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Ligand-Mediated Transcription Elongation Control Using Triplex-Based Padlock Oligonucleotides

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Summary

Triplex-forming oligonucleotides (TFOs) provide useful tools for the artificial regulation of gene expression at the transcriptional level. They can become topologically linked to their DNA target upon circularization, thereby forming very stable triple helical structures. These "padlock oligonucleotides" are able to interfere with transcription elongation when their target site is located in the transcribed region of a gene. In vitro transcription experiments showed that a bacterial RNA polymerase was stopped at the site of triple-helix formation, whereas expression of a reporter gene was inhibited in live cells. In both cases, the padlock oligonucleotide was more efficient at inhibiting transcription elongation than a linear TFO, and the inhibition was observed only in the presence of a triplex stabilizing agent. These results provide new insights into the ligand-modulated locking of padlock oligonucleotides around their DNA target.

Introduction

Triplex-forming oligonucleotides (TFOs) represent an attractive tool for the sequence-specific control of gene expression at the transcriptional level, in the so-called "antigene" strategy [1]. They can bind to the major groove of the DNA double-helix by formation of specific hydrogen bonds between the bases of the TFO and the purine bases within the double-stranded DNA target sequence. The parameters that govern triple-helix formation and stability have been extensively studied [2]. In cells, the activity of TFOs can be compromised by the sensitivity of oligonucleotides toward intracellular nucleases, as well as by the stability of triple helical complexes. The ionic conditions that prevail in the intracellular environnement are different from those which favor triple-helix formation in vitro. Chemical modifications of deoxyoligonucleotides can help overcome some of these constraints [2].

TFOs have been shown to compete with DNA binding proteins in vitro, and inhibition of transcription factor-DNA interactions can interfere with gene expression in cells [3, 4]. There are many fewer reports of transcription inhibition by TFOs that bind downstream of the transcription initiation site. Cell-free transcription assays have shown that micromolar amounts of TFOs can partially inhibit the elongation of transcription by prokaryotic RNA polymerases, such as SP6, T3, or T7 RNA polymerases [5, 6]. By combining the use of a TFO-intercalator conjugate and a triple-helix-stabilizing agent, up to 60% inhibition of SP6 RNA polymerase was achieved [6]. Elongation by eukaryotic RNA polymerases was inhibited more efficiently in cell-free transcription assays (up to 100%), probably because these enzymes are less processive than prokaryotic RNA polymerases [6–8]. All these studies involved chemically modified oligodeoxynucleotides. The most efficient way of blocking elongation by an RNA polymerase was to crosslink the TFO to its DNA target using a TFO-psoralen conjugate, especially in cells [9–11].

We have recently shown that a TFO could be circularized around its target, using a short template oligonucleotide and T4 DNA ligase, yielding a so-called padlock oligonucleotide [12]. These padlock oligonucleotides may have various applications as a double-stranded DNA labeling method and in gene therapy [13–15]. The effects of the padlock oligonucleotide on DNA-protein interactions have been investigated using a restriction enzyme cleavage assay [16]. We have shown that under conditions where the triple helix was stable, the padlock oligonucleotide inhibits cleavage by the restriction enzyme, and that removal of the triplex stabilizing agent was sufficient to restore the cleavage, which suggests that the padlock oligonucleotide could be displaced from its binding site.

In this paper, we have examined the effects of padlock oligonucleotides on transcription elongation in a cell-free system using a bacterial RNA polymerase, and in a cultured mammalian cell line using a reporter gene. We showed that in both systems, the padlock oligonucleotide was able to strongly inhibit transcription elongation, under conditions where a linear TFO had a very limited efficiency. This effect was dependent on the presence of a triplex stabilizing agent.

Results

Inhibition of a Bacterial RNA Polymerase

To examine the ability of a padlock oligonucleotide to inhibit transcription elongation, we first chose to use a cell-free transcription assay. The pGA2 plasmid was obtained by insertion of a triplex target sequence in the multi-cloning-site (mcs) of pBluescript SK+ (Figure 1) [16]. This mcs is located between promoters for two commonly used prokaryotic RNA polymerases, the bacteriophage T3 and T7 RNA polymerases. We had previously shown that a TFO could be efficiently catenated to the pGA2 plasmid and was able to inhibit cleavage of the plasmid by the restriction endonuclease EcoNI

