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Sequence-specific inhibition of a transcription factor by circular dumbbell DNA oligonucleotides

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Abstract The inhibition of specific transcription regulatory proteins is a new approach to control gene expression. The transcriptional activities of DNA-binding proteins can be inhibited by the use of double-stranded oligonucleotides that compete for the binding to their specific target sequences in promoters and enhancers. We used nicked (NDODN-κB) and circular (CDODN-κB) dumbbell DNA oligonucleotides containing a NF-κB binding site to analyze the inhibition of the NF-κB-dependent activation of the human immunodeficiency virus type-1 (HIV-1) enhancer. The dumbbell DNA oligonucleotides are stable, short segments of double-stranded DNA with closed nucleotide loops on each end, which confer resistance to exonucleases. The dumbbell and other oligonucleotides (decoys) with the NF-κB sequence were found to compete with the native strand for NF-κB binding. The circular dumbbell and double-stranded phosphorothioate oligonucleotides competed with the native strand for binding to the NF-κB binding proteins, while the nicked NF-κB dumbbell was a less effective competitor. In Jurkat T-cells, the dumbbell and other oligonucleotides were tested for their ability to block the activation of the plasmid HIV-NL4-3 Luc. The CDODN-κB strongly inhibits the specific transcriptional regulatory proteins, as compared with the NDODN-κB and the double stranded phosphodiester oligonucleotides. On the other hand, the double stranded phosphorothioate oligonucleotides could also block this activation, but the effect was non-specific. The circular (CDODN) dumbbell oligonucleotides may efficiently compete for the binding of specific transcription factors within cells, thus providing anti-HIV-1 or other therapeutic effects.

Key words: DNA dumbbell; Decoy; Inhibition of transcription factor (NF-κB); Jurkat cell; Luciferase assay; Anti-HIV

1. Introduction

The *cis*-acting regulatory elements that control retroviral gene expression are thought normally to reside in the long terminal repeat (LTR) of the virus, particularly within the U3 region. To regulate their gene expression specifically, the viruses have evolved their own regulatory gene products. These viral *trans*-activators are also dependent upon the cellular factors that interact with the viral proteins and the *cis*-acting DNA and RNA regulatory elements to further modulate retroviral gene expression. A variety of cellular transcription factors contribute to the constitutive expression of retroviruses. Among these cellular factors, Sp1 plays an important role [1], and three Sp1 sites are proximal to the TATA box from the HIV-1 long terminal repeat (LTR). Of these three sites, the most upstream site represents the best consensus sequence and is activated by in vitro transcription [1,2]. Several other *cis*-acting regulatory elements are found near the TATA box and the transcriptional initiation site. The NF-κB regulatory element is repeated twice in the HIV-1 enhancer immediately upstream of the three Sp1 sites. The immune response gene, NF-κB, has emerged as a unique model for gene product regulation, which is both adaptable and responsive to cellular activation [3–5]. NF-κB is found in many tissues but its activity is of primary importance within the immune system. As an endpoint for many signal transduction pathways [4], NF-κB participates in the induction of genes that encode immunoglobulins, immunoreceptors, cell adhesion molecules, cytokines, immune growth factors and acute phase proteins. Bound by the inhibitor, IκB, NF-κB is found in the cytoplasm in an inactive form that, once activated, is rapidly turned over in response to signals transduced from the cell surface [6,7]. NF-κB is able to bind in vitro to synthetic double stranded oligonucleotides, which can be used as sequence-specific DNA decoys for the selective sequestering of transcription factors, rendering then unavailable to bind to their target DNA [8–10]. As final result, transcriptional activation is blocked. Several double-stranded DNAs, such as phosphodiester oligonucleotide duplexes [11–13], an αβ-anomeric oligonucleotide [14], phosphorothioate oligonucleotide duplexes [15] and dumbbell oligonucleotides [16,17] have been utilized as decoys for transcription factors. The oligonucleotide duplexes with phosphorothioate backbones exhibit several advantages over the other forms, including relatively high nuclease resistance. Double-stranded phosphorothioate oligonucleotides containing the binding sites for NF-κB or Oct-1 were shown to inhibit HIV-CAT or Oct-CAT expression when cotransfected with these reporter vectors in an Epstein-Barr virus-transformed B-cell line [14]. However, the double-stranded phosphorothioate oligonucleotides have also shown sequence-independent effects, due to non-sequence specific protein binding [18]. On the other hand, circular dumbbell oligonucleotides have increased nuclease resistance [19,20] and the uptake [20] into cells, as compared with the nicked dumbbell- and the linear antisense phosphodiester oligonucleotides. The DNA dumbbell oligonucleotides containing the binding sites for the liver-enriched transcription factor hepatocyte nuclear factor 1 (HNF-1) and the X-box of the DRA promoter were shown to inhibit in vitro transcription

