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Transcription factor decoy oligonucleotides modified with locked nucleic acids: an *in vitro* study to reconcile biostability with binding affinity

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ABSTRACT

Double-stranded oligonucleotides (ODNs) containing the consensus binding sequence of a transcription factor provide a rationally designed tool to manipulate gene expression at the transcriptional level by the decoy approach. However, modifications introduced into oligonucleotides to increase stability quite often do not guarantee that transcription factor affinity and/or specificity of recognition are retained. We have previously evaluated the use of locked nucleic acids (LNA) in the design of decoy molecules for the transcription factor κB. Oligonucleotides containing LNA substitutions displayed high resistance to exo- and endonucleolytic degradation, with LNA–DNA mix-mers being more stable than LNA–DNA–LNA gap-mers. However, insertion of internal LNA bases resulted in a loss of affinity for the transcription factor. This latter effect apparently depended on positioning of the internal LNA substitutions. Indeed, here we demonstrate that intra- and inter-strand positioning of internal LNAs has to be carefully considered to maintain affinity and achieve high stability, respectively. Unfortunately, our data also indicate that LNA positioning is not the only parameter affecting transcription factor binding, the interference in part being dependent on the intrinsic conformational properties of this nucleotide analog. To circumvent this problem, the successful use of an α-L-*ribo*-configured LNA is demonstrated, indicating LNA–DNA–α-L-LNA molecules as promising new decoy agents.

INTRODUCTION

The discovery that altered gene expression is an important component in the pathophysiology of many human diseases has provided unprecedented opportunities for the development of new therapeutics targeting disease processes at the level of gene function and control (1). It is well known that tissue- and stimulus-specific gene expression is initiated and primarily regulated at the transcriptional level by sequence-specific protein/DNA interactions involving specialized proteins, termed transcription factors, with nucleotide regulatory elements found in gene promoters and enhancers (*cis* elements) (2,3). Therefore, as gene regulators, transcription factors have become an attractive target for potential therapeutic intervention (4–6). Indeed, a growing number of transcription factors that regulate changes in gene expression during the pathogenesis of a wide range of disorders have been identified.

A large number of physical and biochemical studies have established that transcription factors display substantial diversity regarding both their structures and activation mechanisms, which are unique and hence suitable for the development of strategies for the selective targeting of these proteins (7). One such strategy is the decoy approach, which provides a very direct and simple way to selectively inhibit the activity of any transcription factor simply knowing minimal structural features of the target, namely its DNA binding sequence (8–13). Conceptually, the approach is based on the evidence that each transcription factor or family of transcription factors recognizes specific DNA sequences termed the consensus binding sequence. Significantly, this type of protein/DNA interaction also occurs in the absence of the surrounding genomic DNA. Therefore, synthetic oligodeoxynucleotides (ODNs) corresponding to the consensus binding sequence of a specific transcription factor have the potential to efficiently compete with the endogenous *cis* elements for transcription factor binding, allowing gene expression to be specifically modulated.

Although transcription factor decoys were initially used as a tool to study gene expression, their *in vivo* effectiveness has prompted the consideration of their use in therapy. E2F decoys are the first double-stranded DNA molecules which have reached the stage of clinical trials, providing the very first evidence that human gene expression can be modulated for therapeutic purposes using transcription factor decoys (14). However, naturally unmodified DNA molecules are subject to

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relatively rapid degradation, primarily through the action of 3′-exonucleases, but also as a result of endonuclease attack (15). Therefore, in view of a more extensive clinical application, ODN decoys are now routinely modified to enhance their biostability so as to improve their pharmacological properties. For this purpose, a variety of nucleic acid analogs containing modifications in the nucleobase, the sugar ring or the phosphodiester backbone have been synthesized and some of them, such as phosphorothioates and peptide nucleic acids (PNA), tested as decoy molecules (16,17). However, a major issue in this effort is represented by the possibility that benefits in terms of nuclease resistance may be negated by a decrease in the affinity and/or specificity of transcription factor binding to its target sequence. Fully modified phosphorothioate oligonucleotides and PNA decoys have been demonstrated to be poorly specific or unable to form stable complexes with the target factor, respectively (17,18). These results highlight that for optimum activity decoy ODNs should possess a combination of various properties rather than exclusively either increased stability toward nucleases or high affinity for the target transcription factor. Since the nature of the nucleotide and internucleotide linkages dictate the biophysical, biochemical and biological properties of oligonucleotides, the positioning of nucleotide analogs in a given sequence is crucial to the outcome and should be chosen carefully.

Despite impressive gains in structural biology over the past years, the effects produced by nucleotide modification on DNA/protein interactions are still difficult to predict. For this reason, in an attempt to reach the optimal decoy design, several research groups have followed the most obvious path, i.e. to keep modifications outside the transcription factor consensus binding sequence. In principle, this approach provides a certain degree of stability toward nuclease action and at the same time excludes interference with transcription factor binding. In this view, terminally phosphorothioate-modified molecules and PNA–DNA chimeras have recently been proposed as alternative decoy agents (19,20).

We have previously investigated the use of a new class of nucleic acid analogs, termed locked nucleic acids (LNAs), in the design of decoy oligonucleotides targeting nuclear transcription factor κB (NF-κB) (21). LNA bases are nucleotide analogs containing a methylene linkage between the 2′ oxygen and the 4′ carbon of the ribose ring (22–24). Thus, oligomers that contain LNA bases have a native phosphate backbone. Furthermore, LNAs are assembled using conventional phosphoramidite chemistry, allowing LNA nucleotides to be easily interspersed among DNA nucleotides, permitting the properties of the chimerical LNA–DNA molecules to be fine tuned for optimized decoy drug development (23,24). Our previous results demonstrated that inclusion of at least two terminal LNA monomers in a decoy molecule, outside the NF-κB binding sequence (κB site), appreciably increased protection against nuclease digestion without interfering with transcription factor binding (21). Significant further stabilization was achieved when LNA monomers were included in the κB site as well. However, replacement of internal nucleotides with LNAs resulted in a loss of affinity of NF-κB for its target sequence. Interestingly, this latter effect was apparently dependent on positioning of the internal LNA substitutions (21). Thus, in an attempt to reconcile biostability with affinity, the possible role of the intra- and inter-strand positioning of LNAs in affecting NF-κB binding and in conferring resistance to nuclease digestion has been further investigated here.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides containing LNA substitutions were obtained as HPLC-purified products on the 0.2 μmol scale from Proligo LLC (Boulder, CO and Paris, France). DNA–LNA mix-mers containing terminal LNA and internal α-L-*ribo*-configured LNA (α-L-LNA) monomers were prepared by the phosphoramidite approach as already described (25,26). After deprotection and cleavage from the solid support, DNA–LNA–α-L-LNA oligomers were purified by DMT-on reversed phase chromatography on disposable purification cartridges which includes detritylation. The composition was confirmed by MALDI-MS analysis and the purity (>90%) by capillary gel electrophoresis. Isosequential phosphodiester oligonucleotides, used as controls, were purchased from Sigma Genosys (Cambridge, UK) as HPLC-purified products.

All oligomers were quantitated based on spectrophotometric A_{260} and the conversion factor of 30.4 μg/ml OD_{260} (sense strand) and 32.1 μg/ml OD_{260} (antisense strand) as indicated for the corresponding phosphodiester oligonucleotides by the manufacturer. Upper and reverse complement strands were hybridized in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) to obtain stock solutions of double-stranded decoy molecules at a final concentration of 40 μM. Annealing was performed in a thermocycler according to the following temperature profile: 5 min at 100°C, followed by a temperature reduction to 37°C over 60 min and from 37 to 4°C over 30 min.

Cell stimulation and nuclear extract preparation

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO_2 incubator. Cells were plated at a density of 5×10^5 cells/60 mm diameter culture dish. The day after, cells were stimulated with 1 ng/ml tumor necrosis factor-α (TNF-α) (Boeringher Mannheim Biochemia, Mannheim, Germany) for 1 h at 37°C. After stimulation, cells were washed and harvested with cold phosphate-buffered saline. Nuclear proteins were obtained by low salt/detergent cell lysis followed by high salt extraction of nuclei as previously described (21).

