Exploring Cellular Activity of Locked Nucleic Acid-modified Triplex-forming Oligonucleotides and Defining Its Molecular Basis*

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The design of synthetic non-protein molecules able to control DNA-associated biological functions through their interaction with a specific DNA sequence represents a very attractive approach. Among DNA code-reading molecules, triplex-forming oligonucleotides (TFOs)3 are able to bind to the major groove of oligopyrimidine-oligopurine regions in double-stranded DNA. A series of results have validated triplex-based approaches at the molecular and cellular levels: triplexes have been shown to interfere with transcription (initiation and elongation), replication, repair, and recombination (1, 2). However, the intracellular efficiency of TFOs still has to be improved.

One possible approach consists of increasing the stability of the non-covalent triple helices under physiological conditions to reach binding affinities comparable to those of DNA-associated regulatory proteins. To this end, a variety of chemically modified nucleic acids have been developed. Among them are locked nucleic acids (LNAs) that contain LNA nucleotide monomers, i.e. ribonucleotides with a 2′-O,4′-C-methylene linkage that effects conformational fixation of the furanose ring in a C3′-endo conformation (3, 4). LNA-containing oligonucleotides have been recently shown to enhance triplex stability and to alleviate in part the sequence constraints imposed by the triple helical recognition motifs (5, 6). Only a few hybridization properties of LNA-modified TFOs (TFO/LNAs) have been reported so far, and they all concern (T,C)-containing TFO/LNAs. It has been shown that fully modified TFO/LNAs failed to bind to double-stranded DNA (7, 8), likely due to conformational restraint of TFO/LNA. However, alternating DNA and LNA nucleotides in TFO sequences is appropriate for efficient triplex formation. A study on a 15-mer pyrimidine TFO/LNA provided evidence that LNA-induced triplex stabilization is associated with a slower dissociation rate constant and a less unfavorable entropic contribution compared with the non-modified TFO (9). In previous works, TFO/LNAs have been used only for in vitro assays, including plasmid functionalization (10). However, LNA-modified oligonucleotides are appealing molecules: they have been successfully used as efficient antisense agents, even in vivo (for examples, see Refs. 11–13), and also as decoys (14), aptamers (15), LNAzymes (16), and DNA-correcting agents (17).

In the present work we report the triplex forming properties and intracellular activity of G-containing TFO/LNAs. We characterized the mechanism of triplex stabilization induced by LNA modifications in G-containing TFOs, compared with non-modified isosequential phosphodiester TFOs and with pyrimidine TFO/LNA directed against the same oligopyrimidine-oligopurine target sequence. The dependence of triplex stability on pH was also explored. To address these questions, kinetic and thermodynamic parameters of triplexes were evaluated by surface plasmon resonance. Specificity of TFO/LNA binding was also analyzed, using UV melting experiments, electrophoretic mobility shift assays, and restriction enzymatic protection assay. Finally, we evaluated the capacity of G-containing TFO/LNAs to interfere with biological processes in vitro and in cells, using experimental settings designed to demonstrate a triplex-based mechanism in a quantitative manner. We showed that G-containing TFO/LNAs were active, as measured by inhibition of transcription elongation. Our results support TFO/LNA activity in a cellular context at submicromolar concentrations, which has never been reported before, and represent the first step toward further development of TFO/LNAs as artificial modulators of DNA-associated biological functions.

The abbreviations used are: TFO, triplex-forming oligonucleotide; LNA, locked nucleic acid; TFO/LNA, LNA-modified triplex-forming oligonucleotide; nt, nucleotide(s); GFP, green fluorescent protein; CMV, cytomegalovirus; PPT, polypurine tract; nt, nucleotide(s); SPR, surface plasmon resonance.

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MATERIALS AND METHODS

Oligonucleotides—Oligonucleotide analogues with LNA residues were synthesized as previously described (3) or purchased from Proligo (France SAS). Phosphodiester oligonucleotides were synthesized by Eurogentec; for cellular experiments, the phosphodiester oligonucleotide (150 pmol/site) was modified by incorporation of a propylamine group (named 15TGC/po) to resist nuclease-mediated degradation.

Plasmids—The pSP-F47 plasmid was constructed by insertion of a 780-bp fragment of the human immunodeficiency virus type 1 nef gene containing the oligopyrimidine-oligopurine PPT target sequence between the T7 and SP6 promoters in the pSP73 host vector (Promega), as previously described in detail (18). This system allowed us to run bidirectional in vitro transcription assays.

The pCMV+PPT/luc plasmid derived from the bidirectional expression vectors (pBT Tet vectors; Clontech). These vectors contain the tet-responsive element between two identical minimal CMV promoters (pBI Tet vectors; Clontech). These vectors contain the tet-responsive element between two identical minimal CMV promoters in opposite directions. This system was used to express two reporter genes, the firefly luciferase (Photinus pyralis) gene (luc) and the GFP gene. Expression of both genes is co-regulated by doxycycline. The activator protein (reverse Tet repressor protein, rTetR) binds the tet-responsive element in the presence of doxycycline and activates transcription. This protein was produced from the pTet-On expression plasmid (Clontech). Two 55-bp inserts containing either the wild-type human immunodeficiency virus type 1 polypurine tract target sequence (PPT, 5′-AAAAAGAAAAAGGGGGA-3′) or a mutated sequence (PPTmut, 5′-AAAAAGAAAAAGGGGGA-3′; the four mutations are shown in bold) were synthesized and inserted into the upstream regions of the luciferase and GFP genes, downstream of the transcription start site.

The pRlCMV vector (Promega) contains Renilla luciferase (from the marine organism Renilla reniformis) under the control of the CMV promoter. pRlCMV was used to monitor transfection efficiency.

