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Accessibility of nuclear DNA to triplex-forming oligonucleotides: The integrated HIV-1 provirus as a target

(oligonucleotide–psoralen conjugate/DNA accessibility/competitive PCR/antigene oligonucleotides/HIV)

CARINE GIOVANNANGELI*†, SILVIA DIVIACCO*, VALÉRIE LABROUSSE‡, SERGEI GRYAZNOV§, PIERRE CHARNEAU‡, AND CLAUDE HELENE*

*Laboratoire de Biophysique, Muséum National d'Histoire Naturelle, Institut National de la Santé et de la Recherche Médicale–Unité 201, Centre National de la Recherche Scientifique, Unité Recherche Associée 481, 43 rue Cuvier, 75231 Paris Cedex 05, France; ‡Laboratoire d' Oncologie Virale, Institut Pasteur, Centre National de la Recherche Scientifique, Unité Recherche Associée 1156, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France; and §Lynx Therapeutics, Inc., 3832 Bay Center Place, Hayward, CA 94545

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ABSTRACT **The control of gene transcription by antigene oligonucleotides rests upon the specific recognition of double-helical DNA by triplex-forming oligonucleotides. The development of the antigene strategy requires access to the targeted DNA sequence within the chromatin structure of the cell nucleus. In this sudy we have used HIV-1 chronically infected cells containing the HIV provirus as endogenous genes to demonstrate that the integrated HIV-1 proviral genome is accessible to triplex-forming oligonucleotides within cell nuclei. An oligonucleotide–psoralen conjugate targeted to the polypurine tract (PPT) of the HIV-1 proviral sequence was used as a tool to convert the noncovalent triple-helical complex into a covalent lesion on genomic DNA after UV irradiation of cells. Triplex-derived adducts were analyzed using two different methods. The photo-induced psoralen cross-link prevented cleavage of the target sequence by *Dra*I restriction endonuclease, and the sequence-specific inhibition of cleavage was revealed and quantitated by Southern blot analysis. A quantitative analysis of cross-linking efficiency was also carried out by a competitive PCR-based assay. These two approaches allowed us to demonstrate that a triplex-forming oligonucleotide can recognize and bind specifically to a 15-bp sequence within the chromatin structure of cell nuclei.**

Oligonucleotides can be designed to bind to double-stranded DNA at oligopurine·oligopyrimidine sites where they form a local triple-helical structure (see refs. 1 and 2 for reviews). This provides a general approach to the sequence-specific recognition and targeting of double-stranded DNA for both basic research and therapeutic applications. However, the description of triplex-mediated effects in cell cultures is still rare. Inhibition of gene expression by triplex-forming oligonucleotides has been demonstrated on plasmid targets transiently transfected into living cells (3–7). When a triplex was preformed with the target *in vitro* and then the complex was transfected into mammalian cells, inhibition of gene expression was observed. We have previously shown that transcription of the gene coding for the α-subunit of the interleukin 2 receptor was inhibited when cells were first transfected with a reporter plasmid in the presence of a psoralen–oligonucleotide conjugate and then irradiated (3), or when transfected cells were incubated with an acridine–oligonucleotide conjugate without any irradiation (8, 9). Mutations have been detected on plasmid vectors after irradiation of transfected cells in the presence of psoralen–oligonucleotide conjugates (10). Even in the absence of irradiation a low level of mutation was detected and was attributed to transcription-coupled repair (11). Location of the mutation sites was as it was expected on the basis of triplex formation. Detection of the DNA mutations is a very sensitive method to demonstrate triplex formation, but it does not allow quantitative analysis of the amount of triplex formed within cells. Using dimethyl sulfate footprinting technique, Svinarchuk *et al.* (7) were unable to detect triplex formation in transfected cells that were further incubated with a triplex-forming oligonucleotide. The footprint was detected only when the complex was preformed *in vitro* before transfection.

There have been several reports describing the use of triplex-forming oligonucleotides to inhibit the expression of endogenous genes (12–17). However, none of these reports provided direct evidence for the implication of triplex formation at the target site in the inhibitory activity. Even when there is an indication of triplex formation [based on inhibition of DNase I hypersensitive sites on the c-*myc* gene, (13)], the mechanism of gene inhibition more likely involves transcription factor binding to the G-rich oligonucleotide acting as a decoy (18).