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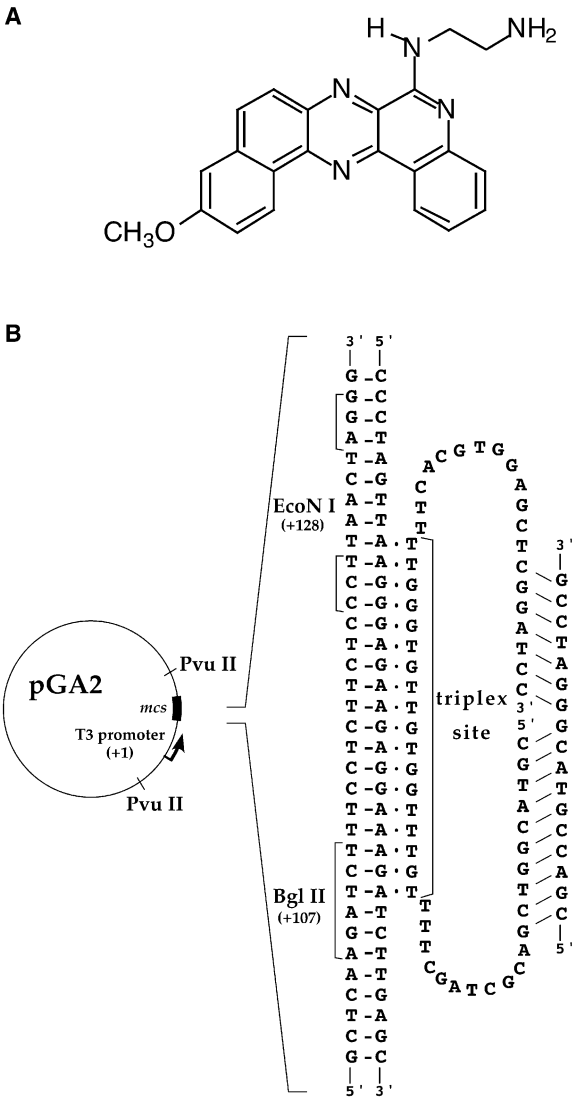

Figure 1. Description of the Chemicals, Plasmid, and Oligonucleotides Used in the In Vitro Transcription Assay

(A) Chemical structure of BQQ. (B) The 59-mer oligonucleotide TFO1 can form a triple helix by binding to an 18 bp oligopyrimidine•oligopurine target sequence, located in the multi-cloning-site (mcs) of the pBluescript SK+ plasmid, downstream from a T3 RNA polymerase promoter. The extremities of TFO1 can hybridize to the 17-mer oligonucleotide, thereby allowing its circularization by an enzymatic ligation. The sites for the restriction enzymes PvuII, BglII, and EcoNI are also indicated.

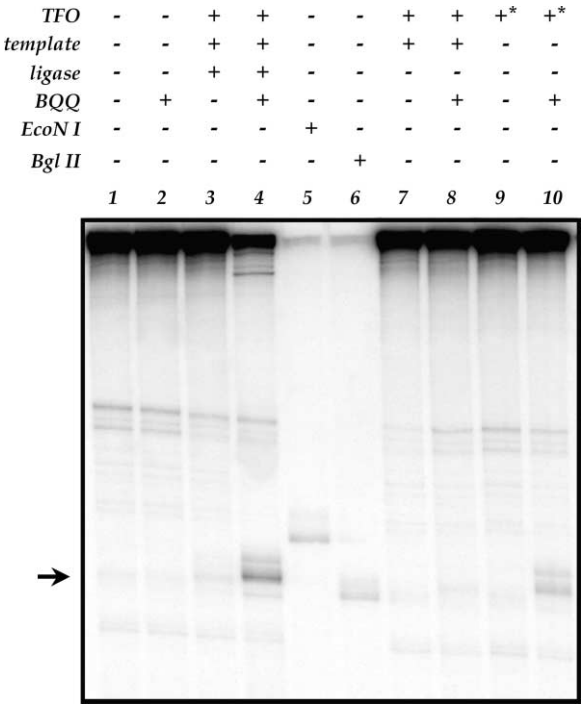

Figure 2. Analysis of T3 RNA Polymerase Elongation by Denaturing Gel Electrophoresis

In vitro transcription was carried out as described in the Experimental Procedures section and above the gel. The circularization reaction was carried out in the presence of the template oligonucleotide and ligase, before spermidine precipitation (lanes 3 and 4). In lane 5 and 6, the pGA2 plasmid had been linearized with the indicated enzymes. In lanes 7 and 8, the ligase had been omitted in the circularization reaction, but the plasmid had been purified as in lanes 3 and 4. In lanes 9 and 10, the plasmid was incubated with the linear TFO (100 nM) before the transcription reaction was started. No template or ligase was added in these last samples.