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driven by these DNA-binding proteins in a concentration-specific manner [16,17].

We are interested in the antisense and decoy abilities of the circular dumbbell oligonucleotides. In particular, we have analyzed the circular dumbbell oligonucleotides for their sequence-specific inhibition of transcription factor activity. As a model system, we have studied the NF-κB-dependent activation of the HIV-1 enhancer. Here we show that the circular dumbbell oligonucleotides containing the NF-κB binding sites are able to specifically inhibit the NF-κB-dependent activation of the HIV-1 enhancer in vitro. The circular dumbbell oligonucleotide strongly inhibits the specific transcriptional regulatory proteins compared to the nicked dumbbell oligonucleotide and the double stranded phosphodiester oligonucleotides. However, the double-stranded phosphorothioate oligonucleotides show non-specific binding of transcription factors.

2. Materials and methods

2.1. Oligonucleotide synthesis

The oligonucleotides were synthesized by means of the phosphoramidite approach using an Applied Biosystems DNA synthesizer, Model 392. The 5′-phosphorylated oligonucleotides were synthesized using a dimethoxytrityl-hexa-ethyloxy-glycol-2-cyanoethyl-*N*,*N*-diisopropyl phosphoramidite as the phosphorylating agent. The oligonucleotide derivatives were purified by polyacrylamide gel electrophoresis (PAGE) or by reverse phase HPLC chromatography. The extinction coefficients of the oligonucleotides were determined by calculating the theoretical extinction coefficients as the sum of the nucleosides and multiplying with the experimentally determined enzymatic hypochromicity. The 5′-phosphorylated nicked dumbbell RNA/DNA chimeric oligonucleotide (0.2 A_{260}) was incubated in 10 μl of 50 mM Tris-HCl, pH 7.5 and 10 mM $MgCl_2$ at 90°C for 5 min and then at 15°C for 2 min. The solution was incubated with 1 μl of 100 mM dATP, 1 μl of 25 μg bovine serum albumin (BSA)/ml and 40 units of T4 DNA ligase at 37°C for 30 min [21]. The reaction mixture was extracted with an equal volume of water-saturated chloroform-phenol (1:1, v/v), and was precipitated with ethanol. The circular oligonucleotides were purified by denaturing PAGE (20% polyacrylamide containing 7 M urea). The band corresponding to the desired oligonucleotide was eluted from the gels with water and was precipitated with ethanol. The identity of each of the ligated dumbbells was verified by phosphodiesterase protection mapping.

2.2. 3′-End digoxigenin (DIG) labeled DNA

The DNAs (100 pmol) and 1 mM DIG-11-dd-UTP (Roche Diagnostics K. K., Germany) in 10 μl of tailing buffer containing 1 M Tris-HCl, pH 6.6, 1 M potassium cacodylate, 1.25 mg/ml BSA and 25 mM $CoCl_2$, were mixed with 1 μl of 50 U/μl terminal transferase and were kept at 37°C for 1 h. The reaction was quenched with 1 μl of a 20 mg/ml glycogen solution and 200 μl of 0.2 mM EDTA (pH 8.0). The labeled-DNA was precipitated with 2.5 μl of 4 M $LiCl_2$ and 75 μl of pre-chilled (−20°C) 99% ethanol. The 3′-end digoxigenin (DIG) labeled DNA was purified by reversed phase HPLC with a linear gradient of 5–50% acetonitrile in 0.1 M triethylammonium acetate (pH 7.0). Non-labeled DNAs were synthesized on the DNA/RNA synthesizer and were purified by, 7 M urea denaturing 20% PAGE.