In the present study we demonstrate that a triplex-forming oligonucleotide, directed against the HIV-1 polypurine tract, can specifically recognize and bind its 15 bp target located on nuclear DNA involved in the intact supranucleosomal structure of chromatin. This is based on a procedure we have developed using an oligonucleotide–psoralen conjugate as a tool to trap the triple-helical complex formed in the cell nucleus. This approach exploits the photochemical reaction of psoralen with the DNA strands at the triplex site which converts a noncovalent triplex into a covalent one and results in a localized damage on genomic DNA. Two different assays were used to quantitatively analyze the oligonucleotide–psoralen cross-links to genomic DNA. These procedures allowed us to examine triplex formation within the cell nuclei directly and to evaluate both the specificity and the efficiency of the reaction.

MATERIALS AND METHODS

Oligonucleotides. The unmodified oligodeoxynucleotides used as PCR primers in this study were obtained from Eurogentec (Brussels) (see sequences in legend of Fig. 3*A*). Oligonucleotide analogues with N3′ → P5′ phosphoramidate (np) linkages were synthesized as described (19).

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Abbreviations: PPT, polypurine tract; po, phosphodiester; np, phosphoramidate.

†To whom reprint requests should be addressed. e-mail: giovanna@mnhn.fr.

Psoralen-modified oligonucleotides (Pso-15TCG, see sequence on Fig. 1) were prepared by coupling the psoralen C6 phosphoramidite derivative of 4′-hydroxymethyl-4,5′,8-trimethylpsoralen (Glen Research, Sterling, VA) to the 5′ end of the oligonucleotide containing phosphodiester (po) or np linkages. The po oligonucleotides used in this study were 3′ modified to resist nuclease-mediated degradation by incorporation of either a triethyleneglycol or a propylamine group. Similar results were obtained with either modification.

Cell Cultures. Cells were grown in RMPI 1640 medium (GIBCO/BRL) supplemented with 50 units/ml of penicillin, 50 μg/ml of streptomycin, 2 mM glutamine, and 10% heat-inactivated fetal bovine serum, at 37°C in a 95% air/5% CO_2 atmosphere. Cells were routinely maintained at a density of 0.25–2× 10^6 cells/ml.

All the cells used in this study were chronically infected cells containing HIV-1 proviral DNA integrated within the cellular genome. The U1 cell line, derived from the U937 cell line, was obtained from American Type Culture Collection (20, 21). It does not contain any detectable amount of unintegrated HIV (21) but contains two copies of the HIV proviral genome per cell.

Cell Permeabilization and Triplex Binding Assay. Exponentially growing cells were harvested by centrifugation and washed with ice-cold PBS. All subsequent operations were performed on ice with precooled buffers. The permeabilization protocol was a modification of the previously described digitonin method (22–25). Cells were resuspended in a buffer containing 10 mM Tris·HCl (pH 7.5), 10 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, and 50 μg/ml digitonin, and incubated for 5 min. After washing twice with digitonin-free buffer, cells were resuspended at 2 × 10^7 cells/ml in triplex-forming buffer containing 10 mM Tris·HCl (pH 7.0), 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM EDTA. Cells were examined with respect to permeabilization efficiency and nuclear integrity (by phase-contrast microscopy after staining with trypan blue, or by confoncal microscopy after labeling using an oligonucleotide–fluorescein conjugate and/or propidium iodide).

Triplex-Induced Adduct Formation. Irradiation with UV light was performed with a 365-nm monochromatic system: a flood lamp (100 W) in a Bioblock (Illkrich, France) housing system was used for irradiation. The dose of 365-nm radiation delivered to the samples was measured using a radiometer/photometer (Bioblock): the typical fluence was 5–10 mW/cm^2. In all cases, samples were irradiated after incubation in the triplex-forming buffer (10 mM Tris-HCl, pH 7.0/50 mM NaCl/10 mM $MgCl_2$/1 mM EDTA). Just after irradiation, triplex-induced adducts were analyzed by *Dra*I protection or competitive PCR assays, as indicated.