[16]. This inhibition was relieved upon removal of the triplex stabilizing agent. The 5′-phosphorylated TFO was added in excess to the pGA2 plasmid in the presence of the triple-helix-specific ligand BQQ, and padlock formation was achieved by hybridizing the 3′- and 5′-ends of the TFO to a 17-mer template oligonucleotide, and then adding T4 DNA ligase and incubating at 37°C for 1 hr. The mixture had to be heated and slowly cooled to allow for triple-helix formation and template hybridization in a precircularization complex, before addition of the ligase. Removal of the TFO in excess and of the triplex-stabilizing agent was achieved by selective precipitation of the plasmid in the presence of spermidine.

The yield of padlock attachment was first measured using the previously described gel shift assay [13]. Briefly, the plasmid was treated by the restriction enzyme PvuII, which can cleave the plasmid at two sites located on both sides of the mcs, 511 bp from each other. Attachment of the padlock oligonucleotide resulted in a band shift of the shortest restriction fragment. The yield of padlock formation was estimated to be 90% (not shown).

In vitro transcription reactions were then conducted with unmodified and padlock-modified plasmids, both in the absence and presence of BQQ (Figure 2). The intact circular plasmid was used in order to preserve the topological link between the plasmid and the TFO. Labeled transcripts were analyzed by denaturing polyacrylamide gel electrophoresis. In vitro transcription carried out on the supercoiled pGA2 plasmid led to the formation of a long transcript, which migrated very high in the gel (Figure 2, lane 1). Only a few shorter transcripts were also observed, which are likely due to pausing of the RNA polymerase. Addition of BQQ to the plasmid had no effect on the pattern and quantity of transcription

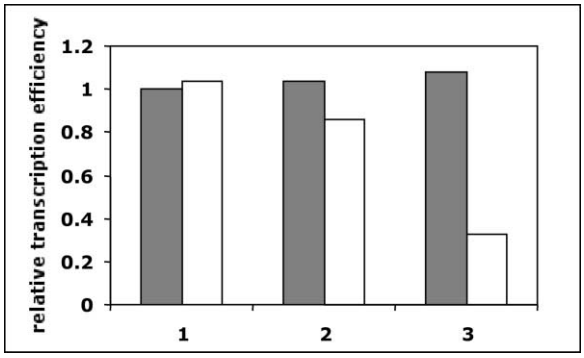

Figure 3. TFO inhibition on T3 RNA Polymerase Elongation

The normalized amount of long transcripts is presented in the presence (white bars) and absence (gray bars) of BQQ, for the plasmid alone (1), for the plasmid in the presence of the linear TFO (2), and for the padlock-modified plasmid (3). The normalization is carried out with respect to the unmodified plasmid in the absence of BQQ. This particular experiment is representative of three different experiments.

products (lane 2). When the padlock-modified plasmid was transcribed in the absence of BQQ (lane 3), the products and yields were the same as with the nonmodified plasmid (lane 1). When both the padlock oligonucleotide and BQQ were present (lane 4), the amount of fully elongated transcript decreased by approximately 80% (Figure 3), while the appearence of a short band could be detected in the gel (see arrow on Figure 2). The length of this band was analyzed by comparing it to the size of transcription products obtained with a plasmid previously linearized with either BglII (Lane 6) or EcoNI (lane 5), which cut the plasmid on both sides of the oligopyrimidine•oligopurine sequence used for triplex formation (see Figure 1). The aborted transcript was slightly longer than that obtained with the BglII digested plasmid, which suggests that the RNA polymerase was blocked by the padlock oligonucleotide bound to its expected target site. Transcription reactions were also conducted with pGA2 plasmid that had been treated like the padlock-modified plasmid, but without the ligase in the reaction. No inhibition of transcription could be observed (lane 8). Experiments carried out in the presence of a 10-fold excess of the linear TFO showed a very weak band at the same position as that observed with the padlock oligonucleotide, only in the presence of BQQ (lane 10). The yield of the transcription was decreased, but to a much lower extent than with the padlock oligonucleotide, as shown by quantification of the long products (Figure 3). T3 RNA polymerase uses the strand containing the oligopurine sequence as template strand. An in vitro transcription assay was also performed using T7 RNA polymerase, which transcribes the other DNA strand. A similar arrest in transcription was observed (not shown).