2.3. In vitro competition.

Four femtomol of DIG-labeled duplex and 4.4 ng of the NF-κB protein (Promega, USA) were incubated in the presence of increasing concentrations of non-labeled oligonucleotides in 17 μl of binding buffer, containing 10 mM HEPES, pH 7.9, 50 mM KCI, 1 μg poly (I-C) in 10% glycerol and 5 mM dithiothreitol, and the mixture was incubated at room temperature for 15 min. The NF-κB complexes with the duplex were separated from the unbound duplex by electrophoresis on 6% non-denaturing polyacrylamide gels containing 0.5×TBE (40 mM Tris-borate, pH 8.3 and 2 mM EDTA). The electrophoresis was carried out in 0.5×TBE buffer for 2 h at 4°C. The relative amounts of the free and bound DNA were determined by a gel analysis system, LAS-1000 (Fuji Photo Film, Japan).

2.4. Transfections and transient Luc assay

The HIV-NL4-3 Luc vector was generated by substituting *nef* gene sequences of the HIV-1 NL4-3 genome with the firefly luciferase gene and deleting the envelope gene sequences [22]. Aliquots of 3×10^5 Jurkat cells/ml were plated in 24-well plates the day before transfection. The plasmid pNL4-3-Luc (3.3 μg) and the oligonucleotides (22 μg) were cotransfected with FuGENE-6 (Roche Diagnostics K. K., Germany), using the manufacturer's protocol. After the transfection and an incubation for 4 h, the cells were incubated for an additional 24 h with PMA (40 nM, Sigma, USA) and PHA (2 μg/ml, Pharmacia, USA).

Luciferase assay. To analyze the luciferase expression, the cells were washed twice with phosphate-buffered saline, and were lysed with 100 μl of 1×luciferase lysis buffer (Toyo ink, Japan). Ten microliters of each lysate were subjected to the luciferase assay (Toyo ink Japan), which was monitored with a Lumat 9507 luminometer (EG and Berthod, Germany) [23].

3. Results and discussion

3.1. Specific binding of the NF-κB to a circular dumbbell oligonucleotide with the NF-κB target site.

To demonstrate that the phosphodiester and phosphorothioate oligonucleotide duplexes and the dumbbell oligonucleotides interact strongly enough with the NF-κB to act as decoys, an in vitro competition assay was performed. For this purpose we chose NF-κB transcription factor, which binds two NF-κB recognition sequences derived from the long terminal repeat of HIV-1 [4]. We first performed the electrophoretic mobility shift assays of human NF-κB towards an NF-κB site in a double stranded oligonucleotide (ds-κB, Fig. 1). Constant amounts of the DIG-labeled NF-κB probe oligonu-

