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Targeting chromosomal sites with locked nucleic acid-modified triplex-forming oligonucleotides: study of efficiency dependence on DNA nuclear environment

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ABSTRACT

Triplex-forming oligonucleotides (TFOs) are synthetic DNA code-reading molecules that have been demonstrated to function to some extent in chromatin within cell nuclei. Here we have investigated the impact of DNA nuclear environment on the efficiency of TFO binding. For this study we have used locked nucleic acid-containing TFOs (TFO/LNAs) and we report the development of a rapid PCR-based method to quantify triplex formation. We have first compared triplex formation on genes located at different genomic sites and containing the same oligopyrimidine•oligopurine sequence. We have shown that efficient TFO binding is possible on both types of genes, expressed and silent. Then we have further investigated when gene transcription may influence triplex formation in chromatin. We have identified situations where for a given gene, increase of transcriptional activity leads to enhanced TFO binding: this was observed for silent or weakly expressed genes that are not or are only slightly accessible to TFO. Such a transcriptional dependence was observed for integrated and endogenous loci, and chemical and biological activations of transcription. Finally, we provide evidence that TFO binding is sequence-specific as measured on mutated target sequences and that up to 50% of chromosomal targets can be covered by the TFO/LNA in living cells.

INTRODUCTION

Sequence-selective compounds that target chromosomal DNA provide strategies for probing and modulating gene structure and function in living systems. Since nuclear DNA is typically bound to histones and tightly packed into chromatin, the binding and activity of these reagents must occur in this environment. One strategy to target determined genomic sites is based on triplex-forming oligonucelotides (TFOs). TFOs recognize oligopyrimidine•oligopurine sequences in DNA by binding in the major groove of the double-helix and base triplets are formed via Hoogsteen hydrogen bounds with the oligopurine strand. Structural requirements influence the design of TFOs and has led to the classification in different subtypes: TFOs containing C and T nucleotides, also named pyrimidine TFOs, bind in parallel orientation via Hoogsteen hydrogen bonds [(T,C)-motif] and TFOs containing G and A or T nucleotides [(G,A)- and (G,T)-motif] mainly bind in antiparallel orientation via reverse Hoogsteen bonds. TFOs have been shown to interfere with DNA-associated functions such as transcription, replication, repair and recombination [for reviews see (1,2)]. In addition to studies that demonstrate site-specific activity in a cellular context and which then support the ability of TFOs to target chromosomal sites, direct demonstrations of triplex formation in cell nuclei have also been reported (3–11). In some of these studies, quantitative assessments of TFO target binding has been developed [for a review see (1)]. These works show that in a cellular environment there are still many impediments to efficient triplex formation. Chromatin structure is one of them since it may preclude access to target sequences. However the mechanisms enabling TFO binding to DNA sequences in chromatin are still largely unexplored. To address this question, *in vitro* studies using reconstituted mono- and di-nucleosomes have been performed and have shown that TFOs did not form triplexes on sequences already covered by nucleosomes, except at sites located towards the extremities of the nucleosomal DNA fragments [for a review see (12)]. Concerning the mechanisms of TFO binding to chromosomal DNA in cells, the data are very few and sometimes apparently

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inconsistent: one study has described that cell cycle could influence triplex formation (7) and transcriptional dependence was observed in one system (8) and not in two others (3,5).

In this report the objectives are to provide mechanistic insights to the targeting of chromosomal DNA with TFOs, in order to enhance the potency of triplex-based strategies and to propose novel applications of TFOs. We chose to characterize the impact of target DNA environment on triplex formation, by studying TFO binding in two types of situations. TFO binding was evaluated on the same oligopyrimidine•oligopurine target sequence located, (i) at different genomic sites, so that binding in different chromosomal contexts was tested, or (ii) within a determined gene in different culture conditions, so that binding in controlled DNA states was evaluated. On one hand we show for the first time that triplex formation can take place efficiently on a silent gene. On the other hand, we clarify how and when triplex formation may depend on the level of gene transcription. Finally we demonstrate that the binding is sequence-specific, as evaluated on mutated target sequences, and that up to 50% of the chromosomal targets can be covered by the TFO in living cells. For this study pyrimidine locked nucleic acid-containing TFOs (TFO/LNAs) were used because it was demonstrated previously that TFO/LNAs improve triplex stability and bioactivity (13–15). Our results encourage further developments of TFO/LNAs as reagents that target chromosomal DNA in cells, for probing chromatin structure and function and for inducing site-directed sequence modifications.