UV Absorption Melting Experiments—All thermal denaturation experiments were performed in a 10 mM sodium cacodylate buffer (at the indicated pH value containing 10 mM GTP, 150 mM NaCl, and 0.5 mM dithiothreitol; 1 unit/µl RNase inhibitor in presence of 500 µM HEPES, pH 7.4, 50 mM NaCl, 10 mM MgCl₂, 0.5 mM spermine, and 10% sucrose (at room temperature over-night). The non-denaturing polyacrylamide gel (15%) was run at 37 °C using the ImageQuant software (Amersham Biosciences). The level of degradation was monitored by scanning the gel with a PhosphorImager, and results were quantified by integration with ImageQuant software.

In Vitro Transcription Assay—Transcription assays were performed using the pSP-F47 plasmid that contains the PPT sequence overlapping one of the seven Drai sites. The transcription initiation was monitored by annealing of PPT hairpin duplexes to TFO. The transcription was initiated by addition of 25 units of phage RNA polymerase (T7; or SP6) which were previously described in (18).

Sprays—The pCMV(PPT/luc plasmid contains the PPT sequence overlapping one of the seven Drai sites. The transcription initiation was monitored by annealing of PPT hairpin duplexes to TFO. The transcription was initiated by addition of 25 units of phage RNA polymerase (T7; or SP6) which were previously described in (18).

Electrophoresis Mobility Shift Assay—PPT or mutPPT hairpin intramolecular duplexes were 5′-end-labeled with [32P]ATP (3000 Ci/mmol; Amerham Biosciences) by T4 polynucleotide kinase (Promega). The duplex (50 mM) was incubated with increasing concentrations of TFO in a buffer containing 50 mM HEPES, pH 7.2, 150 mM NaCl, 10 mM MgCl₂, 0.5 mM spermine, and 10% sucrose (at room temperature overnight). The non-denaturing polyacrylamide gel (15%) was run at 37 °C in a buffer containing 50 mM HEPES, pH 7.2, and 5 mM MgCl₂. Gels were scanned with a PhosphorImager, and results were quantified using the ImageQuant software (Amerham Biosciences). The level of complex formation was estimated by the TFO concentration at which 50% of complexes was formed (C50).

Restriction Enzyme (Dral) Protection Assay—The pCMV+PPT/luc plasmid possesses the PPT sequence overlapping one of the seven PPT sites. It was used as a substrate for a restriction enzyme protection assay. For the cleavage assay, the pCMV+PPT/luc plasmid was incubated at 37 °C for 20 min with increasing amounts of oligonucleotides in 50 mM HEPES, pH 7.2, 50 mM NaCl, 10 mM MgCl₂, and 0.5 mM spermine in the presence of Dral enzyme. The fragments generated by Dral cleavage in the absence of TFO are 3654, 1936, 1718, 692, 633, and 19 bp long. A 3654-bp fragment corresponding to the addition of the 1936- and 1718 bp fragments was obtained when TFO-induced inhibition of Dral cleavage occurred. The extent of trinucleotide- mediated inhibition of Dral cleavage was assessed by gel electrophoresis (0.8% agarose gel) and quantitated. The TFO concentration that gave 50% inhibition (IC50) was then evaluated.

In Vitro Transcription Assay—Transcription assays were performed using the pSP-F47 plasmid that contains the PPT sequence between the T7 and SP6 promoters. The plasmid was linearized by BseMI for T7 transcription and synthesis of purine/PPT-containing RNA (600 nt long) or by Bsu36I for synthesis of pyrimidine/PPT-containing RNA (597 nt long). The linearized plasmids (0.5 µg) were used for in vitro transcription assays in the presence of increasing amounts of TFOs in a buffer containing 40 mM Tris-HCl, pH 7.2; 6 mM MgCl₂; 2 mM spermidine; 4 mM dithiothreitol; 1 unit/µl RNase inhibitor in presence of 500 µM ATP, CTP, and UTP, 100 µM GTP, and 0.3 µM [32P]GTP. Transcription was initiated by addition of 25 units of phage RNA polymerase (T7; or SP6).
In 10 mM cacodylate, 150 mM sodium chloride, and 10 mM magnesium chloride, pH 7. Strand concentrations: 1

duplex absorbance from that of triplex formed with either 15TCG(2)/LNA (●) or 16TC/LNA TFO (●). Note the two

y axes with different scales to better visualize the triplex-induced transition. B, UV melting profiles obtained after subtraction of the PPT target
duplex absorbance from that of triplex formed with either 15TCG(2)/LNA (●) or 16TC/LNA TFO (●). All UV melting experiments were performed
in 10 mM cacodylate, 150 mM sodium chloride, and 10 mM magnesium chloride, pH 7. Strand concentrations: 1 μM PPT duplex and 1.5 μM TFO.

After 5 min at 37 °C, transcription reactions were terminated by ethanol precipitation (10 volumes of ethanol was added to samples with 0.3
M sodium acetate and 15 μg of glycojen). The transcription products were analyzed by electrophoresis on 6% polyacrylamide gels, and quantifi-
cations (±10%) were obtained by PhosphorImager analysis. The percentages were corrected for transcript length effects, taking into
account that the transcripts were uniformly radiolabeled.

Cell Cultures and Transient Expression Assay—The P4-CCR5 cells were derived from HeLa cells (22) and maintained in Dulbecco’s modified
Eagle’s medium supplemented with 10% fetal bovine serum.