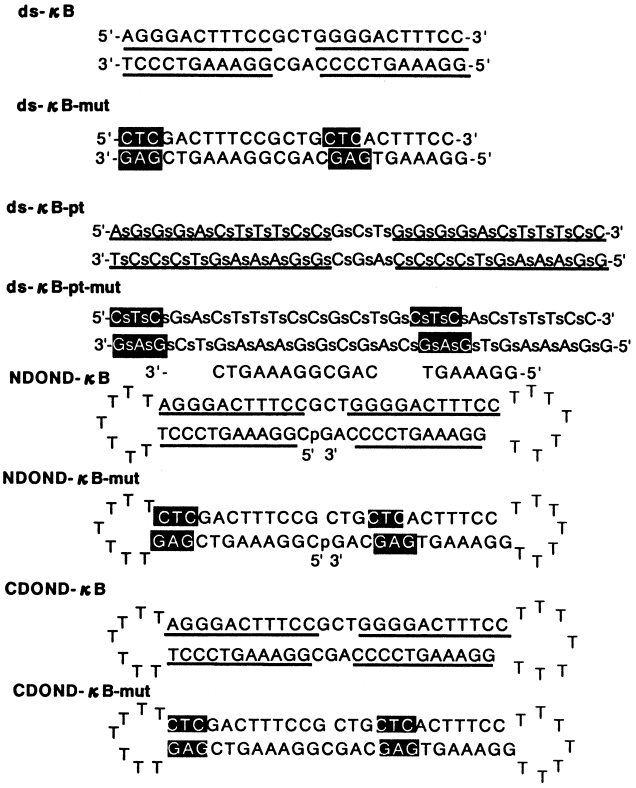

Fig. 1. The structure and the sequences of the oligonucleotides used in this study, as described in the text. The two NF-κB recognition sequences are underlined in each oligonucleotide. Mutated sequences are boxed. pt: phosphorothioate group; ds: double-stranded.

cleotide (DIG-ds-κB) were first added to increasing amounts of the competitor oligonucleotides (ds-κB, NDOND-κB and CDODN-κB). We found that an increase in the non-labeled-ds-κB decreased the intensity of the retarded band, which corresponded to the complex formed by the human NF-κB protein (Fig. 2A, lanes 3–6). A 1000-fold molar excess of the non-labeled competitor, ds-κB, almost completely competed for the human NF-κB binding to DIG-ds-κB. When the nicked dumbbell oligonucleotide, NDOND-κB, was used in place of ds-κB, as little as a 10-fold molar excess of the non-labeled competitor, NDOND-κB, completely competed for the human NF-κB binding to DIG-ds-κB (Fig. 2B). On the other hand, the addition of a 1000-fold molar excess of the control oligonucleotide, the NDOND-κB-mut oligonucleotide containing mutated bases in the human NF-κB site, did not compete with the DIG-labeled ds-κB for human NF-κB binding (Fig. 2C). Our data suggest that the dumbbell oligonucleotides may exhibit sequence-dependent effects due to specific protein binding. Of particular interest, the non-labeled circular dumbbell oligonucleotide, CDODN-κB, successfully competed with DIG-ds-κB for binding to the human NF-κB binding protein. Equimolar amounts of CDODN-κB were able to compete with DIG-ds-κB (Fig. 2D, lane 3). These data indicate that the stability of the double stranded structure in the non-ligated dumbbell is lower than that of the ligated oligonucleotide and former is probably being displaced to a partially single stranded form. This result suggests that the circular dumbbell oligonucleotide (CDODN-κB) has more affinity for the human NF-κB binding protein than the double-stranded DNA (ds-κB) and the nicked dumbbell oligonucleotide (NDODN-κB).

Previous studies by other investigators have shown that the double-stranded phosphorothioate oligonucleotides compete for the binding of the specific transcription factor, NF-κB, and provided anti-viral, immunosuppressive, or other therapeutic effects [15]. Furthermore, phosphorothioate oligonucleotides are highly resistant to degradation by nucleases [24,25], exhibit increased cellular uptake [25,26], and retain their ability to form sequence-specific duplexes, although with reduced stability relative to phosphodiester oligonucleotides [27,28]. To confirm that the protein binding to the double-stranded DNA probe (ds-κBpt) containing phosphorothioate bonds was sequence-specific, an in vitro competition assay was performed. To our surprise, equimolar amounts of the non-labeled competitor ds-κBpt completely competed for human NF-κB binding to DIG-ds-κB (Fig. 2E, lane 3). Control oligonucleotides were prepared for comparison, such as the mutated oligonucleotides, ds-κB-mut, ds-κBpt-mut and CDODN-κB-mut. As shown in Fig. 2E, lanes 7–10, the oligonucleotides (ds-κB-mut and CDODN-κB-mut) containing mutated bases in the human NF-κB sequences were unable to compete for the formation of shifted complexes. However,