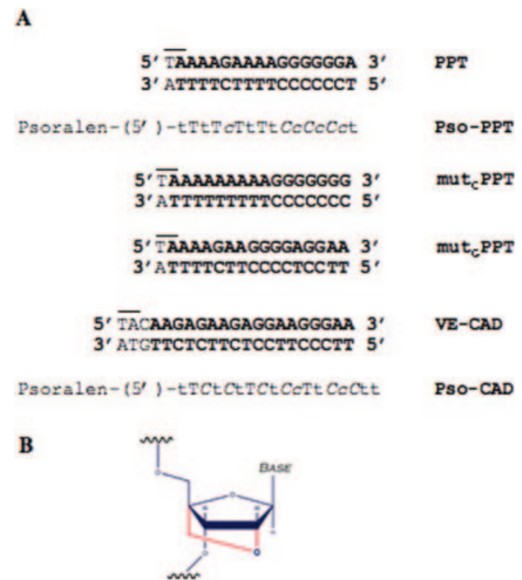

Figure 1. (**A**) Sequences of the oligopyrimidine•oligopurine DNA targets and of the oligonucleotides used in this study. Target sequences are indicated in boldface (PPT and VE-CAD), as well as the mutated sequences used as controls (mut$_C$PPT and mut$_G$PPT). The 5′-TpA site suitable for psoralen photoadduct formation is shown. Sequences of the psoralen-modified TFO directed against the wild-type PPT duplex (Pso-PPT) and the VE-Cadherin gene (Pso-CAD) are described; small letters indicate LNA nucleotides; cytosines in italic (*C* and *c*) are methylated at position 5. (**B**) Chemical structure of LNA modification.

MATERIALS AND METHODS

Oligonucleotides

Psoralen-conjugated oligonucleotide analogues with LNA residues were obtained from Proligo (France SAS) or Eurogentec (Seraing, Belgium). The 5′ end of LNA-modified TFOs, Pso-PPT and Pso-CAD (Figure 1) were modified with a 4,5′,8-trimethylpsoralen via a six or nine carbon linker, respectively.

Cell cultures and specific treatments

The CMV(+)PPT/HeLa cells were derived from HeLa/Tet-on cells (Clontech) that are engineered HeLa cells, stably expressing rTet protein necessary for the expression of doxycycline-inducible promoters. The CMV(+)PPT/HeLa cells stably contained two reporter genes, the firefly luciferase (*Photinus pyralis*) gene (*luc*) and the GFP gene, under the control of a bidirectional doxycycline-inducible CMV promoter. Parental cells, Hela/Tet-on, were electroporated with pPUR (Clontech) and pCMV(+)PPT/luc after Pvu I-linearization and puromycin-resistant clones were selected. The pCMV(+)PPT/luc plasmid derives from the bidirectional Tet-on expression vectors (pBI-Tet vectors, Clontech) and two 55 bp inserts containing either the wild-type HIV-1 polypurine tract target sequence (PPT: 5′-AAAAGAAAAGGGGGGA-3′) or a mutated sequence (mut$_G$PPT: 5′-AAAAGAA$\underline{GG}$GG$\underline{G}$GG$\underline{G}$A-3′, 4 mutations underlined) were cloned in the 5′ transcribed but untranslated regions of the luciferase and GFP genes, downstream of the transcription start site. Then the CMV(+)PPT/HeLa cells contain the PPT and the mut$_G$PPT sequences stably integrated within the cellular genome, upstream reporter genes (*luc* and GFP, respectively). The PPT triplex site in the luciferase gene was found to be integrated as a single copy.

The EA.hy926 cells were derived from fusion of human umbilical vein endothelial cells (HUVEC) with the non-endothelial lung carcinoma A549/8 cell line (gift from E. Dejana, Milano, Italy).

Both cell lines were grown in DMEM (Invitrogen) supplemented with 10% of fetal bovine serum, 2 mM glutamine, 50 U/ml of penicillin and 50 μg/ml of streptomycin. For the culture of the CMV(+)PPT/HeLa cells, addition of 500 μg/ml of G418 and 2.5 μg/ml of puromycin was provided to maintain the integrated target sequences.

When indicated, exponentially growing cells were incubated in the presence of doxycycline (Sigma) necessary for activation of the integrated CMV promoter [1 μg/ml for 24 h (Dox1) to 96 and 120 h (Dox2 and Dox3)], or in the presence of Trichostatin A [TSA (Sigma) at 40 ng/ml for 60 h]. Drug and medium were renewed regularly. For TSA, doses were chosen for CMV(+PPT)/HeLa cells and for EA.hy926 cells to enable progression through multiple cell divisions (16).

Cells permeabilization and triplex binding assays

Pso-TFOs were delivered to cell nuclei using two types of permeabilization procedures.

Digitonin permeabilization: Cell nuclei were prepared according to a digitonin-based protocol, as described

previously (3). In these conditions >90% of cells were permeabilized successfully and then treated with the psoralen-modified TFO and irradiated at 365 nm (4.5 J/cm^2).

Streptolysin-O permeabilization: Streptolysin-O (SLO; a gift from S. Bhakdi, Johannes Gutenberg-Universität, Mainz, Germany) was used to reversibly permeabilize cells. The protocol used here was a modification of a previously described SLO-based method [(17) and Supplementary Data]. Irradiation was performed, 4 h after SLO treatment.