Gel shift competition experiments

Nuclear extracts (0.4 mg/ml) from TNF-α-stimulated HeLa cells were preincubated with 0.2 mg/ml double-stranded non-specific DNA competitor poly(dI·dC) (Amersham Pharmacia Biotech, Piscataway, NJ) for 10 min on ice in binding buffer (20 mM HEPES–KOH, pH 7.9, 0.1 M KCl, 5% v/v glycerol, 0.2 mM EGTA, 0.2 mM EDTA, 1 mM dithiothreitol). Aliquots of the mixture corresponding to 6 μg of nuclear extract were transferred to separate tubes and incubated with increasing concentrations of competitor ODN, either a phosphodiester or a LNA-modified duplex as specified. To this end, stock solutions (40 μM) of double-stranded decoy molecules were serially diluted 1:1.5 in TE buffer and added

to the corresponding tube in order to adjust the concentration of the competitor ODN in the incubation mixture to the final concentration indicated in the figure legends. After incubation for 20 min on ice, a ^{32}P-end-labeled DNA probe (PRDII), corresponding to two copies of the NF-κB binding sequence found in the PRDII domain of the interferon-β (IFN-β) promoter, was added to each tube to a final concentration of 2.5 nM and the incubation was continued for an additional 20 min. Reaction mixtures were then submitted to electrophoretic separation on 5% native polyacrylamide gels (29:1 crosslinked) in Tris–glycine buffer (25 mM Tris base, 192 mM glycine). NF-κB/[^{32}P]PRDII complexes were detected and quantified by exposing the dried gel in a Molecular Imager (Bio-Rad Laboratories, Milan, Italy). All values were fitted to a sigmoidal dose–response equation using Origin 6.0 (Microcal Software) to estimate IC_{50} values.

Serum stability assay

Phosphodiester and LNA-modified oligonucleotides were incubated at 37°C in fresh human serum obtained from blood samples of healthy volunteers. Aliquots of the mixture corresponding to 0.75 μg double-stranded ODN were removed at different incubation times as indicated. Reactions were terminated by addition of EDTA and EGTA to final concentrations of 10 and 5 mM, respectively. Samples were then immediately frozen in dry ice and stored at –80°C. Full-length oligomers were separated from digested ODNs on a non-denaturing 2.5% (w/v) agarose gel. Bands were visualized by ethidium bromide staining using a Gel Doc 1000 apparatus. Densitometric calculations were done with Molecular Analyst imaging software (Bio-Rad Laboratories). The volume density of the band corresponding to full-length oligomers was calculated in each lane with correction for background. Average half-lives and standard deviations from at least three independent experiments for each oligonucleotide were obtained by fitting the data to a single exponential decay function (Origin 6.0; Microcal Software).

RESULTS

Design of the decoy molecules

In a previous study we designed a panel of decoy oligonucleotides modified to various extents and at various positions with LNA bases (21). These oligonucleotides consisted of two κB sites, corresponding to the κB sequence contained in the PRDII domain of the human IFN-β promoter (Fig. 1, PRDII), capped with unrelated extra sequences of 5 nt at both termini (Fig. 1, D+D). Our results demonstrate that insertion of at least two terminal LNA monomers, outside the κB motifs (Fig. 1, B+B), was sufficient to confer appreciable protection against nuclease digestion, without interfering with transcription factor binding (21). However, when LNA substitutions were also positioned within the κB sites, by modifying nucleotides in both strands (Fig. 3, C+C), further increased stability was observed, although a considerable reduction in NF-κB binding affinity was found (21). Interestingly, when considering LNA substitutions in position C separately, the reduction in the NF-κB binding affinity was more pronounced when the LNAs were in the antisense strand (Fig. 1, B+C) rather than in the sense strand (Fig. 1, C+B) (21). From a molecular point of view this effect could be explained by the fact that LNA nucleotides perturb the sugar puckers of the flanking unmodified nucleotides, predominantly in the 3′ direction, from a preferential S-type pucker in DNA duplexes to a mixture of N-type and S-type conformations (27,28). The result is a local rearrangement of the phosphate backbone geometry toward the A-type helix. On the other hand, it is well known that NF-κB recognizes the decameric consensus sequence 5′-GGGRNNYYCC-3′ (N, any nucleotide; R, purine; Y, pyrimidine), where the transcription factor makes base-specific contacts mainly with the conserved G:C pairs at the ends of the site, lying in the major groove (Fig. 1, nucleotides in boxes). Thus, the core of the site, in the minor groove, usually remains open. Only in the case of PRDII do the High Mobility Group (HMG) I(Y) proteins specifically recognize and bind the central AT-rich sequence (29,30). As a consequence, we hypothesized that the differences observed in NF-κB binding affinity for the C+B and B+C molecules could be due to the fact that the conformational modifications introduced by the LNAs in the sense strand (C+B) are mainly propagated to the AT-tract. Conversely, those introduced by the LNAs in antisense (B+C) presumably involve the GC-rich sequence, which is known to be particularly critical for interactions with NF-κB. Therefore, we reasoned that LNA decoys which are more efficiently recognized by NF-κB could be designed by a more convenient positioning of the internal LNA substitutions.

To test this hypothesis, nucleotides in positions C1 and C2 of the sense strand and nucleotides in positions C2 and C3 of the antisense strand were replaced with LNA monomers to obtain the decoy molecules shown in Figures 1 and 3. These positions were chosen in order to preferentially restrict the conformational modifications, introduced by the LNA in the 3′ direction, to the nucleotides of the central AT-rich segment in order to limit to the minor groove any potential structural alteration of the DNA geometry. In fact, it is worth noting that LNA substitutions in positions C2 and C1 of the sense strand and in positions C2 and C3 of the antisense strand are spaced from the first G:C base pair in the 3′ direction by two and three unmodified nucleotides, respectively.

Insertion of LNAs in the κB site differentially affects NF-κB binding affinity depending on their intra-strand positioning

In order to establish whether or not a relationship exists between LNA intra-strand positioning and NF-κB binding affinity, the ability of NF-κB to bind ODN decoys containing internal LNA substitutions in only one strand was evaluated by electrophoretic mobility shift assay (EMSA) (Fig. 1). Because LNA ODNs are not efficiently phosphorylated on the 5′ end (21,31), they could not be used in direct binding assays where high specific activity of the probe is required. For this reason, NF-κB binding to the different LNA decoys was indirectly assessed by means of gel shift competition experiments from which the concentration of each LNA-modified molecule required to displace 50% of the non-modified ^{32}P-labeled probe (IC_{50} value) was determined. Indeed, on the assumption that the competition efficiency is proportional to the binding affinity, the IC_{50} value provides a good measure of the relative affinity of NF-κB for the different LNA ODNs tested with respect to the isosequential unmodified molecule. This type of

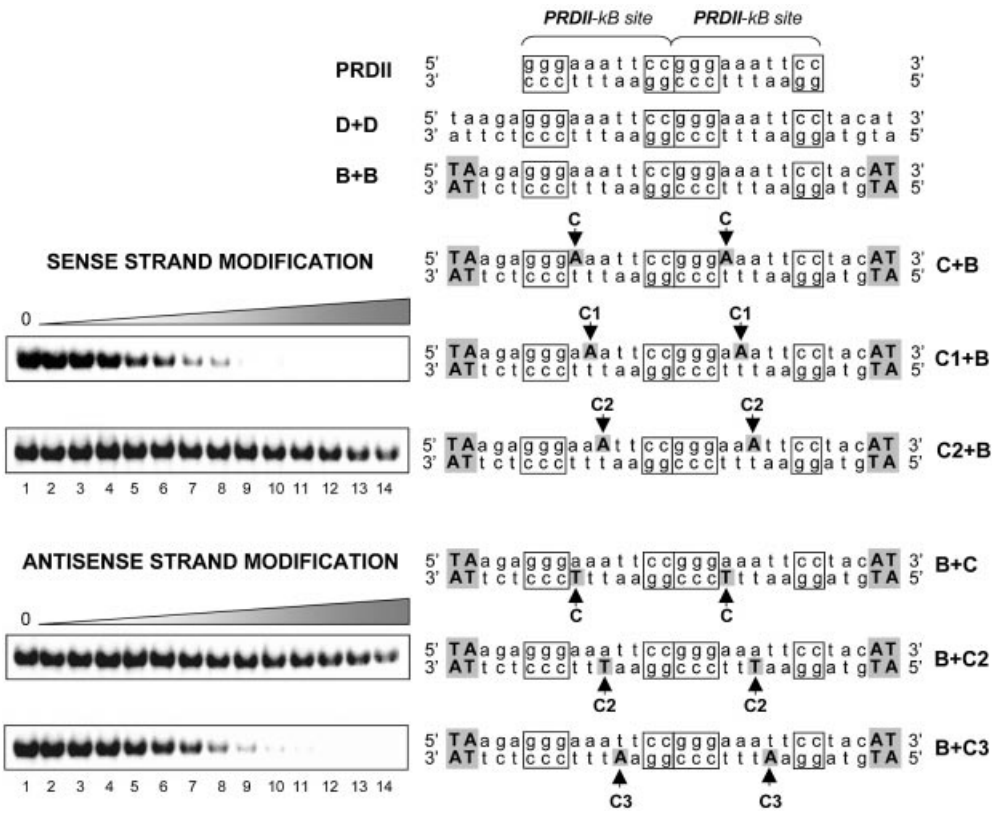

Figure 1. Design of the ODNs containing internal LNA substitutions only in one strand and competition of these molecules for binding of NF-κB to the radiolabeled PRDII probe. Oligonucleotides were derived from an ODN of 30 bp, consisting of two PRDII κB sites in tandem (10 bp each, shown in braces), extended at the terminal ends with unrelated extra sequences of 5 nt (D+D, 30 bp) and modified by replacing the two terminal nucleotides with LNA monomers on both strands (B+B). Lower case, DNA monomers; bold upper case in grey, LNA bases. Besides the LNA end-block, additional LNA substitutions were introduced in the κB sequences at different positions other than C, tested in a previous study, of the sense (C1, C2) or of the antisense (C2, C3) strand, as indicated by the arrows. In replacing internal nucleotides with LNAs, those which establish base-specific contacts with NF-κB (in boxes) have been excluded. The ability of NF-κB to bind these molecules was assessed by gel shift competition experiments as described in Materials and Methods. Only the protein/probe complex is shown. Competitor concentrations in lanes 2–14 were 2.2, 3.3, 5.0, 7.5, 11.3, 16.9, 25.3, 38, 57, 85.8, 128.7, 193 and 289.6 nM. Lanes 1 represent 100% binding of NF-κB to the radiolabeled probe, as obtained by excluding the competitor from the incubation mixture.