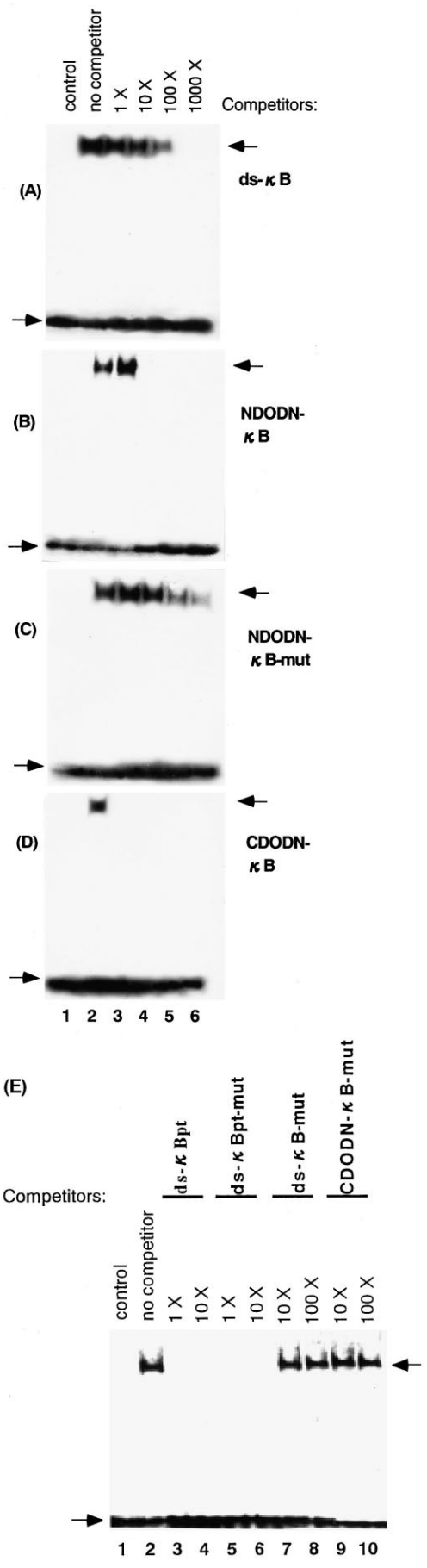

Fig. 2. Competition assay. NF-κB-double stranded oligonucleotide complexes were formed between labeled DIG-ds-NF-κB (4 fmol) and the NF-κB protein (4.4 μg) in the presence of various concentrations of unlabeled oligonucleotides. The competitors were present at 1-, 10-, 100-, and 1000-fold molar excess to the labeled DIG-ds-NF-κB. Arrows indicate the NF-κB complex (right) and free probe (left). A: ds-κB competitor. B: NDODN-κB competitor. C: NDODN-κB-mut competitor. D: CDODN-κB competitor. E: ds-κBpt, ds-κBpt-mut, ds-κB-mut and CDODN-κB-mut competitors.

in the case of the double-stranded mutated DNA containing the phosphorothioate bonds, ds-κBpt-mut, the retarded band corresponding to the complex formed by the human NF-κB protein efficiently was absent (Fig. 2E, lanes 4 and 5). On the other hand, unmodified double-stranded DNA (ds-κB) was an about 100-fold less effective competitor than ds-κBpt and ds-κBpt-mut (Fig. 2A). The results of these competition assays indicate that protein binding to ds-κBpt correlates with the presence of phosphorothioates rather than specific sequence. Consequently, we were unable to assay the phosphorothioate oligonucleotides, ds-κBpt and ds-κBpt-mut, so they could not be used to monitor the binding the human NF-κB protein. Brown et al. [18] have also reported that double stranded phosphorothioate oligonucleotides may exhibit sequence-independent effects due to non-specific protein binding.

3.2. Circular dumbbell oligonucleotides can specifically inhibit luciferase activity in Jurkat cells

The circular dumbbell oligonucleotides containing the binding sites for NF-κB were able to interact sequence specifically with the human NF-κB protein in vitro, while the double stranded phosphorothioate oligonucleotides showed non-specific binding to the human NF-κB protein.