***Dra*I Protection Assay.** Digestion of purified genomic DNA by the *Dra*I restriction enzyme was carried out at 37°C overnight. The fragments generated by cleavage of genomic DNA were separated by electrophoresis in 1% agarose gels in Tris-acetate buffer. Agarose gels were transferred to nylon membranes (N+ hybond; Amersham) using standard procedures. The DNA probe was a restriction fragment (*Sal*I–*Nhe*I) generated by digesting HIV-1 DNA cloned in a plasmid (pBRU; see Fig. 1*A*), and purified by gel electrophoresis. The DNA fragment was labeled by random priming (Amersham kit) and purified on a Sephadex column. Blot quantitations (±10%) were obtained by PhosphorImager analysis.

Competitive PCR Experiments. *Primer choice and competitor construction.* Two sets of three primers were constructed for the HIV sequence as described for other systems (26–28). The localization of these primer sets is shown in Fig. 3*A*, and their nucleotide sequences are given in the legend of Fig. 3. Each primer set consists of (*i*) two 28-nt long oligonucleotides used for PCR amplification (external primers called SX and DX), and (*ii*) one oligonucleotide (internal primer called SX–Comp) used only for competitor construction (see Fig. 3*A*). This primer consists of a 5′ sequence of 28 nt corresponding to one (SX) of the external primers, linked at its 3′ end to a specific sequence complementary to a region within the genomic sequence amplified by the external primers. Competitors for competitive PCR assays were obtained by PCR amplification of purified genomic DNA of U1 cells; they consisted of the amplified product obtained after successive PCRs: first, using the internal primer (SX–Comp) and one of the external primer (DX); second, using the two external primers (SX and DX).

Quantitation. The competitor DNA fragments were quantitated by coamplification of known amounts (10-fold dilutions, followed by progressively closer dilutions in the range of equivalence) of competitor with a fixed amount of genomic DNA of U1 cells, using the external primers. Competitors were then used in competitive PCR assays on partially cross-linked genomic DNA after oligonucleotide treatment to quantitate the efficiency of triplex-induced cross-links (Fig. 3*A*). Conditions for PCR amplifications were as follows: (*i*) 30 sec at 94°C, (*ii*) 30 sec at 55°C, and (*iii*) 30 sec at 72°C. Forty cycles of amplification were performed with 1 unit of *Taq* polymerase (Perkin–Elmer).

RESULTS AND DISCUSSION

Triplex-Forming Oligonucleotide–Psoralen Conjugate as a Tool. The ability for a triplex-forming oligonucleotide to bind its double-stranded target in a cellular environment—i.e., on genomic DNA in its intact supranucleosomal structure in cell nuclei—has not been described as of yet. This is mainly due to the difficulty in detecting such a non-covalent complex by *in situ* footprinting methods. To address this problem we decided to use oligonucleotide–psoralen conjugates. After photoactivation, the triplex formed by oligonucleotide–psoralen conjugate can be converted to a localized irreversible, covalent lesion on genomic DNA that can then be purified and further analyzed.

As a target for triple helix formation we have chosen a 16 bp oligopurine·oligopyrimidine sequence on proviral HIV-1 DNA; this sequence is called PPT and two copies are present in the HIV-1 genome (Fig. 1). An oligonucleotide containing thymines, cytosine, and guanines (15TCG; see sequence in Fig. 1) binds the PPT sequence by triplex formation (29). The use of an oligonucleotide analogue containing N3′ → P5′ np linkages (30) strongly enhanced triplex stability as compared with the isosequential po oligomers (31). After UV irradiation, both the mono-adduct (MA) and the bis-adduct (XL) of Pso-15TCG were obtained with the duplex target, at the 5′-TpA-3′ sequence present at the 5′ end of the oligopurine tract at the duplex–triplex junction (Fig. 1*A*; data not shown). A majority of lesions was bis-adducts (90–95%), while mono-adducts representing 5–10% of the total adducts were obtained on the pyrimidine-containing strand as described (32, 33). Similar results were obtained with the po and the np oligomers.