Dra I digestion of digitonin-permeabilized cells

A buffer containing 50 mM HEPES, pH 7.2, 50 mM NaCl, 0.5 mM spermine, 10 mM $MgCl_2$ and 1 mM EDTA was used to resuspend the digitonin-permeabilized cells and for Dra I digestion. Digestion of permeabilized cells (10^7 cells) was performed at 37°C for 1 h in 250 μl of total sample volume with 200 U/ml of Dra I. DNA was purified and digested for Southern blot analysis.

Analyses of Pso-TFO-induced adducts

Following irradiation triplex-induced adducts were analyzed using different methods.

Dra I protection assay and Southern blotting. Genomic DNA was extracted just after irradiation for purification by standard protocols. For the cleavage protection assay we used a modification of our method described previously [(3) and Supplementary Data].

Quantitative PCR analyses. Two types of quantitative PCR methods, real-time and competitive PCR, were used to quantify the amount of unmodified DNA target after Pso-TFO treatment which can be amplified in contrast to the cross-linked portion (for details see Results). In both cases the amount of target fragments in each sample was compared to the amount of control fragments, obtained from amplification of a control region, as shown in Figure 1 and Supplementary Figure 1. Every quantitative PCR analysis was done at least three times.

Real-time PCR (for luc, GFP, TRβ, CASK, COL4A1 genes): Nuclei or cells were directly resuspended in a lysis buffer containing 10 mM Tris–HCl, pH 8, 0.45% NP40, 0.45% Tween-20 and Proteinase K (100 μg/ml, 2 h at 55°C and 5 min at 95°C) and then subjected to PCR analyses. Analyses were performed with Mx3005P™ Real-Time PCR System (Stratagene) (for details see Supplementary Data).

Competitive PCR (for luc and VE-Cadherin target genes): Purified genomic DNA was subjected to competitive PCR [for details see (18) and Supplementary Data].

Expression analysis of the target genes

At the time of TFO treatment, total cellular RNA were prepared with the RNeasy Mini kit (Qiagen). RNA was quantified by RiboGreen RNA Quantitation Reagent (Molecular Probe). cDNA for RT–PCR analysis was obtained by standard procedures, starting from an equal amount of total RNA for each sample and quantified by real-time PCR, as described above. Such measurements give access to the steady state level of an RNA of interest and is used here, either to evaluate if the gene of interest is expressed or not, or in the case of the doxycycline-inducible luciferase gene, to determine the relative RNA levels following different doxycycline tretaments. The sequences of the primers were described in Supplementary Table 1.

RESULTS

Experimental design

Targets and TFOs. As targets for triplex formation we chose two oligopyrimidine•oligopurine sequences, named PPT and VE-CAD (Figure 1); both sequences end in a 5′-TpA step, which is appropriate for photoaddition by a psoralen moiety. We found that the PPT sequence with the neighbouring 5′-TpA step, 16 bp pair long or a 15 bp long version just lacking the A-3′ (5′-AAAAGAAAAGGGGGG-3′ named PPT_{15}), is present at different genomic loci. We studied TFO binding on two of them: the calcium/calmodulin-dependent serine protein kinase gene (CASK) located on chromosome X and the thyroid hormone receptor beta gene (TRβ) located on chromosome 3. In addition, to manipulate transcription at the TFO target site in genomic DNA, the PPT sequence was introduced downstream a doxycycline-inducible promoter and upstream the luciferase reporter gene (*luc*) and stably integrated in the genome of HeLa/Tet-on cells. Finally, to study the influence of transcriptional activation on TFO binding in another context, a second oligopyrimidine•oligopurine sequence was targeted in the VE-Cadherin gene located on chromosome 16 (VE for Vascular Endothelial).

The TFOs used in this study, Pso-PPT and Pso-CAD (see sequences in Figure 1), were linked to psoralen. They were composed of alternating LNA and DNA nucleotides all along the sequence, with two LNA modifications at the 3′ end to increase resistance to 3′-exonucleases (19). We already described that anti-PPT TFO/LNAs have considerably improved antigene properties compared with the isosequential phosphodiester oligomers (15). Here we used UV irradiation in order to produce covalent triplexes with Pso-PPT and Pso-CAD on the corresponding DNA target. In both cases, photo-adducts were formed at the 5′-TpA-3′ sequence present at the 5′ end of the oligopurine tract and the majority of lesions was *bis*-adducts (∼90%) while a minority of mono-adducts (∼10%) were obtained (data not shown), as already described with other TFO chemistries (20).