P4-CCR5 cells were used for transfection of oligonucleotides and reporter vectors. Transfections were performed using the cationic activ-
ed dendrimer Superfect (Qiagen). Typically, 0.1 μg of pCMV(+/-)−PPT/luc plasmid, 0.1 μg of pTet-On activator plasmid, 0.003125 μg of
pRL-CMV plasmid, and various amounts of oligonucleotides were mixed with Superfect (1.5 μl) in a total volume of 12 μl (serum-free
medium). The mixture was prepared for triplicates and added to P4-
CCR5 cells (13,750 cells/well in a 96-well plate in 88 μl of serum-
containing medium in the presence of doxycycline, which was necessary
for CMV promoter induction). After cell lysis (in 30 μl of Passive Lysis
Buffer; Promega), activities of both luciferases (firefly and Renilla) were
measured in the same cell extract using the dual-luciferase assay kit
(Promega), and GFP expression was measured as well (15

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Buffer; Promega), activities of both luciferases (firefly and Renilla) were
measured in the same cell extract using the dual-luciferase assay kit
(Promega), and GFP expression was measured as well (15 μl of lysate in
80 μl of phosphate-buffered saline). Luciferase and GFP expressions were measured with a luminometer/fluorometer (Victor™-Wallac). The
modulation of firefly luciferase expression by oligonucleotide treatment
was quantitated by evaluation of the firefly/Renilla ratio; the specificity
of triplex-induced inhibition was evaluated by the GFP/Renilla ratio.
Each experiment was repeated at least three times, and values are
presented as the mean of a triplicate (±S.D.) from a representative
experiment.

RNA Analysis—Total cellular RNA was prepared (RNeasy Mini;
Qiagen). Firefly and Renilla luciferase RNAs were subjected to compet-
titive reverse transcription-PCR (for details, see Ref. 23). Briefly two
sets of two PCR primers were designed for each luciferase RNA; two
competitor DNAs (firefly and Renilla) were used and co-amplified with
the RNA sample. For each luciferase, the two amplified products (from
sample and competitor, which are 153- and 192-bp long for firefly and
250- and 294-bp long for Renilla, respectively) were separated using an
8% PAGE in Tris borate-EDTA and quantitated by PhosphorImager
analysis.

RESULTS

Tight and Specific Binding of LNA-modified TFOs in the
(T,C,G)-Motif—In the present work we have studied the binding
and biological properties of a series of LNA-modified TFOs (TFO/LNAs)
directed against a 16-bp-long oligopyrimidine-
oligopurine sequence (named PPT). All the sequences and their
LNA content are shown in Fig. 1. The TFOs (15TCG and 16TC)
have been described previously (25). Both bind parallel to the
purine-containing strand of the duplex by Hoogsteen hydrogen
bonding. Because fully modified TFO/LNA failed to hybridize to
double-stranded DNA (7), only a few LNA modifications (five to
nine) were introduced. For the (T,C,G)-containing sequence,
three TFO/LNAs were designed: the 15TCG(1)/LNA is com-
pared of alternating LNA and DNA nucleotides all along the
sequence (8 LNA/15 nt); the 16TCG/LNA is a modified version of the 15TCG(2)/
sequence (8 LNA/15 nt); the 15TCG(2)/LNA contains LNA mod-
ifications only in the T-rich part of the sequence (5 LNA/15 nt); and
the 16TCG/LNA is a modified version of the 15TCG(2)/
LNA with two additional LNA modifications at the 3′-end of
the sequence to increase resistance to 3′-exonucleases (26), and
it was used in cellular experiments. The 16TCG/LNA sequence is
the pyrimidine analogue of the 15TCG(1)/LNA and was de-
signated as a reference to compare the (T,C)- and (T,C,G)-motifs.
The thermal stability of the different triplexes formed by these
TFO/LNAs and the PPT target sequence was first determined by
UV absorption melting experiments at neutral pH. Representative
UV melting profiles of the triplexes formed by 15TCG/LNA
and 16TC/LNA are shown in Fig. 2; the melting temperature values ($T_m$) are summarized in Table I. The presence of LNA
modifications strongly increases the triplex-forming ability of the
oligomers compared with the isosequential phosphodiester in the
(T,C)-motif (as described previously) (9, 27) and in the (T,C,G)-
motif. At neutral pH and physiological concentrations of mono-
valent cations (150 mM), the highest thermal stability was ob-

TABLE I

Melting temperatures ($T_m$ values) of the different TFOs
with the PPT target duplex

| TFO                  | pH | $T_m$ Target:PPT duplex |
|----------------------|----|-------------------------|
| 15TCG/po             | 7.0| 19                      |
| 15TCG(1)/LNA         | 7.0| 58                      |
| 15TCG(2)/LNA         | 7.0| 55                      |
| 16TC/LNA             | 7.0| 56                      |
| 16TC/C/LNA           | 7.0| 37                      |
| 16TC/LNA             | 6.5| 47                      |
| 16TC/po              | 5.5| 33                      |

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C. Giovannangeli, manuscript in preparation.

Fig. 2. UV denaturation profiles. A, the PPT duplex alone (X) or in the presence of 15TCG(2)/LNA (●) or 16TC/LNA TFO (●). Note the two
y axes with different scales to better visualize the triplex-induced transition. B, UV melting profiles obtained after subtraction of the PPT target
duplex absorbance from that of triplex formed with either 15TCG(2)/LNA (●) or 16TC/LNA TFO (●). All UV melting experiments were performed
in 10 mM cacodylate, 150 mM sodium chloride, and 10 mM magnesium chloride, pH 7. Strand concentrations: 1 μM PPT duplex and 1.5 μM TFO.

2 Each experiment was repeated at least three times, and values are
presented as the mean of a triplicate (±S.D.) from a representative
experiment.