correlation also implies that as the IC_{50} value decreases, NF-κB binding affinity increases and vice versa. Therefore, this approach was used to quantify the effects produced by the insertion of LNA bases in the PRDII κB motif on the NF-κB/DNA interactions, which we intended to investigate in these experiments. Binding assays were performed by incubating crude nuclear extracts from TNF-α-stimulated HeLa cells with increasing concentrations of unlabeled LNA ODNs together with a fixed amount of the [^{32}P]PRDII probe. The concentration of the radiolabeled DNA used in the competition assays was first determined in bandshift titration experiments to make sure that the amount of probe exceeded that of NF-κB present in the incubation mixture (data not shown). A single protein/DNA complex was detected (Fig. 1, lanes 1). Specificity of complex formation was demonstrated by the complete disappearance of the radiographic signal upon addition of a 60 molar excess of a DNA molecule encompassing the κB sequence contained in the enhancer of the immunoglobulin light chain gene (Ig-κB) (data not shown). The same result was not obtained when a mutagenized Ig-κB DNA ODN was used as competitor, confirming that the complex was specific for the NF-κB site (data not shown). The results of the competition experiments are presented in Figure 1, which shows a progressive reduction in the radiographic signal corresponding to the NF-κB/[^{32}P]PRDII complex upon addition of the indicated LNA ODN competitors. Unexpectedly, this reduction was significantly faster for the C1+B and B+C3 molecules with respect to C2+B and B+C2 (Fig. 1), indicating that insertion of LNA monomers in position C2 of the κB site caused a drastic decrease in the capacity of the molecule to act as competitor. Indeed, at the concentrations at which C1+B and B+C3 induced complete disappearance of the radiographic signal, C2+B and B+C2 were still unable to efficiently remove NF-κB from binding with the radiolabeled PRDII DNA (Fig. 1, compare lanes 11–14). In agreement with these observations, the calculated IC_{50} values of C2+B (197.4 nM) and B+C2 (209.7 nM) were 20- and 15-fold higher than those of C1+B (9.67 nM) and B+C3 (13.78 nM), respectively (Table 1). In contrast, the NF-κB binding activity was significantly improved when LNAs in position C were transferred to position C1 in the sense strand, as determined by a decrease of 1.8-fold of the IC_{50} value of C1+B in comparison with that calculated for C+B, under the same experimental conditions (Table 1). Similar results were obtained when LNAs of the antisense strand were moved from position C to position C3, with a 2.2-fold decrease in the IC_{50}

Table 1. Estimated IC_{50} values and half-lives ($t_{1/2}$) of decoy molecules modified to various extent and at various positions with LNA and α-L-LNA in comparison to the unmodified isosequential DNA ODN

ODN name	Modification (type and position)	IC_{50} (nM) ± SE	IC_{50} (relative)	$t_{1/2}$ (h) ± SD	$t_{1/2}$ (relative)
PRDII derived					
D+D	None	10.56 ± 0.63	1.0	13.4 ± 0.8	1.0
B+B[a]	LNA end-block	8.30 ± 1.88	0.8	20.9 ± 1.6	1.5
C+B[a]	LNA end-block + LNA in S	17.68 ± 1.34	1.7	nd	nd
C1+B		9.67 ± 1.80	0.9	nd	nd
C2+B		197.40 ± 25.3	18.7	nd	nd
B+C[a]	LNA end-block+LNA in AS	30.75 ± 3.59	2.9	nd	nd
B+C2		209.70 ± 21.6	19.8	nd	nd
B+C3		13.78 ± 0.76	1.3	nd	nd
Igk derived					
C+B	LNA end-block+LNA in S	8.76 ± 0.64	nd	nd	nd
C2+B		134.80 ± 11.4	nd	nd	nd
PRDII derived					
C+C[a]	LNA end-block +LNA in S+AS	169.90 ± 17.3	16.1	39.5 ± 2.8	2.9
C2+C2		3652.00 ± 493	345.8	34.8 ± 6.6	2.6
C1+C3		93.97 ± 9.86	8.9	19.2 ± 2.2	1.4
C+C2		899.70 ± 113.6	85.2	24.3 ± 2.7	1.8
C+C3		98.64 ± 9.32	9.34	12.5 ± 4.7	0.9
C*+C*	LNA end-block+α-L-LNA in S+AS	24.83 ± 1.88	2.35	40.6 ± 5.8	3.0
C2*+C2*		219.70 ± 29.57	20.8	48.2 ± 18.5	3.6

S, sense strand; AS, antisense strand; relative, relative values normalized to the unmodified DNA; nd, not determined. The half-lives of ODNs containing, besides the end-block, internal LNAs only in one strand have not been determined because, as we have demonstrated in our previous paper (21), a single LNA substitution does not confer higher stability than that obtained with the simple end-block (B+B).
[a]These oligonucleotides have been tested in a previous study (21).

value of B+C3 in comparison with that of B+C (Table 1). Taken together these results demonstrate that NF-κB binding affinity can be fine tuned by appropriate substitution of the nucleotides in the κB sequence taking into account that the degree of interference related to LNA positioning decreased as follows: C2 > C > C1 in the sense strand; C2 > C > C3 in the antisense strand (Table 1).

Insertion of LNAs in position C2 directly affects interactions between NF-κB and its binding sequence

Since in the PRDII κB site nucleotides of the central AT-tract bind HMG I(Y) proteins (32,33), the next step was to evaluate whether the crucial role played by the LNAs in position C2 was an HMG I(Y)-dependent effect. To this end, we designed oligonucleotide decoys in which both the PRDII κB motifs were replaced with the κB consensus sequence (Ig-κB) present in the enhancer of the immunoglobulin light chain gene (Igk) (Fig. 2). The two κB sequences essentially differ in the identity of two of the variable inner base pairs: in the PRDII κB site these are 5′-A<u>AA</u>TT-3′, while in the Ig-κB site they are 5′-A<u>CT</u>TT-3′. Thus, the latter does not bind HMG I(Y) proteins (33). Interestingly, when nucleotides in position C and C2 (sense strand) of the Ig-κB sites were substituted with LNAs, a marked difference in the competitive action of the C+B and C2+B ODNs in gel shift experiments was again found (Fig. 2). In fact, if compared to the C+B ODN, the efficiency of C2+B in competing with the [^{32}P]PRDII probe for NF-κB binding was markedly lower, as demonstrated by an IC_{50} value of C2+B (134.8 nM) 15-fold higher than that of C+B (8.76 nM) (Table 1). These results demonstrate that the LNAs in C2 remain critical in the context of other κB sites, suggesting that replacement of nucleotides in position C2 with LNAs directly affects interactions between NF-κB and its consensus binding sequence.