In order to evaluate the competition efficiency of the circular dumbbell oligonucleotides containing the binding sites for NF-κB, we set up the conditions for cotransfecting Jurkat cells with plasmids with an envelope-defective HIV-1 genome expressing the luciferase gene (HIV-NL4-3 Luc) [22]. We then cotransfected HIV-NL4-3 Luc with oligonucleotides/dumbbells containing the binding sites for NF-κB. When 22 μg of either CDODN-κB or NDODN-κB were added with the HIV-NL4-3 Luc, almost 75% and 50% of the luciferase activity was inhibited, respectively (Fig. 3). It was found that the ligated dumbbell oligonucleotide (CDODN-κB) was more active than the unligated oligonucleotide (NDODN-κB) in blocking the luciferase activity of HIV-NL4-3 Luc [19]. The double stranded oligonucleotide (ds-κB) was much less active, presumably due to degradation by exonucleases. In contrast, under the same conditions, the mutant dumbbells, ds-κB-mut, CDODN-κB-mut, and NDODN-κB-mut, permitted maximal luciferase activity (Fig. 3). These results suggest that the use of dumbbell oligonucleotides results in highly efficient and specific inhibition of gene expression. On the other hand, ds-κBpt was more inhibitory than CDODN-κB. However, the mutant ds-κBpt-mut had inhibitory effects similar to those of ds-κBpt. Given the higher nuclease resistance of the double-stranded phosphorothioate oligonucleotides relative to the circular dumbbell oligonucleotides, the double-stranded phosphorothioate oligonucleotides may prove to be more effective as non-sequence-specific therapeutic agents. Similarly, it has been shown that inhibition of polymerase and RNase H activities by the double-stranded phosphorothioate oligonucleotides is directly proportional to the extent of phosphorothioate modification [29]. However, it has been suggested that the non-specific protein binding by phosphorothioate-modified oligonucleotides may impair their ability to reach the intended therapeutic targets [29–33].

In conclusion, the dumbbell DNA oligonucleotides are stable, short segments of double-stranded DNA with closed nucleotide loops on each end, which confer resistance to exonucleases. The dumbbells and other oligonucleotides (decoys) with the NF-κB sequence were found to compete with the native strand for binding to NF-κB binding proteins. The circular dumbbell and double-stranded phosphorothioate oligonucleotides competed with the native strand for binding to the NF-κB binding proteins, while the nicked NF-κB dumbbell was a less effective competitor. In Jurkat T-cells, the dumbbell and other oligonucleotides were tested for their ability to block the activation of the plasmid HIV-NL4-3 Luc. It was found that the ligated dumbbell oligonucleotide (CDODN-κB) was more active than the unligated oligonucleotide (NDODN-κB) and the unmodified double-stranded oligonucleotide (ds-κB). On the other hand, the double-stranded phosphorothioate oligonucleotides (ds-κBpt) could also block this activation, but the effect correlates with the presence of phosphorothioates rather than specific sequence. The circular dumbbell oligonucleotides can efficiently compete for the binding of specific transcription factors and may provide anti-HIV-1, or other therapeutic effects.

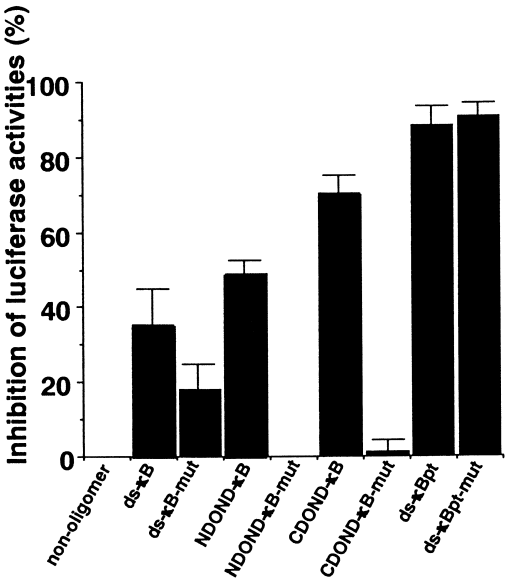

Fig. 3. Inhibition of the NF-κB activity by the oligonucleotides. Jurkat cells were cotransfected with the plasmid HIV-NL4-3-Luc (3.3 μg) and the oligonucleotides (22 μg) with FuGENE-6. The activity of the oligonucleotides is reflected in their ability to down-regulate luciferase activity [23].

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