RNA Analysis—Total cellular RNA was prepared (RNeasy Mini;
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(T,C,G)-Motif—In the present work we have studied the binding
and biological properties of a series of LNA-modified TFOs (TFO/LNAs)
directed against a 16-bp-long oligopyrimidine-
tained with the sequences 15TCG(1)/LNA and 15TCG(2)/LNA ($T_m = 58 \degree C$ and 55 \degree C, respectively): a 21 \degree C and 18 \degree C increase (respectively) in the melting temperature was observed compared with the corresponding pyrimidine sequence 16TC/LNA (respectively) in the melting temperature was observed comparing C50(15TCG(1)/LNA) and C50(15TCG(2)/LNA). Under our experimental conditions (150 mM NaCl), the 15TCG/po (28).

**Table II**

| TFO/LNA | $\Delta T_m$ (°C) | Target: (PPT) | C50 | IC50 |
|---------|-------------------|----------------|-----|------|
|         | PPT target − (mutPPT) target | μM | μM | μM |
| 15TCG(1)/LNA | 58 − 31* = 27 | 0.40 | >2.5 | 0.3 |
| 15TCG(2)/LNA | 55 − 36* = 19 | 0.15 | >2.5 | 0.2 |

$^* T_m$ values of different G-containing TFO with PPT or mutPPT duplexes (see oligopyrimidine-oligouridine sequences in Fig. 1). Data were obtained in 10 mM cacodylate, 150 mM sodium chloride, and 10 mM magnesium chloride, pH 7. Strand concentrations: 1 μM PPT duplex and 1.5 μM TFO. Estimated error in $T_m$ is ±0.5 °C (or ±1 °C for cases indicated by an asterisk, which correspond to a triplex transition presenting a weak hyperchromism).

$^*$ TFO concentrations for 50% triplex formation estimated from gel shift assays (C50 values). Increasing concentrations of TFO/LNAs were incubated in 150 mM sodium chloride, 50 mM HEPES, pH 7.2, 10 mM magnesium chloride, and 0.5 mM spermine, with either PPT or mutPPT duplex. The samples were analyzed by electrophoresis in 15% non-denaturing polyacrylamide gel (50 mM HEPES, pH 7.2, 5 mM magnesium chloride) at 37 °C. Estimated error, ±0.05 μM.

$^c$ Concentration for 50% inhibition of DraI cleavage (IC50 values) by TFO/LNAs. Inhibition of DraI cleavage was evaluated on the pCMV(+)PPT/luc plasmid in 50 mM sodium chloride; 50 mM HEPES, pH 7.2, 10 mM magnesium chloride, and 0.5 mM spermine. Analyses were done by electrophoresis on a 0.8% agarose gel and quantification of DraI digestion profiles. Estimated error, ±0.05 μM.

In order to assess the specificity of duplex recognition by the 15TCG/LNA sequences, we performed three different assays (Table II). In two of them, we compared the TFO/LNA binding to the PPT duplex target and to the mutPPT duplex containing two mutations (5'-AAAAGAAAAAGGAGGA-3'; mutations are shown in bold). First, using UV absorption melting experiments, we showed that 15TCG(1)/LNA and 15TCG(2)/LNA could discriminate between the wild-type and the mutated target with a large decrease in $T_m$ ($\Delta T_m = 27 \pm 19 \degree C$, respectively). It should be noted that nine contiguous triplets could theoretically be formed with the A5G3A5 sequence on both of the targets (PPT and mutPPT). Second, the specificity of triplex formation was confirmed by gel shift experiments. Increasing amounts of the different TFO/LNAs were incubated in the presence of the PPT or mutPPT duplexes at 37 °C and neutral pH (see “Materials and Methods”). TFO concentrations required for 50% of complex formation (C50) were estimated. Estimated error, ±0.05 μM.

The association and dissociation rate constants of the triplexes formed with the G-containing 15TCG/po (28). This enzyme cleaves at the junction of the triplex helix site (TTT AAA), and triplex formation inhibits DNA cleavage (29). The pCMV(+) PPT/luc plasmid contains the PPT site and seven DraI sites. In the presence of TFO, DraI cleavage was impaired only on the site overlapping the PPT sequence: inhibition of cleavage at this site led to the appearance of a unique longer 3654-bp fragment and the disappearance of the 1936- and 1718-bp fragments. TFO concentrations required for 50% inhibition (IC50) of DraI cleavage were determined from the concentration dependence of cleavage inhibition for the two 15TCG/LNA TFOs (IC50(15TCG(1)/LNA) ~ 0.2 μM; IC50(15TCG(2)/LNA) ~ 0.2 μM). Under the same conditions, the isosequential phosphodiester TFO did not inhibit cleavage at $37 \degree C$ up to a concentration of 10 μM. The level of DraI cleavage inhibition specifically at the triplex site induced by TFOs reflects both the specificity and the stability of triplex formation.

These results obtained with G-containing TFO/LNAs in different experimental settings support a strong increase in stability induced by LNA modification and a specific binding of TFO/LNAs to the PPT duplex target.

**Kinetics and Energetics of Complexes Formed with LNA-modified TFOs in the (T,C,G)-Motif**—To investigate the origin of the high stability of triplexes formed with G-containing TFO/LNAs, we performed kinetic and thermodynamic analyses.