The intra- and inter-strand positioning of LNA substitutions have additive effects on NF-κB binding

Insertion of LNA substitutions on both strands is expected to have more pronounced effects on the geometry of the DNA duplex than substitutions on a single strand since the total number of LNA bases introduced in the molecule increases. In addition, the LNA inter-strand positioning could also confer different degrees of interference according to whether LNA bases occupy complementary or non-complementary positions and how these latter are spaced along the sequence. For these reasons, the ability of NF-κB to bind molecules containing internal LNAs on both strands and at different positions was evaluated as well. Again, NF-κB displayed a markedly lower affinity for oligonucleotides containing LNAs in position C2 when compared to the others (Fig. 3, compare C2+C2 and C+C2 with C1+C3 and C+C3). Indeed, IC_{50} values of these molecules were shifted to the micromolar concentration range (Fig. 4 and Table 1). In contrast, estimated IC_{50} values of C1+C3 and C+C3 molecules were 93.97 and 98.64 nM, respectively (Fig. 4 and Table 1). These values were not significantly different, indicating that transfer of the LNAs from position C to C1 of the sense strand had no effects on NF-κB binding. According to the above established intra-strand rules, this result is unexpected, unless a contribution of inter-strand positioning is invoked. Although in terms of intra-strand positioning substitutions in C (sense strand) interfere with NF-κB binding more than those in C1 (sense strand), C is separated from C3 by two unmodified nucleotides in C+C3, while C1 is separated from C3 by only one unmodified nucleotide in C1+C3 (Fig. 3). A significantly increased affinity for NF-κB was observed only when LNAs in position C of the antisense strand were moved to C3. This is demonstrated by a 1.7-fold decrease in the IC_{50} value of C+C3 in comparison with that calculated for the previously tested C+C molecule

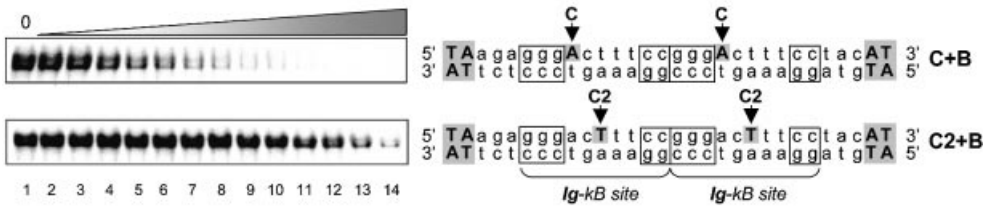

Figure 2. Gel shift competition experiments with LNA ODNs in which the PRDII κB site has been replaced with the Ig-κB site. LNA ODNs were designed by replacing the internal PRDII κB sites with the κB motif found in the enhancer of the immunoglobulin light chain gene (Ig-κB, in braces), while the extra sequences having the LNA end-block were maintained unchanged. Nucleotides of the Ig-κB sequences on the sense strand which occupy the positions corresponding to C or C2 on the sense strand of the PRDII κB motifs were replaced with LNAs (bold upper case in grey). The ability of NF-κB to bind these molecules was tested in gel shift competition experiments as described in Materials and Methods. Only the protein/probe complex is shown. Competitor concentrations in lanes 2–14 were 2.2, 3.3, 5.0, 7.5, 11.3, 16.9, 25.3, 38, 57, 85.8, 128.7, 193 and 289.6 nM. In lanes 1, 100% binding of NF-κB to the radiolabeled probe is shown, as determined by exclusion of the competitor from the incubation mixture.

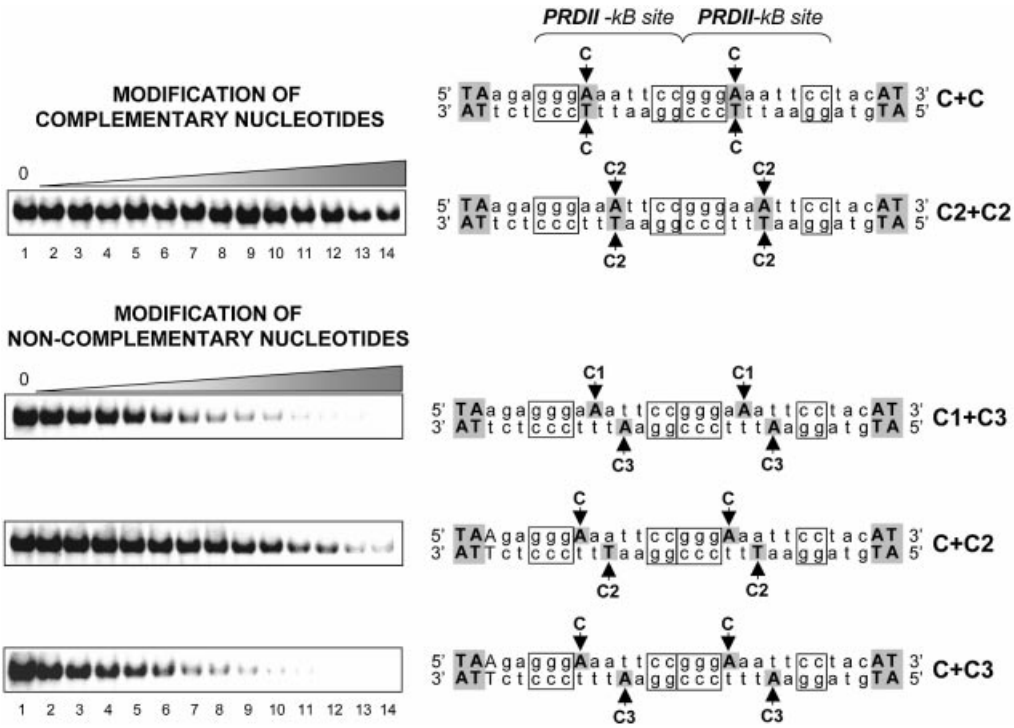

Figure 3. Design of the ODNs containing internal LNA substitutions on both strands and competition of these molecules for binding of NF-κB to the radiolabeled PRDII probe. LNA ODNs with internal substitutions on both strands were obtained by annealing oligomers containing LNAs in position C, C1, C2 of the sense strand with oligonucleotides containing LNAs in position C, C2, C3 of the antisense strand to generate the double-stranded molecules shown in the figure. LNA substitutions involved either complementary (C+C and C2+C2) or non-complementary (C1+C3, C+C2 and C+C3) nucleobases. Lower case, DNA monomers; bold upper case in grey, LNA bases. The ability of NF-κB to bind these molecules was tested in gel shift competition experiments as described in Materials and Methods. Only the protein/probe complex is shown. Competitor concentrations in lanes 2–14 were 38, 57, 85.8, 128.7, 193, 289.6, 434.4, 651.5, 977, 1466, 2200, 3300 and 4950 nM. In lanes 1, 100% binding of NF-κB to the radiolabeled probe is shown as determined by exclusion of the competitor from the incubation mixture.

(Table 1). In this case also a cooperative role of the inter- and intra-strand positioning might contribute to increase the affinity of NF-κB for C+C3 with respect to C+C, with the LNA substitutions in this latter case being positioned on complementary nucleotides (Fig. 3). C+C3 and C1+C3 were the molecules with internal LNA substitutions on both strands with the best NF-κB affinities of all the decoys tested (Fig. 4 and Table 1).

Increased resistance to nuclease degradation depends on the inter-strand positioning of the LNA substitutions in the κB site

Since the ideal decoy molecule should not only be efficiently recognized by the target transcription factor but also stable in biological media, the susceptibility to nuclease degradation of the LNA decoys was evaluated by incubating these molecules

in human serum. Based on our previous observation that introduction of additional internal LNAs in only one strand is not sufficient to confer a higher stability with respect to that obtained with a simple LNA end-block (21), only molecules where internal LNA substitutions were placed in both strands have been tested in these experiments. Aliquots of the mixture were taken at the indicated time points and analyzed by electrophoresis on non-denaturing agarose gels followed by ethidium bromide staining (results of a typical experiment are shown in Fig. 5). Disappearance of the double-stranded decoy molecule was considered proof of degradation by serum nucleases. The area of the uncleaved fraction was densitometrically evaluated and plotted as a function of the incubation time. Figure 5 shows the results for the ODNs containing, other than the LNA end-block, additional internal LNA substitutions in both strands in different positions, involving either complementary (Fig. 3, C+C and C2+C2) or non-complementary (Fig. 3, C1+C3, C+C2 and C+C3) nucleotides, in comparison with the results obtained for end-blocked ODN B+B (Fig. 1) as well as the reference DNA molecule D+D (Fig. 1). Due to the higher resistance to nuclease degradation of double-stranded DNA with respect to single-stranded species, more than 44 h incubation in serum were required to achieve almost complete degradation of the unmodified oligonucleotide D+D (Fig. 5). At the same time point 30 and 50% of the initial fractions of B+B and C+C, respectively, were still intact (Fig. 5). Rates of degradation similar to those of C+C were obtained for C2+C2, which contained complementary LNA substitutions as well (Fig. 5). In agreement with our previous observations, the LNA end-block increased the half-life of the decoy in serum from 13.4 to 20.9 h, while insertion of additional LNA substitutions in the κB sites conferred a significant further degree of stabilization as demonstrated by half-lives of C+C and C2+C2 of 39.5 and 34.8 h, respectively (Table 1). Surprisingly, molecules containing, in addition to the LNA end-block, internal LNA substitutions in non-complementary

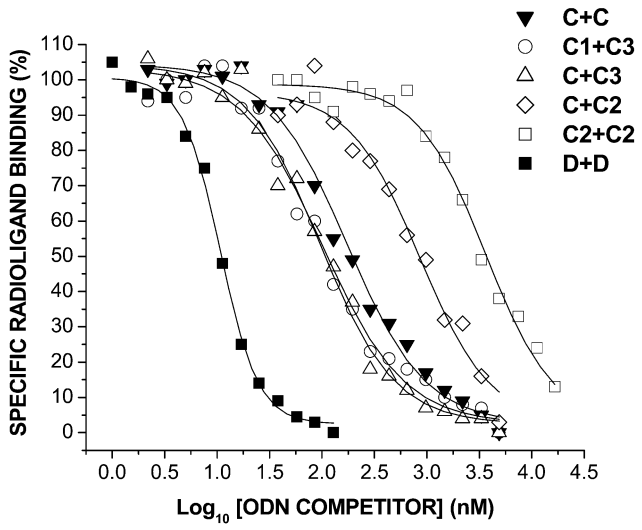

Figure 4. Determination of the IC_{50} values. IC_{50} values of the LNA-modified ODNs and of the phophodiester ODN D+D (control) were estimated by gel shift competition experiments in a suitable concentration range for each molecule. Quantitative evaluation of NF-κB/[^{32}P]PRDII complex formation in the presence of increasing concentrations of each ODN competitor was performed in a molecular imager. Data were expressed as percent binding relative to that determined in the absence of the competitor. IC_{50} values were calculated by plotting these data as a function of $\log_{10}$ of the competitor concentration (nM) and fitting to a dose–response curve. Curve fitting was performed with Origin 6.0 Microcal Software. The 50% inhibitory concentrations (nM) ± SE are reported in Table 1.