Inhibition of Transcription in Mammalian Cells

In order to investigate the effects of padlock oligonucleotides on gene transcription in mammalian cells, we looked for the presence of oligopyrimidine•oligopurine sequences in commonly used reported genes, and

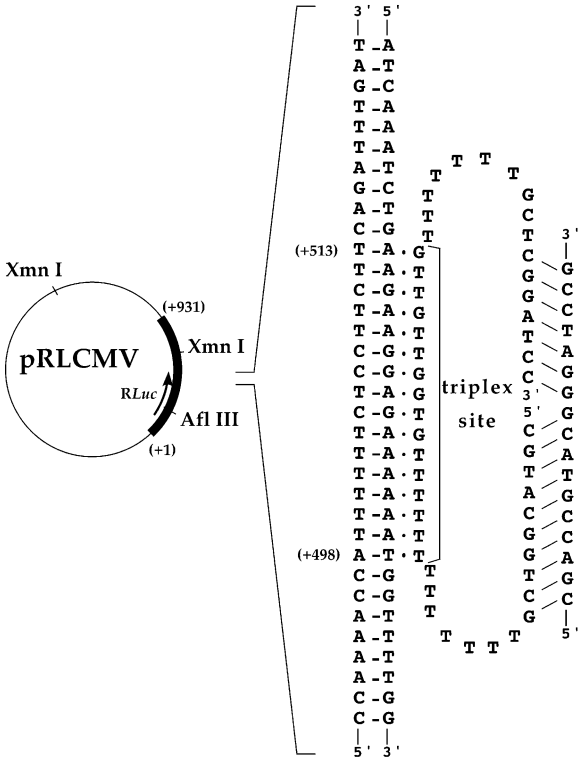

Figure 4. Description of the Plasmid and Oligonucleotides Used in the Cellular Experiments

Sequences of the 50-mer TFO2 and the 17-mer template are shown. The 50-mer oligonucleotide can form a triple helix by binding to a 16 bp oligopyrimidine•oligopurine target sequence, located in the transcribed region of the *Renilla* luciferase gene. The sites for the restriction enzymes AflIII and XmnI are also indicated.

found an appropriate sequence in the *Renilla* luciferase gene on the pRLCMV plasmid (Promega). The 16-bp oligopyrimidine•oligopurine sequence was located between position +498 and +513 downstream of the start site for transcription of the *Renilla* Luciferase reporter gene. TFO2 was designed to bind to this sequence and to form a triple helix made of T.AxT and C.GxG triplets (Figure 4). Padlock formation was achieved in vitro as described above. The yield of padlock attachment was measured by a gel shift assay using XmnI and AflIII as restriction enzymes. About 90% of the 348 bp fragment containing the target sequence was shifted, showing that at least 90% of the plasmids were carrying a padlock oligonucleotide at the expected site (Figure 5).

HeLa cells were cotransfected with both the pGL3c and pRLCMV plasmids. The pGL3c plasmid expresses Firefly Luciferase under the control of an SV40 promoter, and was cotransfected with pRLCMV in order to check for the influence of the presence of BQQ on transgene expression. Transfection was carried out with the nonmodified pRLCMV plasmid or the plasmid that has been padlock-modified in vitro. These transfection were carried out after or without addition of the triplex stabilizing agent BQQ. After 24 hr, the activity of both firefly and *Renilla* luciferases was measured in cell extracts. The level of expression of *Renilla* luciferase was the same in the absence and presence of BQQ when the non-

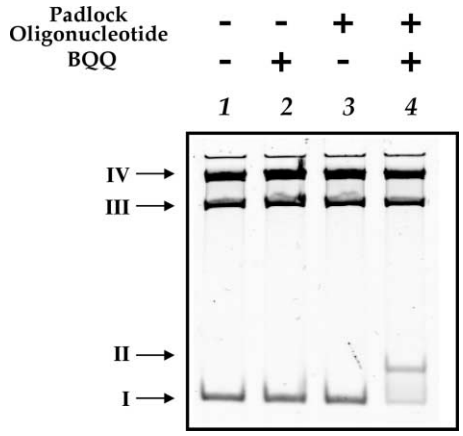

Figure 5. Measurement of the Yield of Padlock Formation by a Gel-Shift Assay

Plasmids were treated in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of an excess of 5′-phosphorylated TFO to allow for padlock formation, as described in the Experimental Procedures section. After purification by spermidine precipitation, samples were heated in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 2 μM BQQ and digested by XmnI and AflIII to produce a short restriction fragment containing the target sequence. Samples were then loaded on a 6% native polyacrylamide gel containing 10 mM $MgCl_2$, stained with SybrGreen I, and imaged with a camera. I, III, and IV refer to the restriction fragments of 348, 978, and 2753 bp, respectively. II corresponds to fragment I shifted by the presence of a catenated padlock oligonucleotide.