Triplex Detection on Genomic DNA. We first used naked genomic DNA as a target for Pso-15TCG (po and np). Genomic DNA was isolated from persistently infected U1 cells containing two integrated copies of the HIV-1 genome per cell. The U1 genomic DNA contains many *Dra*I cleavage sites (5′-TTT ↓ AAA-3′). The products of the *Dra*I cleavage reaction in the HIV-1 region of the U1 genome around the PPT triplex site are indicated on Fig. 1*A*. Two DNA fragments (1068 and 2402 bp long) appeared after Southern blot analysis with a probe covering the PPT site. They arise from *Dra*I cleavage at three sites, one of which overlaps the triplex formation site (Fig. 1*A*). If triplex formation inhibits *Dra*I cleavage at the PPT site the 1068- and 2402-bp fragments should disappear and give rise to a longer fragment, 3470 (= 1068 + 2402) bp long. Triplex specificity can be demonstrated

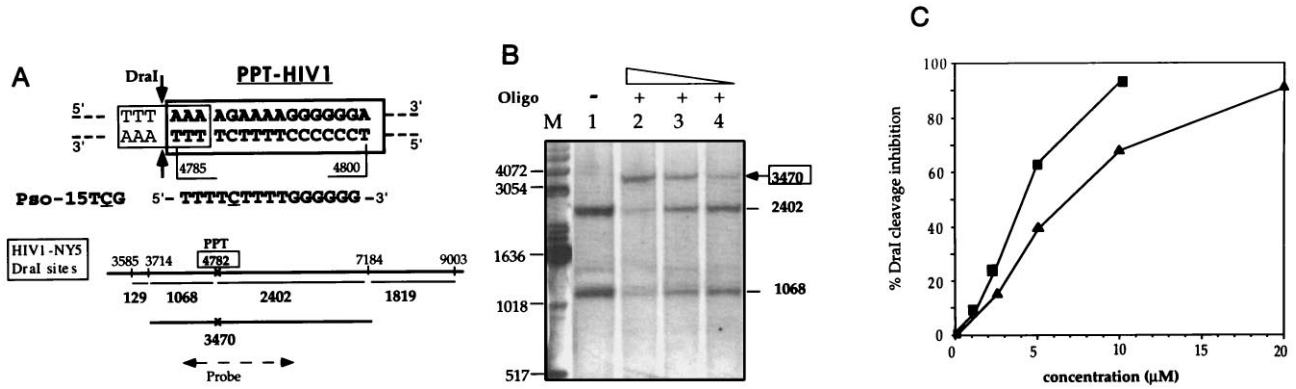

FIG. 1. *Dra*I protection assay on naked genomic DNA. (*A Upper*) The 16-bp oligopurine·oligopyrimidine sequence we have chosen as a target for triple helix formation is indicated in boldface type. It is present in the HIV-1 genome and is called PPT. The PPT triplex site overlaps the *Dra*I recognition sequence (thin line); the two arrows indicate the sites of *Dra*I cleavage. The sequence of the psoralen-modified third strand is indicated. Underlined cytosine (C) indicates a 5 methyl cytosine. Pso- indicates a psoralen derivative attached to the 5′ phosphate (see *Materials and Methods*). (*A Lower*) *Dra*I sites around the PPT triplex site (which is shown by a cross and boxed) present in the *pol* gene (HIV-NY5 sequence). sequence). The lengths of the fragments obtained after *Dra*I cleavage are indicated. Location of the DNA probe (1902 bp long) used for Southern blot analysis is shown (broken arrow); the probe hybridizes with the 1068- and 2402-bp fragments over 897 and 1005 nt, respectively. (*B*) Naked genomic DNA (0.8 μg/μl) of U1 cells was irradiated (in the triplex-binding buffer) in the absence (lane 1) and in the presence of different concentrations of Pso-15TCG(po) (lane 2, 10 μM; lane 3, 5 μM; lane 4, 2.5 μM); the genomic DNA was then analyzed by *Dra*I protection assay. Lane M, DNA marker (GIBCO/BRL). Fragments lengths (in base pairs) are indicated near the gel. (*C*) Quantitation of *Dra*I cleavage inhibition at the PPT triplex site on the naked genomic DNA (as described in Fig. 2*B*) as a function of oligonucleotide concentration (■, np; ▲, po). The ratio of the number of counts at the 3470-bp fragment to the sum of the 3470-, 2402-, and 1068-bp fragments are shown.

by complete lack of protection of the two neighboring *Dra*I sites, which means the absence of either 1197 (= 1068 + 129) or 4221 (= 2402 + 1819) bp fragments.