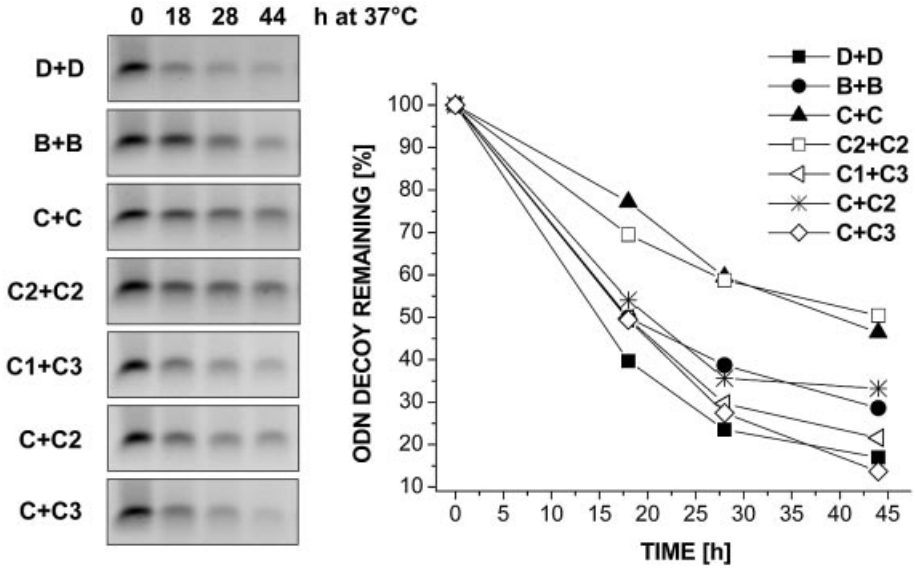

Figure 5. Stability of LNA-modified oligonucleotides in human serum. LNA-modified (B+B, C+C, C2+C2, C1+C3, C+C2, C+C3) and control phosphodiester (D+D) decoy molecules were incubated in human serum at 37°C. Aliquots were taken at the time points indicated and then submitted to non-denaturing electrophoretic separation on 2.5% (w/v) agarose gels (a typical experiment is shown). Detection and quantification of the ethidium bromide stained bands corresponding to full-length oligomers were performed in a Gel Doc 1000 apparatus equipped with Molecular Analyst software. The volume density for the time 0 sample was set as the reference value for each incubation. Relative values for the other time point samples in the corresponding incubation were calculated on the basis of this reference value and plotted as a function of incubation time.

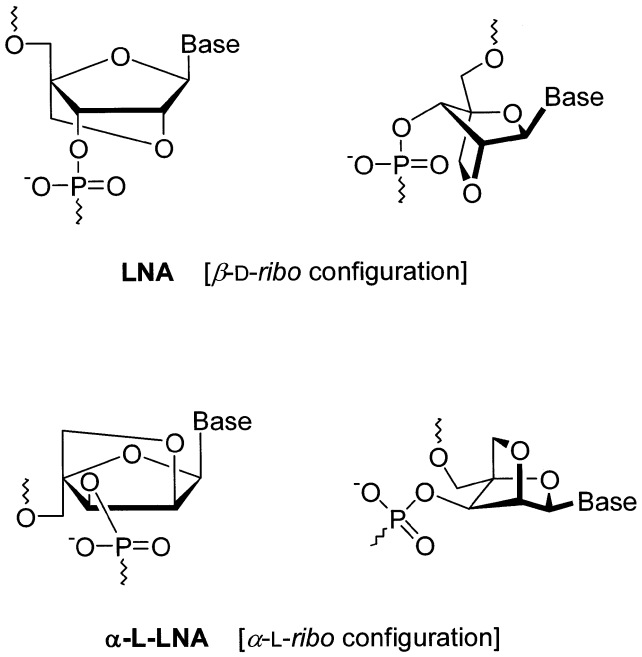

Figure 6. Structures of the nucleotide monomers of LNA and α-L-LNA and sketches of their locked N-type furanose conformations.

positions had half-lives not significantly different (C1+C3, C+C2) or even lower (C+C3) than that of the end-blocked molecule (B+B) (Table 1). These findings demonstrate that in a DNA duplex non-pairing LNA bases are not protective (C1+C3, C+C2), even becoming destabilizing as the space between the LNA bases increases (C+C3). Therefore, it can be concluded that inter-strand positioning of the internal LNA substitutions is a critical parameter for ODN stability, requiring the replacement of complementary nucleotides with LNAs as *conditio sine qua non* biostability cannot be further improved.

Insertion of α-L-LNA monomers in the κB site increases NF-κB binding affinity although the intra-strand positioning effect remains

Recently, a diastereoisomeric form of LNA, α-L-LNA (α-L-*ribo*-configured LNA) has been introduced (26). Both LNA and α-L-LNA monomers are efficiently locked in an N-type conformation (Fig. 6). However, when hybridized to complementary DNA, the LNA has been recognized as an RNA mimic that drives the duplex towards an A-type helix. In contrast, NMR and CD spectroscopic studies of α-L-LNA–DNA duplexes have shown that the local native B-like double-stranded DNA structure is preserved. These results point to α-L-LNA being better described as a DNA mimic (34,35). Based on this evidence, we hypothesized that substitution of nucleotides in the κB sites with this LNA diastereoisomer could strongly limit interference with transcription factor binding. For this purpose we have designed two decoy ODNs in which the LNA end-block was maintained, while nucleotides in the κB site positions C and C2 were replaced with α-L-LNA (Fig. 7, C*+C* and C2*+C2*). The efficiency of binding of NF-κB to these molecules was assayed in gel shift competition experiments (Fig. 7 shows a typical experiment)

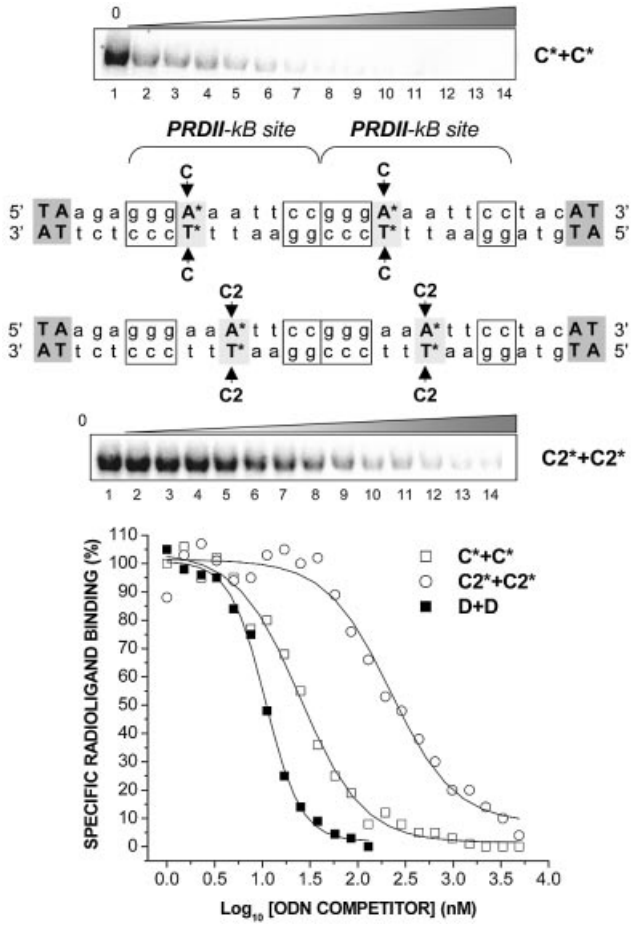

Figure 7. LNA end-blocked ODN decoys containing internal α-L-LNA substitutions: design and estimation of the IC_{50} values. C*+C* and C2*+C2* ODN decoys, in which the internal LNA monomers present in the C+C and C2+C2 molecules, respectively, were replaced with α-L-LNA nucleotides (bold upper case letters with asterisk in light grey) were synthesized and tested in gel shift competition experiments. Only the protein/probe complex is shown. Competitor concentrations in lanes 2–14 were 38, 57, 85.8, 128.7, 193, 289.6, 434.4, 651.5, 977, 1466, 2200, 3300 and 4950 nM. In lanes 1, 100% binding of NF-κB to the radiolabeled probe is shown. Lower case, DNA monomers; bold upper case in grey, LNA bases. IC_{50} values of these molecules were determined by assaying a broader range of concentrations with respect to those shown in the figure. Data obtained were plotted as a function of decoy ODN concentration (log_{10} [nM]) and fitted to a dose–response function (graph on the bottom). The 50% inhibitory concentrations (nM) ± SE are reported in Table 1. In the graph, the unmodified D+D ODN shown in Figure 4 is reported again to allow direct comparison.