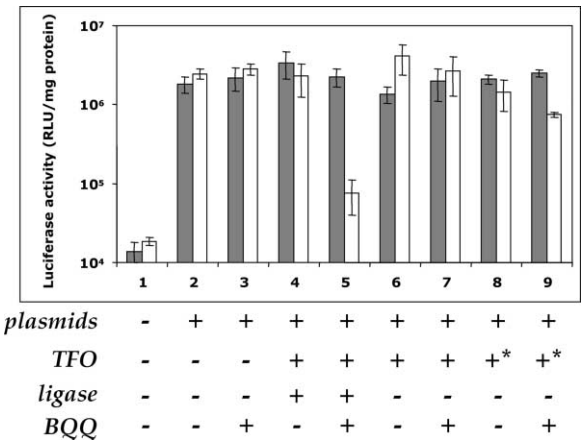

Figure 6. Specific Inhibition of *Renilla* Luciferase Expression by the Padlock Oligonucleotide

Luciferase expression was measured 24 hr after cotransfection of HeLa cells with both pGL3c and pRLCMV, and normalized for protein content (n = 3 in all cases). The expression of both *Renilla* luciferase (white bars) and firefly luciferase (gray bars) is shown. (1) No plasmid. (2 and 3) Unmodified pGL3c and pRLCMV plasmids. (4 and 5) The pRLCMV plasmid was modified with the padlock oligonucleotide. (6 and 7) The pRLCMV plasmid was treated as for padlock modification, except that the DNA ligase was not added. (8 and 9) The unmodified pRLCMV was prehybridized with the linear TFO (as indicated by an asterisk) at a concentration of 1 μM. The plasmids were prepared in the absence (2, 4, 6, and 8) or presence (3, 5, 7, and 9) of BQQ. Each experiment was performed in triplicate. Vertical bars represent the standard error of the mean of the three samples. This panel shows one experiment representative of three different experiments.

modified pRLCMV plasmid was used (Figure 6, samples 2 and 3). Expression of *Renilla* luciferase was specifically inhibited by 90% when the padlock-modified pRLCMV plasmid was transfected after addition of BQQ (Figure 6, sample 5). This inhibition was not observed when the pRLCMV plasmid did not carry a padlock oligonucleotide (Figure 6, sample 3), when BQQ was omitted (Figure 6, sample 4), or when the plasmid had been treated as for padlock attachment, but without the ligase in the reaction (Figure 6, sample 7). Transfection was also conducted in the presence of linear TFO2 at a concentration of up to 1 μM in the transfection medium (Figure 6, sample 9). No significant decrease in *Renilla* luciferase expression was observed. All these data suggest that in the presence of BQQ the padlock oligonucleotide was able to form a triple helical complex that inhibited transcription of the *Renilla* luciferase gene specifically. The formation of this triple helical complex as well as gene expression inhibition was observed only in the presence of the triplex stabilizing agent BQQ. Moreover, the padlock oligonucleotide was much more efficient than a linear oligonucleotide at blocking transcription elongation in this cellular transcription system.

Discussion

The purpose of this study was to investigate the effects of padlock oligonucleotides on transcription elongation by RNA polymerases. First, a cell-free transcription assay showed that a padlock oligonucleotide that had been bound to a triplex site located downstream of the start site was able to arrest transcription elongation by T3 RNA polymerase and led to the production of a truncated RNA reflecting the position of the binding site for the padlock oligonucleotide. This happened only in the presence of the triplex stabilizing agent BQQ, and inhibition was much higher when the TFO had been circularized around its target compared with a linear TFO. These results are consistent with previous reports showing that GT-rich TFOs that have been locked around their target sequence in the presence of the triplex stabilizing agent BQQ can inhibit DNA cleavage by a restriction endonuclease. We show here that this locking strategy results in complexes that are not displaced by highly processive enzymes such as RNA polymerases.

The effects of padlock oligonucleotides on transcription elongation were also investigated in cells. Previous experiments had shown that padlock oligonucleotides did not interfere with gene expression when they were targeted to sequences located outside of reporter genes [14]. We selected a 16 bp oligopyrimidine•oligopurine sequence in the *Renilla* luciferase gene which appeared to be a good candidate for triple-helix formation. This sequence was located 500 bp downstream from the transcription start site. We designed a TFO that could be efficiently catenated around this target sequence. A very efficient inhibition of gene expression was observed in cells for the plasmid that had been padlock modified and incubated with the triplex stabilizing agent. Importantly, it should be noted that excess unbound oligonucleotides were removed in our assay. Expression of the *Renilla* luciferase gene by the unmodified plasmid as

well as expression of the firefly luciferase gene carried by the cotransfected pGL3c plasmid were not affected by the presence of BQQ.