The kinetics of triplex formation was studied by SPR experiments for the 15TCG/LNAs and compared to both the 16TC/LNA and 15TCG/po. The PPT duplex was captured on the presence of the PPT or mutPPT duplexes at 37 °C: 10 concentrations of TFO (0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, and 12 μM) were used in injection experiments to determine the association ($k_{app}$) and dissociation ($k_{off}$) rate constants. The association rate constant was deduced from the apparent association rate constant $k_{app} = k_{on}(TFO) + k_{off}$ For all the studied TFOs, we obtained a linear dependence of the $k_{app}$ with respect to the concentration of TFO (data not shown), consistent with a simple two-state model. The calculated values of the kinetic constants are reported in Table III.

The association and dissociation rate constants of the triplexes formed with the G-containing 15TCG(1)/LNA (and 15TCG(2)/LNA) were 5.33 × 10^6 (1.1 × 10^6) M⁻¹ s⁻¹ and 4.5 × 10⁻⁵ (1.3 × 10⁻⁴) s⁻¹, with these latter $k_{off}$ values corresponding to a lifetime ($\tau = ln2/k_{off}$) as long as 9.5 h (and 1.5 h). Compared with the pyrimidine 16TC/LNA, triplex formation with the 15TCG/LNAs exhibited an equivalent association rate ($k_{on}(16TC/LNA) = 0.9 × 10^{13}$ M⁻¹ s⁻¹), but a much higher dissociation rate constant ($k_{off}(16TC/LNA) = 0.9 × 10^{13}$ M⁻¹ s⁻¹), which overlaps the triplex site on 3 bp (Fig. 1). This enzyme cleaves at the junction of the triplex helix site (TTT AAA).
slower dissociation (~10–35-fold decrease in $k_{\text{off}}$ ($k_{\text{off}}$(16TC/LNA) = $1.5 \times 10^{-3}$ s$^{-1}$). Thus, this decrease in the dissociation rate constants is the major reason for the enhanced stability of triplexes formed with the two G-containing TFO/LNAs compared with the one formed with the pyrimidine TFO/LNA ($K_d$(37 °C-16TC/LNA) = $0.1 \mu M$; $K_d$(37 °C-15TCG/LNA) = $1.7 \mu M$; $K_d$, equilibrium constant of triplex dissociation).

The comparison of the two 15TCG/LNAs (15TCG(1)/LNA and 15TCG(2)/LNA), which contain the same 5′-sequence (5′-tTtTcTtTt; LNA is shown in lowercase letters) and differ only by the presence of three LNA modifications in the G-rich 3′-end region resulted in a slightly slower association rate (2-fold decrease in $k_{\text{on}}$) and in a longer lifetime of the triplex (3.5-fold decrease in $k_{\text{on}}$). As a result, the two 15TCG/LNAs exhibited very similar equilibrium constants ($K_d$(37 °C) = $0.1 \mu M$). The observed 2-fold decrease in $k_{\text{on}}$ could be explained by unfavorable conformations of the single-strand 15TCG(1)/LNA, possibly including structures generated by self-association.

The pH-induced effects on kinetic and equilibrium constants of triplexes formed with TFO/LNAs (15TCG(2)/LNA and 16TC/LNA) were studied (Fig. 3B). Changes in pH values between pH 6.5 and pH 7 affected the stability of triplex formed with 15TCG(2)/LNA (6-fold increase in $K_d$ at 0.5 unit of pH) less than that of the one formed with 16TC/LNA (14-fold increase in $K_d$ at 0.5 unit of pH), as expected considering the different C content of the TFOs (one in 15TCG and seven in 16TC). In this pH range (pH 6.5–7), $K_d$ appeared to be mainly driven by $k_{\text{off}}$ for both types of triplexes (formed with 15TCG(2)/LNA or 16TC/LNA). Such a trend has already been reported for phosphodiester pyrimidine TFOs (21).

To investigate the kinetic mechanisms of LNA-induced triplex stabilization, we compared the 15TCG/LNA TFOs to the isosequential phosphodiester 15TCG/po. Due to weak triplex stability with the unmodified TFO ($T_m = 19 ^\circ C$), SPR measurements were performed at 10 °C, as compared with 37 °C with TFO/LNAs. The decrease in $K_d$ for the 15TCG/LNAs was mainly associated with a decrease in the dissociation rate constant (Table III). For 15TCG(2)/LNA, we interpolated the Arrhenius plots of $k_{\text{off}}$ and $k_{\text{on}}$ (Fig. 4) to estimate their values at 10 °C ($k_{\text{off}}$(10 °C) = $1.9 \times 10^{-5}$ s$^{-1}$, $K_d$(10 °C) = $2.4 \times 10^{-8}$ M). We could thus evaluate the LNA-induced effect compared with the isosequential phosphodiester TFO, namely, a 68-fold increase in affinity, associated with a 162-fold decrease in dissociation rate constant.

![Image](http://www.jbc.org/Downloaded from http://www.jbc.org/)

**TABLE III**

| TFO     | $T$ °C | pH | $k_{\text{on}}$ M$^{-1}$ s$^{-1}$ | $k_{\text{off}}$ s$^{-1}$ | $r = \ln 2/k_{\text{off}}$ s | $K_d = k_{\text{off}}/k_{\text{on}}$ | $K_d$ M |
|---------|-------|----|-------------------------------|---------------------------|-----------------------------|--------------------------------|--------|
| 15TCG/po| 10    | 7.0 | $0.33 \times 10^0$ ($r^2 = 0.98$) | $1.3 \times 10^{-2}$ ($4 \times 10^{-4}$) | 5.3 $\times 10^2$ | 3.9 $\times 10^{-6}$ |
| 15TCG(1)/LNA | 37  | 7.0 | $0.53 \times 10^0$ ($r^2 = 0.99$) | $4.5 \times 10^{-3}$ ($2 \times 10^{-6}$) | 1.53 $\times 10^1$ | 0.8 $\times 10^{-7}$ |
| 15TCG(2)/LNA | 37  | 7.0 | $1.1 \times 10^0$ ($r^2 = 0.98$) | $1.3 \times 10^{-3}$ ($3 \times 10^{-7}$) | 5.3 $\times 10^1$ | 1.2 $\times 10^{-7}$ |
| 16TC/LNA  | 37  | 7.0 | $0.9 \times 10^0$ ($r^2 = 0.99$) | $1.5 \times 10^{-3}$ ($2.5 \times 10^{-4}$) | 4.5 $\times 10^2$ | 1.7 $\times 10^{-6}$ |