Quantification of triplex formation in genomic DNA. Our goal is to characterize triplex formation in a quantitative manner in different chromatin contexts. For this purpose we developed a PCR-based approach that allows quantification of triplex formation whatever the targeted site in the genome. Briefly our method is based on the fact that cross-linked DNA molecules such as the ones obtained after treatment by Pso-TFO and irradiation, are not substrates for PCR amplification using primers located on each site of the cross-link. Real-time PCR was used to quantify the amount of amplified products in samples treated by Pso-TFO and irradiated. Two sets of primers were used: one for amplification of the target region and another one for amplification of a control region lacking the target site, as schematically described on Figure 2B. Then the relative PCR inhibition (target region/control region) might reflect the level of triplex-induced cross-links. In order to characterize this dependence we

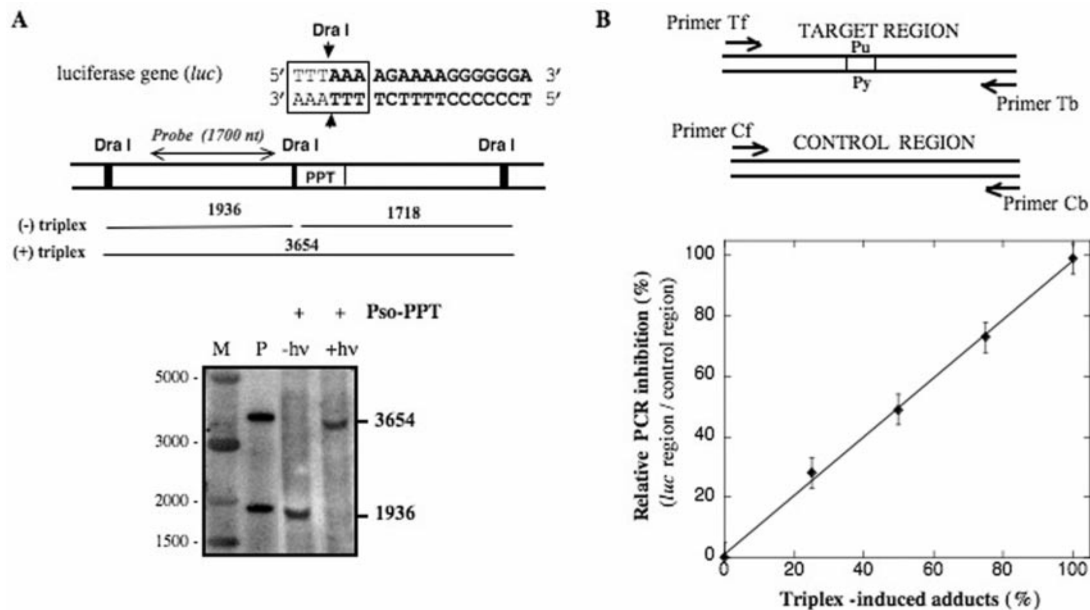

Figure 2. Quantification of triplex formation. (**A**) Quantification of triplex formation by Dra I protection assay. (Upper panel) The 16 bp oligopyrimidine•oligopurine target sequence (PPT) present upstream the luciferase gene overlaps a Dra I recognition sequence (boxed); the two arrows indicate the sites of Dra I cleavage. Dra I sites around the PPT sequence are shown and the lengths of the fragments in base pairs obtained after Dra I cleavage are indicated. Location of the RNA probe used for Southern blot analysis is shown. (Lower panel) Naked genomic DNA of CMV(+PPT)/HeLa cells was treated with Pso-PPT and irradiated (+hν) or not (−hν); then the DNA was analyzed by Dra I protection assay (Supplementary Data). DNA markers: (lane M), 1 kb DNA marker (New England Biolabs); (lane P), pCMV(+PPT)/luc plasmid that was used to generate the CMV(+PPT)/HeLa cells, was treated by Pso-PPT, irradiated and cleaved by Dra I. (**B**) Quantitative PCR analysis of site-directed inter-strand lesions. (Upper panel) Schematic representation of the quantitative PCR analysis method. Regions containing the oligopyrimidine•oligopurine target sequence (Py•Pu) were amplified by quantitative PCR with primers flanking the Py•Pu sequence (primers Tf and Tb). The amounts of these amplified products ('target fragments') were compared to that of the amplified products obtained in the same sample from a control region ('control fragments') (primers Cf and Cb). (Lower panel) The genomic DNA that was 100% modified by Pso-PPT adducts at the PPT site in the luciferase locus (sample corresponding to lane +hν in Figure 2A) was mixed in various proportions with untreated genomic DNA of CMV(+PPT)/HeLa cells (1:0; 1:0.25; 1:0.5; 1:0.75; 0:1) in order to obtain defined amounts of triplex-induced adducts (with 100, 75, 50, 25, 0%). Relative PCR inhibition (amount of amplified *luc* target fragments/amount of amplified control fragments) is reported as a function of the amount of triplex-induced adducts in the sample (measured by Dra I protection assay).

prepared genomic DNA with determined amounts of cross-links and measured the relative PCR inhibition as described above (Figure 2). The samples were prepared starting from 100% cross-linked genomic DNA that was further mixed with untreated genomic DNA in appropriate ratios (0:1, 1:4, 1:2, 3:4, 1:0 matching 0, 25, 50, 75 or 100% of triplex-induced adducts). We demonstrated that relative PCR inhibition was linearly correlated to the percentage of triplex-induced lesions, that were measured by a well-validated method, protection from enzyme cleavage and Southern blot analysis. We also verified that the same amounts of triplex-induced adducts were obtained with the real-time PCR developed here and the competitive PCR assay we reported previously (3). It is of importance that no inhibition was detected when irradiation was omitted, demonstrating that this type of analysis only measured the covalently psoralen-modified DNA targets and not reversible triplex formation that could occur independently of irradiation.