The oligonucleotide that has been circularized around its target appears to be much more efficient at blocking transcription than its linear counterpart, both in a cell-free assay and in cells. These results confirm previously published data showing that linear unmodified TFOs are not strong inhibitors of transcription elongation, even when they form stable triple helices [5, 6]. This may be explained by the fact that the polymerase is able to stall at the triplex site, and resume polymerization as soon as the TFO dissociates. This dissociation may be enhanced by the RNA polymerase [6]. Padlock oligonucleotide can lower the level of fully elongated RNA transcripts with efficiency up to 80%. With a total yield of the padlock formation reaction of approximately 90%, this means that the inhibition is as strong as the one observed with TFOs that have been crosslinked to their target by a psoralen molecule (about 90%). These observations suggest that the lifetime of the complex between the target and the padlock oligonucleotide is longer than the lifetime of a complex formed with a linear oligonucleotide, or that the triple helix made by the padlock oligonucleotide is less easily dissociated by the RNA polymerase. One can also postulate that it takes less time for the dissociated padlock oligonucleotide to reassociate with its target than it takes for the polymerase to resume its activity. In the absence of triplex stabilizing agent, the padlock oligonucleotide may have slid away from its binding site, but the RNA polymerase may also have gone through the circular oligonucleotide or pushed it to the end of the transcribed region. Single molecule experiments may be used to answer this question.

Triplex-mediated inhibition of gene expression in cells requires TFOs with a sufficient nuclease resistance and binding strength under cellular conditions. Cellular studies are often performed with plasmid-harbored genes, and triple helix is usually formed before transfection or microinjection of the plasmid by preincubation of the plasmid with the TFO under favorable conditions [17–19]. Chemical modifications of the TFOs are always required for efficient inhibition of gene expression [3, 19] except for some G-rich oligos that form very stable triple helices [4]. In some reported cases, covalent linking of the TFO to the target site was a requirement for inhibition of gene expression [10, 11, 20]. The results we obtained in cells may be explained by a better resistance of the circular TFO to 3′-exonucleases, and by the physical link between the plasmid and the TFO. But the results of the cell-free assay suggest that the enhanced inhibition induced by circularizing the oligonucleotide could also be due to the intramolecular nature of the complex, or to steric reasons. Some triplex-mediated covalent modifications, such as DNA alkylation, are at least partially repaired in cells [21]. Psoralen crosslinks seem to be much less efficiently repaired [10, 22]. It would be interesting to investigate the interactions between padlock oligonucleotides and the DNA-repair machineries.

There are only a few reported examples of linear TFOs that can inhibit elongation by RNA polymerases, either in vitro [5–8] or in cells [19, 23]. An efficient blockage of transcription elongation was observed using PNA oligonucleotides, in an in vitro transcription assay with prokaryotic RNA polymerases [24] and in eukaryotic cells [25]. Synthetic polyamides made of N-methylimidazole and N-methylpyrrole amino acids, which bind in the minor groove of DNA, were not able to abolish in vitro transcription elongation by T7 RNA polymerase on a histone-free DNA [26]. Circularizing a TFO around its DNA target represents a new way to increase triple helix stability to a level where the complex is almost irreversible without any covalent modification of the target. The circularization reaction is easy to carry out in vitro, and may also be achieved by a chemical pathway [27; Roulon et al., unpublished results]. Interestingly, this complex can be reversed upon removal of the triplex-stabilizing agent. In addition to inhibition of gene expression, TFOs have been used for several other purposes, such as transcriptional activation [28], activation of recombination [29], targeted gene modifications [30, 31], and as a tool for studying DNA-associated functions, such as DNA repair [22, 32, 33] and protein-DNA interactions [9, 34, 35]. The ligand-modulated padlock oligonucleotides described here with their specific features may be used for such purposes.

Other applications may be thought of which would exploit further the concept described here. Endogenous genes can be targeted by TFOs. There has been experimental proof for the ability of TFOs to reach their target within the nucleus [36] and to inhibit transcription factor binding as well as to arrest RNA synthesis [23, 37]. An in vivo circularization method would introduce a topological link between a TFO and its target. It has been reported that RNAs can be circularized around single-stranded RNA targets by an autocatalytic mechanism [38]. Triple helices can be formed with RNA as third strands [39, 40]. Our results point to the interest of applying in vivo circularization methods for targeting double-stranded DNA, which may result in improved TFOs, the same way the circularizable antisense molecules are more efficient than linear ones. In gene therapy, the expression of therapeutic transgenes must be tightly controlled, for example by using tissue-specific promoters. The constitutive expression of a transgene may have cytotoxic effects. Therefore, conditional gene expression systems that can be temporally or spatially regulated are of special interest. This control may be achieved at the posttranscriptional level using low molecular weight compounds [41, 42]. Provided the triplex stabilizing agent can induce triple-helix formation in vivo, padlock-modified plasmids may offer a new method for the control of transgene expression by a small molecule.