and the data obtained were fitted to a sigmoidal dose–response curve in order to determine their respective IC_{50} values. The results obtained clearly demonstrate that insertion of α-L-LNAs in position C of the κB sites drastically reduces interference with transcription factor binding, as indicated by an IC_{50} of 24.83 nM, which is 7-fold lower than the IC_{50} value of the corresponding LNA-modified C+C molecule and only 2-fold higher than the IC_{50} value of the reference DNA molecule D+D (Table 1). Conversely, substitution of the nucleotides in position C2 with α-L-LNA monomers resulted in an IC_{50} value of 219.7 nM, indicating that this nucleotide is crucial and that an intra-strand positioning effect exists for the α-L-LNA as well.

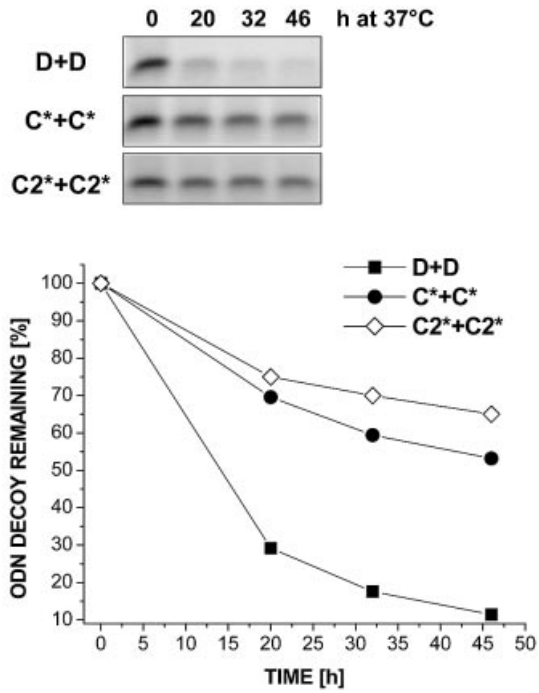

Figure 8. Stability of α-L-LNA-based decoy ODNs in human serum. C*+C* and C2*+C2* decoy ODNs containing internal α-L-LNA substitutions were incubated in human serum at 37°C. The control D+D molecule was analyzed in parallel for comparison. Aliquots of the mixtures were taken at the time points indicated and then submitted to non-denaturing electrophoretic separation on 2.5% (w/v) agarose gels (a typical experiment is shown). Detection and quantification of the ethidium bromide stained bands corresponding to full-length oligomers were performed in a Gel Doc 1000 apparatus equipped with Molecular Analyst software. The volume density for the time 0 sample was set as reference value for each incubation. Relative values for the other time point samples in the corresponding incubation were calculated on the basis of this reference value and plotted as a function of incubation time.

α-L-LNA-modified ODNs are resistant to nuclease degradation

Having found a decoy with optimal binding affinity, we wanted to investigate if insertion of α-L-LNA monomers in the decoy resulted in an increased stability to nuclease degradation, as previously demonstrated for LNA substitutions. To this end, C*+C* and C2*+C2* ODNs were incubated in human serum and their susceptibilities to nuclease action were compared, under the same experimental conditions, to that of the unmodified D+D molecule. As shown in Figure 8, after 46 h incubation 50% of the initial amount of C*+C* and 65% of C2*+C2* was still uncleaved, while only 10% of the full-length DNA molecule D+D was left (Fig. 8). Consistent with these observations, the half-lives of these molecules were 3- and 3.6-fold higher than that of the isosequential DNA ODN D+D (Table 1). Interestingly, C*+C* and C2*+C2* displayed half-lives of 40.6 and 48.2 h, respectively, which were very similar to that of the corresponding LNA-modified molecules C+C and C2+C2 (Table 1), demonstrating that replacement of internal LNAs with α-L-LNAs guaranteed the same degree of protection against nuclease digestion.

DISCUSSION

The use of synthetic ODNs as drugs is a relatively new concept in the field of pharmacology. Indeed, several studies involving oligonucleotide-based therapeutics are already in the preclinical or clinical stages of development. The key objective in the field, however, remains the identification of oligonucleotide analogs providing high *in vivo* efficacy. When pursuing structural modifications to improve the pharmacological properties of a DNA molecule, two main goals should be achieved: increased resistance to nuclease degradation and high affinity for the target. These requirements are difficult to reconcile in a decoy ODN, where the ideal modification should, on the one hand, prevent nuclease degradation and, on the other, preserve the molecular interactions with the target transcription factor, in order to retain both the affinity and the specificity of recognition. Regarding decoy strategies as gene therapy, one of the major concerns is non-specific effects of fully phosphorothioate-substituted ODNs, which have been widely used in basic research (8,36). Thus, by analogy with antisense molecules, decoy ODNs where only terminal phosphodiester internucleoside linkages are replaced by phosphorothioate linkages have been proposed because of their decreased propensity for non-specific protein binding (18). Nevertheless, these molecules may still cause toxicity (37). In addition, terminal phosphorothioation is only expected to protect ODNs from exonuclease degradation, while having little effect on endonuclease activity, which plays an important role in ODN metabolism (38,39). Attempts to overcome some of the drawbacks associated with ODN phosphorothioation, i.e. non-specific binding and toxicity, have recently involved the use of circular dumb-bell DNA molecules because of their proven resistance to exonucleases (40). These ODNs are constructed by the circularization of the 3′ and 5′ ends of the oligonucleotides and enzymatic ligation. However, further advances in this direction will certainly require the use of more sophisticated DNA chemical modifications, which have the potential to provide a more exhaustive approach to the problem. In this view, PNA- and LNA-based double-stranded oligonucleotides are currently under investigation as alternative decoy agents. With respect to phosphorothioate, LNA-modified oligonucleotides have clearly been proved to be more resistant to nuclease attack and less toxic and, therefore, more suitable in the design of decoy molecules (41). Terminal modification with LNA as well as PNA bases is indeed sufficient to make ODN decoys more resistant to exonuclease and, to a certain extent, endonuclease degradation (21,42). This latter effect is thought to be due to the propagation to the unmodified internal DNA fragment of the structural modifications introduced at the terminal ends, since both LNA and PNA drive the duplex to adopt a conformation which differs from the standard DNA/DNA B-type geometry (27,43). In general, the magnitude of stabilization increases as the number of terminal modifications increases. However, depending on the extent of the modifications introduced, a parallel decrease in the ability of the transcription factor to recognize its target sequence is observed. In this regard, PNA gap-mers have been shown to be resistant to both exo- and endonuclease degradation only when modifications involve both terminal ends of the duplex (42). Unfortunately, these molecules are inefficient as decoys

against the transcription factor Sp1 (20). In contrast, when PNA end-blocks are positioned on only one strand, resistance to DNase I cleavage was only slightly improved (42), although these molecules were equally efficient as DNA/DNA ODNs in inhibiting Sp1 activation (20). These results highlight the fact that the percentage of nucleotide analogs introduced in a sequence by the end-capping strategy can be critical for transcription factor binding to the central consensus sequence. To address these concerns, the possibility of directly protecting the internal DNA segment by inserting additional LNA bases in the binding site has been investigated here. Based on our previous observation that introduction of LNA bases in the κB site further increased ODN stability, although causing a loss of NF-κB binding affinity (21), a comprehensive analysis of the LNA intra- and inter-strand positioning effect on ODN biostability and affinity of the target transcription factor has been performed here to address the problem of reconciling these two different aspects. The results presented in this paper demonstrate that a strict correlation between positioning of the internal LNA substitutions and NF-κB binding affinity exists. Introduction of LNA bases at different positions in the κB site indeed resulted in a different degree of perturbation in the interactions between the transcription factor and its modified target sequence. Nevertheless, by combining the most appropriate intra- and inter-strand LNA positionings, the IC_{50} of the resulting molecules C1+C3 and C+C3 was still 9-fold higher than that calculated for the reference DNA ODN, indicating that NF-κB affinity could not be further improved (Table 1). Indeed, when LNA nucleotides are incorporated several times in duplexes, as in our case, a cooperative effect of the local conformational changes introduced by each substitution has been observed (27). On the whole, the result is a change in the overall duplex conformation towards an A-type geometry, which is obviously not optimal to sustain interactions between NF-κB and LNA–DNA mix-mers. In addition to the correct geometry of the duplex, sufficient flexibility of the DNA molecule is necessary to support the conformational changes required for NF-κB binding. Indeed, co-crystal structures of NF-κB bound to different κB sites have shown that NF-κB induces DNA bending (33). Of particular relevance is the fact that in PRDII, the AT-tract appears to be the primary determinant of DNA bending, although these central bases make minimal contacts with the transcription factor (32). This is analogous to other cases in which non-contacted base pairs can influence protein binding affinity (44). Since LNAs are locked in the 3′-*endo* conformation they do not possess flexibility. Thus, it cannot be excluded that flexibility of the molecule could also be compromised when central nucleotides are replaced with LNAs.