Targeting the same sequence present in different chromosomal contexts

We exploited the fact that the same oligopyrimidine• oligopurine sequence is present at various locations in the human genome to investigate the influence of different chromosomal contexts on the efficiency of TFO binding. Indeed we chose to study three genes that contain the PPT sequence in the transcribed region and have different expression status in HeLa cells: the CASK gene is expressed, the TRβ and the integrated luciferase locus (*luc*) are not. In addition, two mutated oligopyrimidine•oligopurine genomic targets, mut_CPPT in COL4A1 gene and mut_GPPT in GFP, were used to establish TFO binding specificity (see sequences in Figure 1). The PCR-based analysis described above was used to determine the Pso-PPT-induced adducts in these three target genes containing the PPT_{15} sequence, as well as on the two mutated target sequences.

On naked genomic DNA, we demonstrate that the Pso-PPT can recognize its double-stranded target present at different locations within genomic DNA in a sequence-specific manner and that complete coverage of the three target sites can be obtained (Figure 3A). The specificity of TFO-induced lesions at the PPT sequence in genomic DNA was established by the use of another pyrimidine psoralen-conjugate TFO (Pso-CAD) and of the two mutated target sequences described above. Under conditions allowing almost complete modification of the PPT sequence by the Pso-PPT, no detectable triplex formation was obtained, either with the Pso-CAD at the PPT site or with the Pso-PPT at the mut_GPPT target, and around 20% PCR inhibition was measured with the Pso-PPT at the mut_CPPT target. It is noteworthy that the COL4A1 gene was originally chosen as one of the PPT_{15} containing gene.

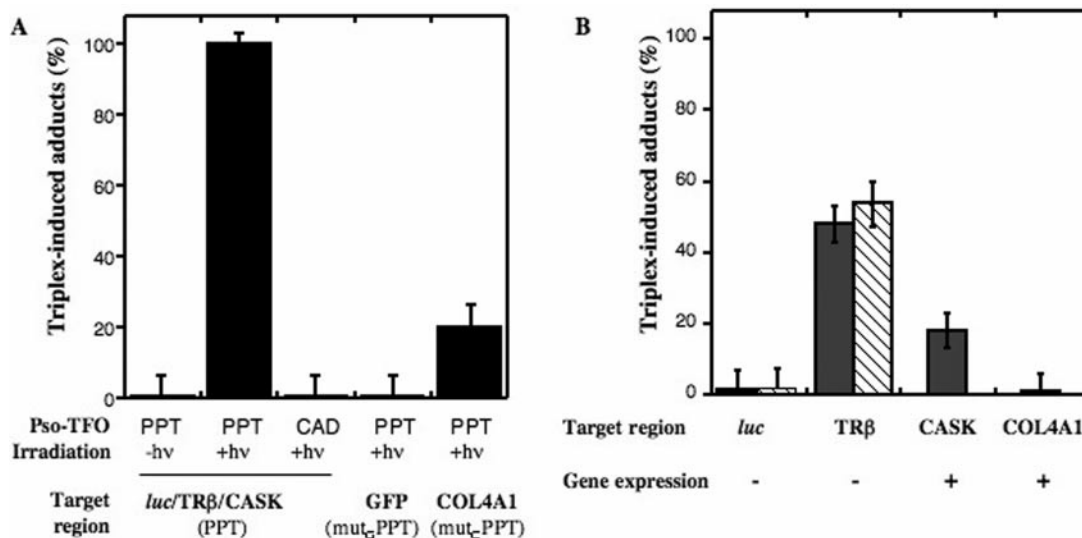

Figure 3. Efficient TFO binding is not correlated to gene expression at the target site. Triplex formation was evaluated on PPT sequences present at different genomic loci. The target regions that were PCR-amplified are: *luc* (integrated luciferase locus), TRβ and CASK genes that all contain the PPT_{15} sequence, GFP (integrated GFP locus) and COL4A1 genes that contain a mutated PPT sequence (mut_CPPT and mut_GPPT, respectively) (see precise genomic locations in Supplementary Data). (**A**) Genomic DNA of CMV(+PPT)/HeLa cells was treated with Pso-PPT or Pso-CAD TFO, with or without irradiation and the amount of cross-linked targets was evaluated. (**B**) CMV(+PPT)/HeLa cells were treated and permeabilized in the presence of Pso-PPT (10 μM) and irradiated. The percentages of triplex-induced adducts on the different genomic targets were reported. Equivalent levels of triplex formation were obtained in nuclei preparations (digitonin-permeabilized cells; closed columns) and in whole cells when Pso-PPT was delivered by SLO permeabilization (hatched columns). The expression status of the target genes was indicated: (+) expressed gene; (−) no detectable expression.

