging oxygen is replaced with sulfur, and this phosphorothioate internucleotide linkage has been among the most widely examined oligonucleotide backbone modifications (*36*).

We have shown that triplex formation by the cyclin D1 promoter inhibits the binding of Sp1 recombinant protein and inhibits transcription of the luciferase reporter gene driven by cyclin D1 promoter in intact cells. We have not confirmed that the inhibition of cyclin D1 transcription is due to the inhibition of DNA binding by this specific nuclear protein. Therefore, it is also possible that inhibition of cyclin D1 transcription by CD18P.S.ap may result from blocking the assembly of the preinitiation complex either by bending or stiffening the target duplex in a way that might be antagonistic to the promoter function or by a combination of these two effects.

The identification and characterization of genes that are involved in human disease have provided important targets for modulation of gene expression. Because of their specificity to selectively inhibit transcription of their target genes in intact cells, triplex forming oligonucleotides could be potential therapeutic agents. We have identified an oligonucleotide targeted to the human cyclin D1 promoter and demonstrated that the TFO binds to its target in a sequence specific manner and inhibits Sp1 binding and the transcription in HeLa cells. The data presented in this report suggest the potential future application of this oligonucleotide to the specific modulation of cyclin D1 expression in vivo.

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