Significance

Circular oligonucleotides can be assembled around specific double-stranded DNA sequences by using triple-helix formation and an enzymatic circularization reaction. Ligand-modulated padlock oligonucleotides have been previsouly shown to inhibit the cleavage of a plasmid by a restriction enzyme. In this study, their effects on transcription elongation were investigated. Our results provide evidence for the sequence-specific blocking of transcription elongation by padlock

oligonucleotides on plasmid-harbored genes. This blockage was observed both in vitro and in live cells that had been transfected with the padlock-modified plasmid. The padlock oligonucleotides were more efficient than linear TFOs at inhibiting transcription elongation by prokaryotic and eukaryotic RNA polymerases. This inhibition was observed only in the presence of a triplex stabilizing agent. These results set the basis of new strategies for the in vitro formation of very stable triple helical DNA complexes that do not involve covalent modification of the double-stranded DNA target. They can be used for interfering efficiently with various DNA-related biological machineries, but also pave the way for new artificial gene regulation mechanisms and single molecule studies.

Experimental Procedures

Plasmids, Oligonucleotides and Triple-Helix-Specific Ligand

Plasmid pGA2, which was derived from pBluescript SK+ by insertion of a triplex target site in the multi-cloning-site (mcs), has already been described [16]. Plasmids pRLCMV and pGL3c were obtained from Promega. The sequences of the oligonucleotides used in this study are the following ones: 5′-CGTACGGTCGACGCTAGCTTTTG TTTGGTGTTGTGGGTTTTCACGTGGAGCTCGGATCC-3′ for the 59-mer triplex-forming oligonucleotide TFO1, 5′-CGTACGGTCGTTTTTT TTTTTTGTGGTTGTTGTTTTTTTGCTCGGATCC-3′ for the 50-mer oligonucleotide TFO2, and 5′-CGACCGTACGGGATCCG-3′ for the 17-mer template oligonucleotide used in the circularization experiments (see Figures 1 and 4). The sequence of the so-called TRAP oligonucleotide, which forms an intramolecular triple helix and is used to remove BQQ from the samples, is the following: 5′-CTTTCCTTC TCTCCTTTTTGGAGAGAAGGAAAGTTTTTGTTTGGTTGTGTGG-3′. All these oligonucleotides were obtained from Eurogentec (Seraing, Belgium). Their concentration was calculated using a nearest-neighbor model for absorption coefficients. Synthesis of the triplex stabilizing agent BQQ (6-[3-(dimethylamino)propyl]amino-11-methoxybenzo[f]quino-[3,4-b]quinoxaline) has been described elsewhere [43].

TFO1 and TFO2 were 5′-phosphorylated using the following protocol: 300 pmol of oligonucleotide were incubated in 50 μl of T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, and 25 μg/mL BSA [pH 7.8] at 25°C, NEB) with 10 units T4 PNK, for 1 hr 30 min at 37°C. The phosphorylated oligonucleotides were used without further purification.

Padlock Formation

To assemble the padlock oligonucleotides and the plasmids, the 5′-phosphorylated TFO (400 nM) was mixed with 200 nM (4 μg in 10 μL) of plasmid and 20 μM BQQ in T4 DNA ligase buffer. This mixture was heated to 80°C and slowly cooled down to 37°C. Then, 600 nM of the 17-mer oligonucleotide template and 133 units of T4 DNA ligase (NEB) were added to achieve circularization of the oligonucleotide around the plasmid.

To remove BQQ from the triplex, the mixture (10 μL) was first diluted to 50 μl so that the final buffer concentration was 40 mM Tris-HCl (pH 8.0), 80 mM NaCl, 16 mM $MgCl_2$, and 5 μM TRAP oligonucleotide. The samples were heated to 80°C and then slowly cooled to 37°C. Then, 50 μl of 40 mM spermidine was added and the samples were left for 30 min at room temperature with frequent vortex mixing, in order to allow plasmid compaction. After centrifugation, the pellet was washed with 200 μl of a solution containing 50% isopropanol, 10 mM $MgCl_2$, 300 mM NaCl, and 25 mM EDTA as previously described. This treatment resulted in complete elimination of the free oligonucleotides. Plasmid was resuspended in 20 μl Tris-EDTA buffer (10 mM Tris [pH 8.0] and 1 mM EDTA).

Gel-Shift Experiments

One microgram of pGA2 was diluted in NEB2 buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol [pH 7.9] at 25°C, and NEB) containing 2 μM BQQ. The samples were heated to 80°C and slowly cooled to 37°C. Then, 5 μg of PvuII was added to the sample. The total volume was 20 μL. Digestion was conducted for 2 hr at 37°C. Samples were mixed with 5 μl of 50% glycerol and loaded on nondenaturing polyacrylamide gels. The 6% polyacrylamide gel was prepared with a 29:1 acrylamide to bisacrylamide ratio. The gel and the buffer contained TBM (100 mM Tris, 90 mM boric acid, 1 mM EDTA [pH 8.3], and 10 mM $MgCl_2$). They were run at 4°C, stained for 15 min with SybrGreen (Molecular Probes), and imaged by UV transillumination. For the pRLCMV plasmid, the process was essentially the same except that 5 μg of both AflIII and XmnI were used instead of PvuII.