**Fig. 3. SPR experiments.** A, typical corrected SPR sensorgrams of triplex formation and dissociation obtained with the 15TCG(1)/LNA at different concentrations (0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, and 12 μM, from bottom to top) at 37 °C, pH 7. B, pH-induced effects on triplexes containing TFO/LNAs. The values of ($-\ln K_d$) (equilibrium dissociation constant) and ($-\ln k_{\text{off}}$) (dissociation rate constant) are shown for the different triplexes, as indicated. These values were obtained at 37 °C for the TFO/LNAs and at 10 °C for the 15TCG/po ($\ddagger$); in the latter case the triplex is unstable at 37 °C under the conditions applied. All data were obtained in a buffer containing 10 mM cacodylate, 150 mM sodium chloride, 10 mM magnesium chloride, and 0.0005% P20.
Our kinetic data demonstrate that the tight stabilization observed for triplexes formed with G-containing TFO/LNAs was mainly due to a longer lifetime of the triplexes (in the range of 1 h, at 37 °C) compared with phosphodiester TFO (in the range of 100 s). In contrast, the association rate was marginally affected. These results support the data obtained with another pyrimidine TFO/LNA sequence (9) and extend them to G-containing TFO/LNAs.

To further understand the origin of the triplex stabilization observed with 15TCG(2)/LNA, the entropy and enthalpy changes were evaluated and compared with those for both the isosequential phosphodiester (15TCG/po) and 16TC/LNA. For the TFO/LNAs, UV melting profiles did not allow an accurate determination of thermodynamic parameters; indeed, either the melting of the triplex was too close to that of the duplex (15TCG/po) or the transition was poorly cooperative (16TC/LNA) (see Fig. 2). Instead, SPR experiments were carried out at different temperatures (20 °C, 25 °C, 30 °C, and 37 °C) under conditions allowing triplex formation with the 15TCG/LNA and 16TC/LNA (see Fig. 3). The values of the equilibrium dissociation constant \( K_d \) were calculated as the ratio of the kinetic rate constants \( k_{on} \) and \( k_{off} \), the enthalpy and entropy changes \( \Delta H^0 \) and \( \Delta S^0 \) associated with triplex formation were calculated from a linear fitting of the Arrhenius plots of association and dissociation rate constants \( k_{on} \) and \( k_{off} \). The correlation coefficients of linear regression were \( r^2 \) and \( r^2 \). For 16TC/po, the thermodynamic parameters were deduced from UV melting experiments (van’t Hoff plots: \( r^2 \) and \( r^2 \)). Enthalpy \( \Delta H^0 \) and entropy \( \Delta S^0 \) values of TFO association are reported. The estimated \( T_m^0 \) values were estimated from Arrhenius plots as the temperatures at which \( K_d = 1 \mu M \) (Fig. 4B). The measured \( T_m^0 \) values from Table I are indicated for comparison.

| TFO                  | \( \Delta H^0 \) | \( \Delta S^0 \) | \( T_m^0 \) | \( T_m^0 \) |
|---------------------|-----------------|-----------------|-------------|-------------|
| 15TCG/po (pH 7.0)   | 15              | 17              | 55.5        | 55          |
| 16TC/LNA (pH 7.0)   | 48              | 136             |             |             |
| 16TC/LNA (pH 6.5)   | 24              | 45              | 50          | 47          |
| 16TC/po (pH 6.0)    | 66              | 135             |             |             |

The thermodynamic constant parameters of triplexes formed with TFO/LNAs and TFO/po in the (T, C, G)- and (T, C)-motifs (TFO/pos in the (T, C, G)- and (T, C)-motifs) were determined. For 15TCG/po and 16TC/po, the thermodynamic parameters were deduced from UV melting experiments (van’t Hoff plots: \( r^2 \) and \( r^2 \)). Enthalpy \( \Delta H^0 \) and entropy \( \Delta S^0 \) values of TFO association are reported. The estimated \( T_m^0 \) values were estimated from Arrhenius plots as the temperatures at which \( K_d = 1 \mu M \) (Fig. 4B). The measured \( T_m^0 \) values from Table I are indicated for comparison.
and G-containing TFO, as obtained by SPR experiments, are consistent with the corresponding UV melting profiles, featuring weakly cooperative transitions (Fig. 2). It can be noted that SPR experiments did allow determination of $\Delta H^\circ$ (and $\Delta S^\circ$) values as low as 10–20 kcal mol$^{-1}$ (and cal K$^{-1}$ mol$^{-1}$, respectively); it could have been hard to obtain these values from UV spectroscopic experiments or calorimetry measurements.