In comparison with the LNA monomer, its diastereoisomeric form, α-L-LNA, has the advantage of leaving the unmodified part of the molecule unperturbed when it is incorporated into a DNA duplex (35). The result is that the native overall B-type structure is retained so that conformational perturbations are excluded. Indeed, insertion of α-L-LNAs in position C of the κB site permitted almost complete reversal of the adverse effect of LNAs on NF-κB binding. Considering that α-L-LNAs act as a B-type mimic, it can be concluded that perturbations of the interactions between NF-κB and its binding sequence in the C+C molecule mainly occurred at the conformational level, being counteracted by the replacement of internal LNAs with α-L-LNAs.

Unexpectedly, the same conclusions cannot be drawn for position C2. Indeed, insertion of LNAs in C2 strongly perturbed NF-κB binding affinity. At present, the reason why the nucleotide in position C2 is more critical than the nucleotide in position C when replaced with an LNA nucleotide is not clear, for several reasons. Firstly, this nucleotide does not establish base-specific contacts with the transcription factor in any of the NF-κB/DNA crystal structures resolved to date (30,45). Secondly, we have shown that the nucleotide remains critical in the context of the Ig-κB site, which is known not to bind HMG I(Y) proteins, excluding the possibility that the loss of affinity for NF-κB was indirectly produced by perturbation of the HMG I(Y)/DNA interactions. Thirdly, from a theoretical point of view, the conformational changes introduced by the LNA in position C2 on the global duplex geometry should be comparable to those induced by the LNAs in other positions. In contrast to what has been observed for the nucleotide in position C, replacement of internal LNAs in position C2 with α-L-LNAs only partially improved the ability of NF-κB to bind the modified κB sequence, suggesting that other properties of the DNA molecule which do not only concern conformation are affected by LNAs at C2.

In this paper we also report that while the intra- and inter-strand positioning of LNAs is important for NF-κB binding affinity, their inter-strand positioning is critical for stability. Indeed, serum stability assays have confirmed our previous evidence that ODNs containing additional internal LNAs in both strands are more stable than end-blocked LNA ODNs (Table 1). Similar results have been obtained by Wahlestedt *et al*., who showed a significantly higher half-life of antisense LNA–DNA mix-mers than isosequential all-phosphorothioate ODNs and a lesser stability of LNA–DNA gap-mers (41). However, here we show that, in the context of a double-stranded ODN, improved stability can be achieved only when internal LNA substitutions are placed in complementary positions. As a consequence, by excluding the C2+C2 molecule because of its very poor NF-κB affinity, C+C was the only molecule tested with a significantly increased stability in comparison with the LNA end-modified (B+B) ODN, but also with a lower affinity (Table 1). The 16-fold decrease in affinity of C+C could not be of secondary importance in terms of activity considering that the predicted binding affinity of NF-κB is extremely high due to the low abundance of this transcription factor within cells (46). However, we here demonstrate that the problem of increasing stability while maintaining high binding affinity can be solved by replacing nucleotides at position C in the κB sequence with α-L-LNAs. Significantly, introduction of α-L-LNAs conferred the same degree of stability as obtained with LNAs, while they did not interfere with NF-κB binding. Indeed, the competition efficiency of the C*+C* ODN containing α-L-LNAs was only 2-fold lower than that of the corresponding DNA ODN, allowing us to predict a K_d value of the same order of magnitude as the unmodified ODN (Table 1).

In conclusion, this study highlights the importance of LNA positioning in a decoy molecule when substitutions are also introduced within the transcription factor binding sequence in order to improve resistance to nuclease digestion. This work

has revealed which nucleotides are most crucial for NF-κB binding when replaced with LNAs or their diastereoisomeric forms, α-L-LNAs. At the same time, we have identified those nucleotides which are not crucial, implying that these residues could be modified to increase nuclease resistance. However, we also show that LNA interference is not only dependent on positioning, but also on intrinsic conformational properties. In this context, α-L-LNAs, as DNA mimics, permit minimization of this latter effect, while maintaining high stability. This evidence emphasizes the importance of addressing the appropriate chemical modification to overcome many of the problems which limit engineering of ODN decoys to the end-capping. To our knowledge, the results presented here are the very first report on the feasibility of nucleotide modifications in the binding site of a decoy molecule without loss of affinity and/or specificity and provide further insights into the usage of LNAs and their positioning in the design of decoy ODNs. The results obtained with α-L-LNA-modified ODNs encourage further studies focused on testing their efficacy in cellular experimental systems. In this regard, delivery of α-L-LNA-based decoys against NF-κB to the monocyte/macrophagic compartment are in progress with the goal of achieving long-term modulation of NF-κB activation and as a consequence controlled release of pro-inflammatory mediators in pathological conditions such as chronic inflammation and autoimmunity (47).