In Vitro Transcription Assay

0.2 μg of the pGA2 plasmid (modified or not with the padlock oligonucleotide) was mixed in 5 μl of 80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 20 mM NaCl, and 10 mM DTT. The final concentration of plasmid was 10 nM. In some samples, the linear TFO1 was added so that the final concentration was 100 nM. One microliter of 200 μM BQQ was added, the volume was brought to 8 μl, the sample was heated to 80°C, and slowly cooled down to 37°C. Then, the tubes were transfered into ice and 1 μl (5 units) of T3 RNA polymerase was added. The transcription reaction was initiated by addition of 1 μl of the NTP mix (500 μM ATP, CTP, UTP, 100 μM GTP and 0.5 to 1 μCie α^{32}P-GTP). It was stopped after 15 min by ethanol precipitation. Samples were resuspended in a formamide loading buffer and analyzed in a 8% denaturing polyacrylamide gel.

Reporter Gene Expression in Mammalian Cells

HeLa cells (ATCC HB 8056, Rockville, MA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% heat inactivated FBS. The culture medium was supplemented with nonessential amino acids, 2 mM L-glutamine (Gibco), and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). Cells were grown at 37°C in a 5% CO_2/air incubator. Padlock-modified pRLCMV plasmid was prepared as described above, and treated as indicated for removal of BQQ.

One day before the transfection assay, cells were harvested by treatment with PET (PBS with 0.02% [w/v] EDTA and 2.5 μg/mL trypsin) at 37°C for 5 min and resuspended in the culture medium supplemented with 2 μM of BQQ at 1.10^5 cells/mL. 96-well culture plates were then seeded with 100 μl per well (10^4 cells) of the suspension of cells.

The polyamidodendrimer Superfect (Qiagen) was chosen for its ability to transfect efficiently many cell types in vitro. In order to ensure triple-helix formation between the padlock oligonucleotide and its target sequence, the padlock-modified pRLCMV plasmid (0.6 μg) was first heated to 80°C and slowly cooled down to 37°C in the presence of 66 μM of BQQ in a total volume of 10 μl of NEB 2 buffer. For control experiments, the unmodified pRLCMV plasmid was treated identically. Some control samples were heated in the absence of BQQ. The treated plasmid was diluted in 20 μl of cell culture medium devoid of serum and of antibiotics and mixed with 3 μg of Superfect in 20 μl of the same medium. Cells were cotransfected with pGL3c which was used as a control for transfection efficiency. The pGL3c plasmid was prepared in parallel by mixing 1.2 μg of plasmid with 6 μg of Superfect in a total volume of 60 μl of serum and antibiotic-free DMEM. In both cases, the Superfect:DNA mass ratio was 5:1. Both formulations were gently mixed, kept 30 min at room temperature and diluted with 115 μl of culture medium containing serum and antibiotics. Complexes of pRLCMV (165 μL) were finally mixed with complexes of pGL3c (175 μL). The final BQQ concentration was 1.94 μM. Samples were vortexed briefly, cells were washed with PBS, and 105 μl of the coformulation was loaded onto the cells (three different wells with 0.2 μg pRLCMV and 0.4 μg pGL3c each). After 2 hr, the transfection medium was removed and replaced by culture medium supplemented with 2 μM BQQ, and the cells were grown for 24 hr. For some experiments, the linear TFO2 was added in the transfection medium at a final concentration of 1 μM and the sample was heated to 80°C, then slowly cooled down to 37°C.

Firefly and *Renilla* luciferase activity were quantitated using the dual-luciferase kit (Promega) according to the instructions of the manufacturer. The cells were washed twice with PBS and lysed with

50 μl of cell lysis reagent for 30 min at room temperature, with gentle agitation. Luciferase expression was measured on 20 μl of lysate using the Luciferase Assay Reagent. Light emission was measured by integration over 10 s using a multilabel counter Victor² (EG&G Wallac, Evry, France). Light emission was normalized to the protein concentration determined using the Biorad Protein Assay (Biorad). Briefly, 250 μl of a 5× dilution of the supplied dye reagent concentrate was added to 5 μl of cell lysate and the absorbance at 595 nm was measured in 96-well microtiter plates using the multilabel counter.

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