Indeed the central mutation in the mut_CPPT sequence was a single polymorphism that was not mentioned in the genome database and that we directly sequenced to understand the unexpected low TFO binding at this locus. The other target sequences were then also sequenced and were shown to contain the expected oligopyrimidine•oligopurine sequence.

Following digitonin permeabilization, we found that Pso-PPT-mediated triplex was formed on the different target genes with various efficiencies (Figure 3B): 45% at TRβ, 15% at CASK and 0% at non-induced *luc*; no binding was detected on the mutated mut_CPPT sequence present on COL4A1 gene. We observed that a silent gene can be either inaccessible to TFO as it is the case for the non-induced *luc* gene, or more surprisingly, highly accessible as it is the case for the TRβ gene. It can also be noticed that the silent TRβ gene is more efficiently targeted than the expressed CASK gene. On the basis of these data it is tempting to suggest that the transcription itself is dispensable at some chromosomal sites for chromatin accessibility to TFOs and that efficient TFO binding is not necessarily correlated to gene expression at the target site.

Targeting a determined chromosomal site in different transcriptional states

Transcription is one of the factors that is known to modify the chromatin structure. We then studied the influence of transcriptional activation and of transcriptional levels of the target gene on TFO binding in three situations: (i) cell type-dependent expression; (ii) manipulation of transcriptional level by treatment with a chemical, here doxycycline; and (iii) induction of transcriptional activation by Trichostatin A (TSA) treatment.

The VE-Cadherin gene was chosen as a target because its expression varied considerably from a cell type to another (21). It is high in endothelial cells as EA.hy926 cells but not detectable in HeLa cells (Figure 4A). These two cell lines were transfected with Pso-CAD via digitonin permeabilization, then irradiated or not. The amount of triplex-induced adducts just after irradiation was estimated by the PCR-based method described previously. Weak binding (∼15%) was detected in non-expressing cells, but it was increased in expressing cells up to 50%.

The *luc* gene was used to study another type of transcriptional activation and also to further evaluate TFO binding at different levels of transcription. CMV(+)PPT/HeLa cells were treated or not with doxycycline in different conditions to induce transcription at different levels, and subsequently permeabilized with digitonin and treated with oligonucleotides (Figure 4B). No binding was detected in the absence of doxycycline-induced transcription. In contrast targeted cross-links were considerably enhanced as soon as transcription was chemically induced at the target site and they increased with the transcriptional level of the *luc* gene. Up to 50% of chromosomal targets can be specifically modified. However we observed that efficiency of triplex formation did not vary linearly with transcriptional activity and did reach a plateau of 50%, observed even for a high increase in RNA levels. It can be noticed that in conditions allowing maximal coverage of the target site, no binding was detected on the mutated mut_GPPT sequence that is present in the same transcriptional context within the GFP gene, also under the control of the CMV doxycycline-inducible promoter.

Finally transcription activation of target genes was induced by treatment with TSA, a well known histone deacetylase inhibitor. The long-term TSA treatment that we used