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REFERENCES

1. Latchman,D.S. (1997) How can we use our growing understanding of gene transcription to discover effective new medicine? *Curr. Opin. Biotechnol.*, **8**, 713–717.
2. Latchman,D.S. (1997) Transcription factors: an overview. *Int. J. Biochem. Cell Biol.*, **29**, 1305–1312.
3. Johnson,W. and Jameson,J.L. (1998) Transcriptional control of gene expression. In Jameson,J.L. (ed.), *Principles of Molecular Medicine*. Humana Press, Totowa, NJ.
4. Latchman,D.S. (2000) Transcription factors as potential targets for therapeutic drugs. *Curr. Pharm. Biotechnol.*, **1**, 57–61.
5. Emery,J.G., Olhlstein,E.H. and Jaye,M. (2001) Therapeutic modulation of transcription factor activity. *Trends Pharmacol. Sci.*, **22**, 233–240.
6. Darnell,J.E.,Jr (2002) Transcription factors as targets for cancer therapy. *Nature Rev. Cancer*, **2**, 740–749.
7. Papavassiliou,A.G. (1998) Transcription-factor-modulating agents: precision and selectivity in drug design. *Mol. Med. Today*, **4**, 358–366.
8. Morishita,R., Higaki,J., Tomita,N. and Ogihara,T. (1998) Application of transcription factor "decoy" strategy as means of gene therapy and study of gene expression in cardiovascular disease. *Circ. Res.*, **82**, 1023–1028.
9. Mann,M.J. and Dzau,V.J. (2000) Therapeutic applications of transcription factor decoy oligonucleotides. *J. Clin. Invest.*, **106**, 1071–1075.
10. Tomita,N., Morishita,R., Tomita,T. and Ogihara,T. (2002) Potential therapeutic applications of decoy oligonucleotides. *Curr. Opin. Mol. Ther.*, **4**, 166–170.
11. Nakamura,H., Morishita,R. and Kaneda,Y. (2002) Molecular therapy via transcriptional regulation with double-stranded oligodeoxynucleotides as decoys. *In Vivo*, **16**, 45–48.
12. Dzau,V.J. (2002) Transcription factor decoy. *Circ. Res.*, **90**, 1234–1236.
13. Tomita,N., Azuma,H., Kaneda,Y., Ogihara,T. and Morishita,R. (2003) Gene therapy with transcription factor decoy oligonucleotides as a potential treatment for cardiovascular disease. *Curr. Drug Targets*, **4**, 339–346.
14. Mann,M., Whittemore,A., Donaldson,M.C., Pollack,M.D., Orav,J. and Dzau,V.J. (1999) *Ex vivo* gene therapy of vascular bypass graft with E2F decoy: the PREVENT single-centre, randomised, controlled trial. *Lancet*, **354**, 1493–1498.
15. Chu,B.C.F. and Orgel,L.E. (1992) The stability of different forms of double-stranded decoy DNA in serum and nuclear extracts. *Nucleic Acids Res.*, **20**, 5857–5858.
16. Bielinska,A., Shivdasani,R.A., Zhang,L. and Nabel,G.J. (1990) Regulation of gene expression with double-stranded phosphorothioate oligonucleotides. *Science*, **250**, 997–1000.
17. Mischiati,C., Borgatti,M., Bianchi,N., Rutigliano,C., Tomassetti,M., Feriotto,G. and Gambari,R. (1999) Interaction of the human NF-kB p52 transcription factor with DNA–PNA hybrids mimicking the NF-kB binding sites of the human immunodeficiency virus type 1 promoter. *J. Biol. Chem.*, **274**, 33114–33122.
18. Brown,D.A., Kang,S.-H., Gryaznov,S.M., DeDionisio,L., Heidenreich,O., Sullivan,S., Xu,X. and Nerenberg,M.I. (1994) Effect of phosphorothioate modification of oligodeoxynucleotides on specific protein binding. *J. Biol. Chem.*, **269**, 26801–26805.
19. Romanelli,A., Pedone,C., Saviano,M., Bianchi,N., Borgatti,M., Mischiati,C. and Gambari,R. (2001) Molecular interactions with nuclear factor-kB (NF-kB) transcription factors of PNA–DNA chimera mimicking NF-kB binding sites. *Eur. J. Biochem.*, **268**, 6066–6075.
20. Borgatti,M., Lampronti,I., Romanelli,A., Pedone,C., Saviano,M., Bianchi,N., Mischiati,C. and Gambari,R. (2003) Transcription factor decoy molecule based on a peptide nucleic acid (PNA)–DNA chimera mimicking Sp1 binding sites. *J. Biol. Chem.*, **278**, 7500–7509.
21. Crinelli,R., Bianchi,M., Gentilini,L. and Magnani,M. (2002) Design and characterization of decoy oligonucleotides containing locked nucleic acids. *Nucleic Acids Res.*, **30**, 2435–2443.
22. Singh,S.K., Nielsen,P., Koshkin,A. and Wengel,J. (1998) LNA (locked nucleic acids): synthesis and high-affinity nucleic acid recognition. *Chem. Commun.*, 455–456.
23. Braasch,D.A. and Corey,D.R. (2000) Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. *Chem. Biol.*, **55**, 1–7.
24. Petersen,M. and Wengel,J. (2003) LNA: a versatile tool for therapeutics and genomics. *Trends Biotechnol.*, **21**, 74–81.
25. Koshkin,A.A., Singh,S.K., Nielsen,P., Rajwanshi,V.K., Kumar,R., Meldgaard,M., Olsen,C.E. and Wengel,J. (1998) LNA (Locked Nucleic Acids): synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclonucleoside monomers, oligomerisation and unprecedented nucleic acid recognition. *Tetrahedron*, **54**, 3607–3630.
26. Sørensen,M.D., Kværnø,L., Bryld,T., Håkansson,A.E., Verbeure,B., Gaubert,G., Herdewijn,P. and Wengel,J. (2002) α-L-*Ribo*-configured locked nucleic acid (α-L-LNA): synthesis and properties. *J. Am. Chem. Soc.*, **124**, 2164–2176.
27. Nielsen,K.E., Singh,S.K., Wengel,J. and Jacobsen,J.P. (2000) Solution structure of an LNA hybridized to DNA: NMR study of the d($CT^LGCT^LT^LCT^LGC$):d(GCAGAAGCAG) duplex containing four locked nucleotides. *Bioconjugate Chem.*, **11**, 228–238.
28. Petersen,M., Nielsen,C.B., Nielsen,K.E., Jensen,G.A., Bondensgaard,K., Singh,S.K., Rajwanshi,V.K., Koshkin,A.A., Dahl,B.M., Wengel,J. and Jacobsen,J.P. (2000) The conformations of locked nucleic acids (LNA). *J. Mol. Recognit.*, **13**, 44–53.
29. Berkowitz,B., Huang,D.B., Chen-Park,F.E., Sigler,P.B. and Ghosh,G. (2002) The X-ray crystal structure of the NF-kB p50·p65 heterodimer bound to the interferon β-kB site. *J. Biol. Chem.*, **277**, 24694–24700.
30. Escalante,C.R., Shen,L., Thanos,D. and Aggarwall,A.K. (2002) Structure of the NF-kB p50/p65 heterodimer bound to the PRDII DNA element from the interferon-β promoter. *Structure*, **10**, 383–391.
31. Grünweller,A., Wyszko,E., Bieber,B., Jahnel,R., Erdmann,V.A. and Kurreck,J. (2003) Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2′-*O*-methyl *RNA*,

phosphorothioate and small interfering RNA. *Nucleic Acids Res.*, **31**, 3185–3193.
32. Thanos,D. and Maniatis,T. (1992) The high mobility group protein HMG I(Y) is required for NF-kappa B-dependent virus induction of the human IFN-β gene. *Cell*, **71**, 777–789.
33. Falvo,J.V., Thanos,D. and Maniatis,T. (1995) Reversal of intrinsic DNA bends in the IFNβ gene enhancer by transcription factors and the architectural protein HMG I(Y). *Cell*, **83**, 1101–1111.
34. Wengel,J., Petersen,M., Nielsen,K.E., Jensen,G.A., Håkansson,A.E., Kumar,R., Sorensen,M.D., Rajwanshi,V.K., Bryld,T. and Jacobsen,J.P. (2001) LNA (locked nucleic acid) and the diastereoisomeric alpha-L-LNA: conformational tuning and high affinity of DNA/RNA targets. *Nucleosides Nucleotides Nucleic Acids*, **20**, 389–396.
35. Nielsen,K.M., Petersen,M., Håkansson,A.E., Wengel,J. and Jacobsen,J.P. (2002) α-L-LNA (α-L-*ribo*-configured locked nucleic acid) recognition of DNA: an NMR spectroscopic study. *Eur. J. Chem.*, **8**, 3001–3009.
36. Crooke,S.T. (2002) Progress in antisense technology: the end of the beginning. *Methods Enzymol.*, **313**, 3–45.
37. Boye,S.M., Pradhan,A.A. and Clarke,P.B. (2002) Evidence for sequence-dependent and reversible non-specific effects of PS-capped antisense treatment after intracerebral administration. *Antisense Nucleic Acid Drug Dev.*, **12**, 95–102.
38. Sands,H., Gorey-Feret,L.J., Ho,S.P., Bao,Y., Cocuzza,A.J., Chidester,D. and Hobbs,F.W. (1995) Biodistribution and metabolism of internally ^{3}H-labeled oligonucleotides. II. 3′,5′-blocked oligonucleotides. *Mol. Pharmacol.*, **47**, 636–646.
39. Uhlmann,E., Peyman,A., Ryte,A., Schmidt,A. and Buddecke,E. (2000) Use of minimally modified antisense oligonucleotides for specific inhibition of gene expression. *Methods Enzymol.*, **313**, 268–284.
40. Hosoya,T., Takeuchi,H., Kanesaka,Y., Yamakawa,H., Miyano-Kurosaki,N., Takai,K., Yamamoto,N. and Takaku,H. (1999) Sequence-specific inhibition of a transcription factor by circular dumbbell DNA oligonucleotides. *FEBS Lett.*, **461**, 136–140.
41. Wahlestedt,C., Salmi,P., Good,L., Kela,J., Johnsson,T., Hökfelt,T., Broberger,C., Porreca,F., Lai,J., Ren,K., Ossipov,M., Koshkin,A., Jakobsen,N., Skouv,J., Oerum,H., Jacobsen,M.H. and Wengel,J. (2000) Potent and non-toxic antisense oligonucleotides containing locked nucleic acids. *Proc. Natl Acad. Sci. USA*, **97**, 5633–5638.
42. Borgatti,M., Romanelli,A., Saviano,M., Pedone,C., Lampronti,I., Breda,L., Nastruzzi,C., Bianchi,N., Mischiati,C. and Gambari,R. (2003) Resistance of decoy PNA-DNA chimera to enzymatic degradation in cellular extracts and serum. *Oncol. Res.*, **13**, 279–287.
43. Saviano,M., Romanelli,A., Bucci,E., Pedone,C., Mischiati,C., Bianchi,N., Feriotto,G., Borgatti,M. and Gambari,R. (2000) Computational procedures to explain the different biological activity of DNA/DNA, DNA/PNA, PNA/PNA hybrid molecules mimicking NF-kB binding sites. *J. Biomol. Struct. Dyn.*, **18**, 353–362.
44. Koudelka,G.V., Harrison,S.C. and Ptashne,M. (1987) Effect of non-contacted bases on the affinity of 434 operator for 434 repressor and Cro. *Nature*, **326**, 886–888.
45. Chen,F.E., Huang,D.-B., Chen,Y.-Q. and Ghosh,G. (1998) Crystal structure of p50-p65 heterodimer of transcription factor NF-kB bound to DNA. *Nature*, **391**, 410–413.
46. Zabel,U., Schreck,R. and Bauerle,P. (1991) DNA binding of purified transcription factor NF-kB. *J. Biol. Chem.*, **266**, 252–260.
47. Magnani,M., Rossi,L., Fraternale,A., Bianchi,M., Antonelli,A., Crinelli,R. and Chiarantini,L. (2002) Erythrocyte-mediated delivery of drugs, peptides and modified oligonucleotides. *Gene Ther.*, **9**, 749–751.