Kinetic SPR measurements were validated by comparison with data from UV melting experiments. UV melting experiments were carried out at duplex and TFO concentrations so that the equilibrium dissociation constant at the melting temperature was 1 μM ($K_{\text{UV}}^\circ$ ($T_m^\circ$) = 1 μM). By interpolating the Arrhenius plots obtained from SPR measurements ($k_{\text{SPR}}$ versus $T_1^\circ$ - $K_d^{\text{UV}}$; $k_{\text{SPR}}$), we extrapolated the temperature $T_m^{\text{SPR}}$ at which $K_d^{\text{SPR}} = 1$ μM. The temperatures deduced from SPR ($T_m^{\text{SPR}}$) agreed with those obtained by UV melting experiments ($T_m^{\text{UV}}$) (Table IV). The consistency of the data obtained separately by the two techniques provides a validation of parameters measured by SPR.

**TFO/LNAs for Arrest of Elongating RNA Polymerases in Vitro**—We evaluated the capacity of TFO/LNAs to interfere with DNA-associated proteins, such as elongating RNA polymerases.

A plasmid with the T7 polymerase promoter upstream of the PPT site was used as a template for transcription assays (Fig. 5). After appropriate plasmid linearization, full-length transcripts (660-nt long) were produced. In the presence of TFO/LNAs, truncated products did appear. To establish whether the truncated fragments corresponded to the arrest of transcription at the PPT site, the transcription arrest was compared with that obtained when the DNA template was cleaved with Drai on the 5′-side of the PPT target sequence (Drai RNA marker: 332-nt long, lane M in Fig. 5B). The truncated transcripts migrated at the same position as the Drai RNA marker, consistent with a physical blockage of RNA synthesis at the PPT site, i.e. the TFO/LNA binding sequence. In the presence of TFO/LNAs, the percentage of truncated fragments increased with TFO concentration. For T7 transcription at 37 °C, the plateau (reached at 10 μM TFO/LNA) corresponded to a percentage of truncated fragments of about 50% with 15TCG(1/PPT/luc) coding for the firefly luciferase; they either contained or lacked (respectively) the PPT sequence in the transcribed and untranslated region of the luc gene (Fig. 6). More
precisely, these constructs differed by the presence or absence of a 55-bp insert containing the wild-type PPT target sequence (see "Materials and Methods"). P4-CCR5 cells were transiently transfected with the pCMV(+/−)PPT/luc plasmid together with pTet-On (expressing the activator protein necessary for transcription from the CMV-inducible promoter) and pRL-CMV plasmid (expressing Renilla luciferase and used for correction of transfection efficiency) and various TFO/LNAs. Both luciferase activities were measured on the same cell extracts. The ratio of luminescence (firefly/Renilla) permits quantification of the modulation of the firefly luciferase expression induced by TFO treatment. In addition, the pCMV(+/−)PPT/luc plasmid did contain a second CMV promoter in the opposite direction controlling the expression of a GFP gene and a mutated version (PPTmut, 5′-AAAAAGGGGAGGAA-3′, the four mutations are shown in bold) was inserted upstream of the GFP gene. Alternatively, there was no insertion of the PPT-containing fragment in the pCMV(+/−)PPT/luc plasmid. B, cell extracts were analyzed for firefly and Renilla luciferases activities 24 h after transfection by a cationic dendrimer (Superfect). The expression vector (pCMV(+/−)PPT/luc) was introduced together with the pRL-CMV plasmid, in the presence or absence of TFO/LNAs. Normalized firefly/Renilla luminescence intensity ratios are reported in histogram with different TFO/LNA concentration treatments. Data are derived from 3 (for 16TCG-cont/LNA and 15TCG/po) to 10 experiments (for other TFO/LNAs). GFP expression was also evaluated on the same cell lysates, and the GFP/Renilla ratio was unaffected by TFO treatments (data not shown). Equivalent results were obtained with the two 15TCG/LNAs (15TCG/LNA(1) and 15TCG/LNA(2)), 15TCG/po 3′-modified by incorporation of a propylamine group.

DISCUSSION

In this work, we have characterized the binding properties and cellular activity of triplexes formed with G-containing
TFO/LNAs. These triplexes were compared with the ones formed with isosequential phosphodiester TFO (15TCG/po) and also with pyrimidine TFO/LNA (16TC/LNA) directed against the same oligopurine-oligopurine PPT target sequence, with the aim of determining the specific influence of LNA modifications in the context of G-containing TFO/LNAs. Actually, thus far, TFO/LNAs have only been used to form triplexes in the (T,C)-motif. Our data, obtained in conditions simulating physiological ones (37 °C, neutral pH), showed a strongly enhanced DNA binding of G-containing TFO/LNAs compared with 15TCG/po and 16TC/LNA. For the G-containing TFO/LNA, 15TCG(2)/LNA, an increase in $T_m$ as high as 7.2 °C per LNA modification was observed, the highest ever reported for modified triplexes. It is noteworthy that, in the present study, all the data were obtained at physiological monocation concentrations (150 mM NaCl). These conditions are known to decrease the stability of triplexes formed with G-containing TFOs: indeed, TFO self-association can compete with triplex formation. For some G-rich LNA-modified oligonucleotides (33), the formation of G-quadruplexes has been observed, which could act as competing structures.

To understand the origins of triplex stabilizations observed with 15TCG/LNAs compared with 15TCG/po and 16TC/LNA, we performed kinetic and thermodynamic analyses. From a kinetic point of view, triplex stabilization induced by G-containing TFO/LNA was mainly due to a decrease in the dissociation rate constants, with residence time in the range of 1–10 h; the association rates were marginally affected. The decrease in the dissociation rates appeared to be correlated with the number of LNA modifications in the TFO, as shown by the comparison of 15TCG/po (no LNA, as a reference) with 15TCG(2)/LNA (five LNAs) and 15TCG(1)/LNA (eight LNAs). The origin of the decreased dissociation rate might be at the triplet level, as suggested previously (31): a lower propensity for bp opening for single triplets in triplexes formed with TFO/LNAs would translate into a lower dissociation rate at the strand level. Finally, it has already been shown by UV melting experiments (8, 27) that a pH increase destabilizes triplexes formed with cytosine-containing TFO/LNAs. Here we reported pH-induced effects (with pH value close to neutral) on kinetics for triplexes formed with TFO/LNAs in the (T,C,G) - and (T,C)-motifs. We showed that the major effect did concern the dissociation process, as reported previously for non-modified triplex in the (T,C)-motif (21): a pH increase was associated with a faster dissociation.