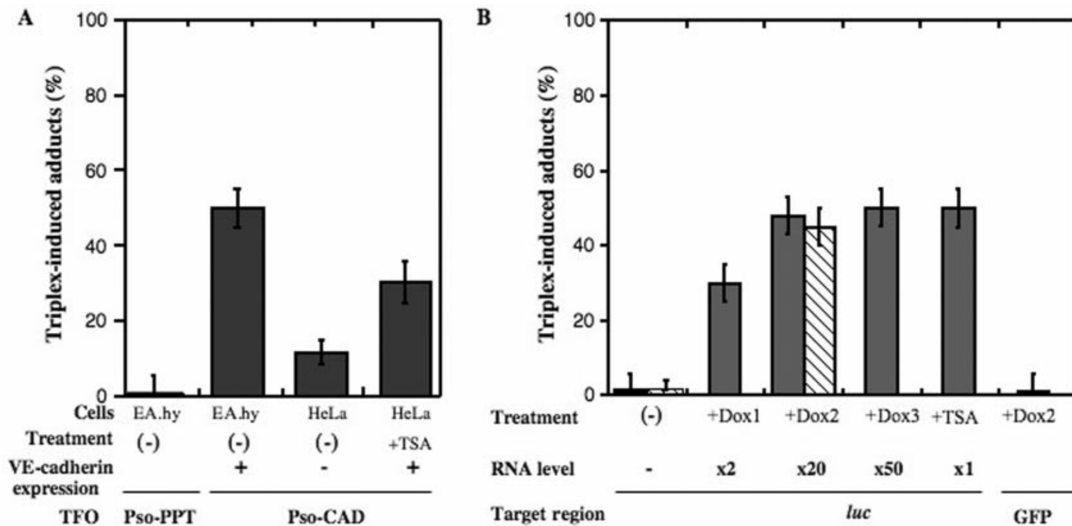

Figure 4. Transcriptional activity influences efficiency of TFO binding in some situations. (**A**) Triplex formation on the VE-Cadherin gene in different cellular contexts. Percentages of triplex-induced adducts in cell nuclei after treatment with the Pso-CAD (5 μM). Two cells lines were used, either endothelial (EA.hy926) or CMV(+PPT)/HeLa cells, in absence (−) or in presence of TSA treatment (+TSA). VE-cadherin expression at the time of TFO administration was indicated: (+) expressed gene; (−) no detectable expression. (**B**) Triplex formation on the integrated target in different transcriptional contexts. Percentages of triplex-induced adducts on the PPT target (luciferase gene) and on the mut_GPPT sequence (GFP gene) in cells (closed columns, cell nuclei; hachted columns, whole cells). Before TFO addition, different treatments were performed, as indicated. Relative luciferase RNA levels determined just before Pso-PPT addition were reported; (−) means that the gene is not expressed, with an RT–PCR signal close to the background level.

(40 ng/ml for 5 days) has been described and characterized previously (16): it has been shown to globally increase histone acetylation, measured using antibodies against histone H4 acetylated at lysine 5 and 8, and to have a marked effect on chromatin structure with the possibility to even unfold highly compact regions. It was used here as a way to induce expression of the silent genes studied above, VE-cadherin and *luc*: for these target genes, such TSA exposure provoked enhancement of gene transcription and induced a substantial improvement in the amounts of TFO-modified targets (2- and >10-fold for VE-Cadherin and *luc*, respectively; Figure 4).

Targeting chromosomal sites in whole cells versus cell nuclei

Works aimed at studying chromatin structure and chromosomal DNA accessibility were mainly performed in nuclei using preparation that maintain chromosomal structure. Such approaches were also largely used to quantify TFO binding in chromatin—including in the present paper—since they enable uptake limitation to be overcome and deliver large amounts of TFO in nuclei by passive diffusion through the nuclear membrane. Here we also evaluated TFO-based chromosomal targeting in whole cells. TFOs were introduced by streptolysin-O (SLO) reversible permeabilization which is among the most efficient method existing at present to deliver oligonucleotides. Thus typically, using an optimized amount of SLO, around 70% of CMV(+)PPT/HeLa cells were successfully permeabilized without marked cell killing (Supplementary Figure 2). TFO binding to the PPT_{15} sequence present on *luc* and TRβ genes was determined in whole cells (Figures 3 and 4, hatched columns). Equivalent results were obtained with binding efficiencies in SLO-permeabilized living cells of 42% for the doxycycline-activated *luc* gene and 52% for TRβ, compared to 48 and 45% in cell nuclei, respectively. Dependence of TFO binding on *luc* gene activation was also observed. These data suggest that targeting of chromosomal sites by TFOs was mainly limited by the nuclear environment of the DNA target and not by the cellular context *per se*.

DISCUSSION

Triplex-induced activities in cells have been largely reported, whereas there are few studies that have attempted to provide direct evidence of TFO binding to chromosomal DNA in nuclei preparations and even fewer in living cells, as described here. In all these studies the triplex fraction in treated cells was from 1 to 30%. Interestingly in two studies the extent of TFO binding was compared in cell nuclei and in whole cells (6,11). Binding was strongly decreased and even abolished in whole cells, most likely due to a reduced TFO concentration, that could be explained either by poor transfection or entrapment by cellular proteins. In our experimental conditions we observed equivalent efficiencies in the two settings, nuclei or cells, supporting that there is no intrinsic cellular limitation to TFO binding besides the chromatin structure at the target site. Concerning efficiency, we showed that >50% of a specific DNA sequence can be successfully targeted when both TFO chemistry and DNA nuclear environment are appropriate. TFO/LNAs were recently shown to be active on transiently transfected templates that might be inefficiently assembled into chromatin if at all (15). Here, they appear to be also efficient to target DNA sequences in a chromosomal environment. However we never observed complete coverage of the target, consistent with an heterogeneity of the chromatin structure in the gene population at the time of analysis (22). Concerning specificity we provide evidence

that irrelevant TFO sequences were inactive and also, highly important and rarely done, that mutated targets, containing mutations, were inefficiently recognized, both in naked DNA and in chromatin (Figures 3 and 4).

Different approaches have been described to quantify TFO binding in genomic DNA. They all make use of TFOs covalently linked to a reactive moiety and measure the amount of triplex-induced modification at the target site. Here we have developed a quantitative PCR-based assay that is robust, consumes little material and is also much more rapid than methods described already, including PCR-related ones. It is based on PCR inhibition produced by an inter-strand DNA lesion, a cross-link in our case, when using primers around the damaged site. The level of PCR inhibition directly reflects the amount of modified targets. We have validated it by comparison with two already established methods, both restriction enzyme protection assay associated with Southern blotting and competitive PCR. Then the described approach will be useful to determine DNA accessibility to TFOs at different genomic sites and to measure a large variety of site-directed DNA lesions, as well.

Using this method, we have explored some of the mechanisms enabling accessibility of chromosomal DNA to TFOs. To address this question we have studied two types of situations that are discussed below.

We have first examined the influence of various chromosomal contexts. The same TFO was used to target the oligopyrimidine•oligopurine PPT sequence that is present in different genes. Binding efficiency did vary from one gene to another and was not correlated with the fact that the gene is expressed or not, since a silent gene, such as TRβ in HeLa cells, can be efficiently targeted. There would be at least two possible explanations for such accessibility to TFO of the silent TRβ sequence. One is that it might be located in an unfolded large-scale chromatin structure produced independently of transcription, as already proposed in other systems (23). A second possibility is that antisense transcription from the strand opposite to the coding strand might occur within the target region, even if such RNA species has not been presently described to our knowledge.

Then we have further characterized the role of transcription, by studying targeting of a given gene by TFOs in different transcriptional contexts. Transcriptional influence on triplex formation in chromatin was already reported but the results are apparently contradictory. In the case of a *lacZ* transgene, an increase in TFO binding was observed in cell nuclei after a 250-fold activation of target gene expression, measured at the protein level (8). However, in two other studies, treatments known to enhance transcription did not affect the level of triplex formation (3,5). In this work we have observed two major situations. On one hand we have demonstrated that transcriptional activation enhanced TFO access to chromatin in the case of a silent gene that is not or weakly accessible to a TFO, such as *luc* and VE-cadherin genes, respectively. Such transcriptional dependence was observed for different types of activation and at integrated and endogenous chromosomal loci; it seems to be a general phenomenon. It must be noticed that we report that it is possible to specifically and efficiently target a gene in its active state without targeting the silent state. Our data support an increased access to TFO of transcribed regions, where the oligopyrimidine•oligopurine targets are located, following transcriptional activation. The role of this latter phenomenon in opening chromatin structure was extensively documented in the promoters but also, even if more rarely, in transcribed regions (24). On the other hand, we have shown that beyond a certain level of transcriptional activity, an additional increase had no impact on efficiency of targeting by TFOs. In fact, targeting an inducible gene whose transcription can be precisely controlled, we observed that TFO binding started to increase with transcriptional activity and finally reached a plateau. These data suggest that for highly transcribed genes, further increase in transcription might have no effect on triplex formation. This type of result can explain the apparently inconsistent data reported previously [(3,5) compared to (8)]. It would be due to the RNA polymerase helicase activity that could remove the triplexes, as described for other helicases, and reduce the probability of triplex formation during the irradiation. It is also relevant in the light of recent findings describing that above a certain level of transcription and RNA pol II density, histones lose contact with DNA and efficient re-establishment of histone–DNA contacts after polymerase passage might not be possible, resulting in a net loss of nucleosomes.

Together it is tempting to suggest that TFO binding is efficient in open chromatin and may occur competitively with nucleosome binding, enlarging *in vitro* data obtained with reconstituted nucleosomes. To support this model, chromatin accessibility of the targeted locus was assessed by using a standard restriction enzyme accessibility test. We chose to study the luciferase locus that is appropriate for Dra I analysis. We showed that chromatin access within this locus paralleled the percentage of triplex-induced adducts: no Dra I cleavage of the *luc* gene was observed in nuclei of non-activated cells, whereas *luc* gene was cleaved after doxycycline activation (Dox2 treatment; data not shown). Finally, we reported a strong increase in TFO binding following TSA treatment that induced transcriptional activation of the target gene, likely by increasing histone acetylation. These data suggest that it may exist a relation between histone acetylation and more generally the nature of histone modifications in the vicinity of the target site and the efficiency of TFO binding. Indeed it appears more and more clearly that the 'histone code' that defines silent, active, accessible chromatin regions is more complicated than expected and likely results in a combination of histone modifications [e.g. (25)]. The combination(s) defining chromatin regions that are accessible to DNA ligands such as TFOs remains to be determined.

In conclusion, the results presented here have practical significance for triplex-based strategies. Among oligonucleotide technologies, TFOs offer unique potential as DNA binding molecules that recognize specific sequences in chromatin. They would be useful both for positioning DNA interacting or reactive compounds, such as anticancer drugs, based on chemical conjugation of these reagents to TFOs, and for probing chromatin structure and function. On one hand, our data suggest that targeting of a gene which is specifically expressed under some physiological conditions, e.g. during tumor development, might occur preferentially in the conditions of active transcription. This will be an important consideration for specific targeting of some tumor-associated

genes in cancer versus normal cells. On the other hand, TFOs can also efficiently access to chromatin at silent or weakly expressed genes and likely at intergenic regions. These results will encourage the use of TFO/LNA as synthetic tools for investigating fundamental questions of chromosome biology at these loci.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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