Energetically, triplex formation with TFO/LNA in the (T,C,G)-motif (15TCG/LNA) was accompanied by a smaller (less negative = unfavorable) enthalpy change that is compensated for by a smaller (less negative = favorable) entropy change, compared with the phosphodiester (T,C,G)-sequence (15TCG/po). Thus, the enhancement in triplex stability was entropic in origin. This feature has already been reported for triplexes formed with TFO/LNA in the (T,C)-motif (9). This could be explained by a conformational restraint of the unbound TFO/LNA (both G-containing and pyrimidine sequences) because the conformational state of free TFO plays an important role in the thermodynamics of triplex formation (34). The increased rigidity of TFO/LNA in the free state, compared with non-modified TFO, may entropically favor its binding on the target duplex. The enthalpic loss induced by TFO/LNA compared with non-modified TFO may be explained in part by a recent NMR structural study (31): the duplex structure may change upon TFO/LNA binding to accommodate nucleotides with different sugar puckers. These structural changes alter the base stacking interactions and the hydrogen bonding pattern, compared with non-modified TFO. The differences in thermodynamic parameters observed with the LNA (T,C,G)-sequence (15TCG(2)/LNA) compared with the LNA (T,C)-sequence (16TC/LNA) could be explained in part by the following: (i) differences in the conformational micro-states of the two types of TFO/LNA when unbound (34); (ii) for entropic contribution, differences in the pattern of solvent water molecules, as already suggested for phosphodiester triplexes in the reverse Hoogsteen (T,G)- and (A,G)-motifs compared with (T,C)-motifs (it is possible that there are more water molecules removed in G-containing triplex motifs than in pyrimidine motifs) (32); and (iii) for enthalpic contribution, a different extent of cytosine protonation.

Finally, LNA modifications facilitated Hoogsteen hydrogen bonding to the duplex when the third strand is in parallel orientation with respect to the the oligopurine target sequence because it was observed with the pyrimidine and G-containing oligomers studied in this report. In contrast, reverse Hoogsteen hydrogen bonding with (G,A)- and (G,T)-containing TFO/LNAs seemed to be disfavored (data not shown). The same type of results (triplex stabilization for parallel Hoogsteen bonding and not for antiparallel reverse Hoogsteen bonding) has already been observed with other TFO modifications associated (as is the case with LNA oligonucleotides) with an RNA-like N-type (C3’-endo type) conformation, namely, phosphorodimides and RNA (25, 35).

The biological activity of these G-containing TFO/LNAs reported here was consistent with the high triplex stability observed under conditions simulating physiological conditions. We evaluated the in vitro and cellular activities of TFO/LNAs using experimental systems designed to demonstrate quantitatively and rapidly a triplex-based mechanism. Their capacity to interfere with transcription elongation was estimated: all the G-containing TFO/LNAs studied here were active under conditions in which the parent phosphodiester oligonucleotide did not exhibit any inhibitory effect. In different experiments, using control oligonucleotides and targets, we could demonstrate the triplex involvement in transcription arrest, both in vitro and in cells. The three different G-containing LNAs tested showed almost the same activity in our assays. It is noticeable that the 15TCG(2)/LNA, which contains only five LNA modifications, was active in cells. A 3’-modification is generally required to protect an oligonucleotide from nulease degradation. The fact that the 15TCG(2)/LNA, which lacks a 3’-modification, was active intracellularly could be explained by a partial self-association, which protected it from nulease degradation but still allowed triplex formation. The efficiency of TFO/LNAs can still be improved by functionalization strategies, as illustrated here with psoralen conjugation: a psoralen conjugate of a TFO/LNA was active within cells at submicromolar concentrations. Inhibitory concentration of TFO/LNAs would likely be lowered in another context, more favorable than our experimental system: indeed, the target sequence was quite short (16 bp), and the cellular activity to inhibit was transcription elongation, which is known to be difficult to arrest. It is likely that TFO/LNAs might efficiently interfere with other biological processes at concentrations lower than those obtained in this study for transcription elongation.

A series of oligonucleotide-based techniques do exist and are efficient for gene knock-down, including antisense and small interfering RNA technologies. Nevertheless, TFOs are unique in their nature, as sequence-specific DNA ligands, for modulation of DNA-mediated biological functions. They have already been used to interfere intracellularly with processes occurring on DNA, such as transcription, repair, or recombination. Manipulation of chromatin structure (36, 37) and positioning of DNA-damaging agents using these agents conjugated with
TFO (24, 38) are other promising applications of DNA code-reading molecules; they have been successfully explored with TFOs, but mainly in vitro. However, today, the major limitation of triplex-based applications is the limited intracellular stability of the complex. This is the reason why it is necessary to identify and characterize novel chemically modified TFOs and to synthesize and compatible with conjugation chemistry, as is the case for the LNA-modified oligonucleotides described here. Our study is the first step for further developments and original use of TFO/LNAs in the anti-gene strategy.

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Exploring Cellular Activity of Locked Nucleic Acid-modified Triplex-forming Oligonucleotides and Defining Its Molecular Basis
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