Protein-free purified genomic DNA was incubated in the absence and in the presence of various concentrations of Pso-15TCG followed by irradiation and digestion with *Dra*I (see *Material and Methods*). In absence of oligonucleotide the 1068- and 2402-bp fragments were observed (Fig. 1*B*, lane 1). In the presence of increasing concentrations of Pso-15TCG the amount of the 1068- and 2402-bp fragments decreased whereas the amount of the 3470-bp fragment increased (Fig. 1*B*, lanes 2–4).

The efficacy of triplex formation was quantitated (Fig. 1*C*): the fraction of 3470-bp fragment reflected the amount of PPT triplex site on genomic DNA covered by the triplex-induced adducts. It is worth mentioning that the percentage of cleavage inhibition reported here did not reflect directly the efficiency of binding of the triplex-forming oligonucleotide to its 15-bp double-stranded target, because it resulted from triplex formation plus photochemical reaction of the Pso-15TCG psoralen moiety. Thus 50% of *Dra*I cleavage inhibition was obtained at micromolar concentrations for both np and po oligonucleotides after photoactivation in the presence of naked genomic DNA (Fig. 1*C*).

The obtained results demonstrated that a 15-nt-long oligonucleotide can specifically recognize its double-stranded target present within genomic DNA, and that complete coverage of the triplex site can be obtained (Fig. 1*C*). Triplex formation on isolated mammalian DNA has been previously reported (34, 35), but never directly quantitated as described above.

Triplex Formation on Chromosomal DNA in Nuclei of Permeabilized Cells. Oligonucleotides, as reported, possess little ability to penetrate the cellular membrane, but are known to passively diffuse through the nuclear pores (36, 37). To overcome this uptake limitation, U1 cells were permeabilized with digitonin: this method allowed permeabilization of the plasma membrane to oligonucleotides, while at the same time retaining the structural integrity of the nucleus (24). After permeabilization, cells were incubated in the triplex-forming buffer in the absence or in the presence of various concentrations of Pso-15TCG then irradiated (or not). Genomic DNA was purified and digested with *Dra*I.

Triplex formation. Triplex formation was detected and quantitated following the *Dra*I protection assay described above. Similar to results on naked DNA, the 3470-bp fragment appeared (Fig. 2*A*); this demonstrated the specific inhibition of *Dra*I cleavage on the PPT triplex site while the other *Dra*I sites were not affected. In the absence of irradiation no inhibition was observed and only the 1068- and 2402-bp fragments were detected. Therefore, the observed inhibition was caused by the photoproducts of Pso-15TCG on the PPT triplex site formed within the nuclei of permeabilized U1 cells.

Efficacy of triplex-induced cross-linking reaction. The efficacy of triplex-induced photoreaction was examined with dose-response experiments (Fig. 2 *A* and *B*). Up to 30% of the PPT site could be protected from *Dra*I cleavage. The np analogue was more efficient compared with the isosequential po as had been observed on naked DNA (Figs. 2 *A* and *B* and 1*C*). These results are consistent with (*i*) the increase in triplex stability of np oligonucleotides as previously described, and (*ii*) the resistance toward nucleases observed with np oligonucleotides under our experimental conditions (data not shown; ref. 38). Also, nuclear proteins have been shown to bind non-sequence-specifically to po oligonucleotides (36). Preliminary experiments have shown that cellular proteins bind np oligomers less efficiently than po oligonucleotides (38). This may lead to the better availability of np than of po oligomers.

Transcriptional state of the target. It is known that transcriptional activation can change chromatin organization including nucleosome disruption or displacement in several systems (39, 40). Phorbol-12 myristate-13 acetate (PMA) treatment strongly enhances viral expression in U1 cells by active transcription of HIV genes and leads to a 10- to 20-fold increase in virus release in the medium (data not shown; ref. 41). Therefore we compared triplex formation in U1 cells (0.5–1 $\times$ 10^6 cells/ml) before and after a treatment with PMA (20 ng/ml), before permeabilization. The same amount of triplex formed with the Pso-15TCG(np) was detected without and with PMA induction (data not shown). Thus the accessibility of the PPT site in U1 nuclei does not seem to be influenced by the level of transcription rate, at least averaged over the irradiation time. The targeted sequence presented here is located in the coding region of the *pol* gene but it belongs to a 500-bp fragment which has been shown to exhibit

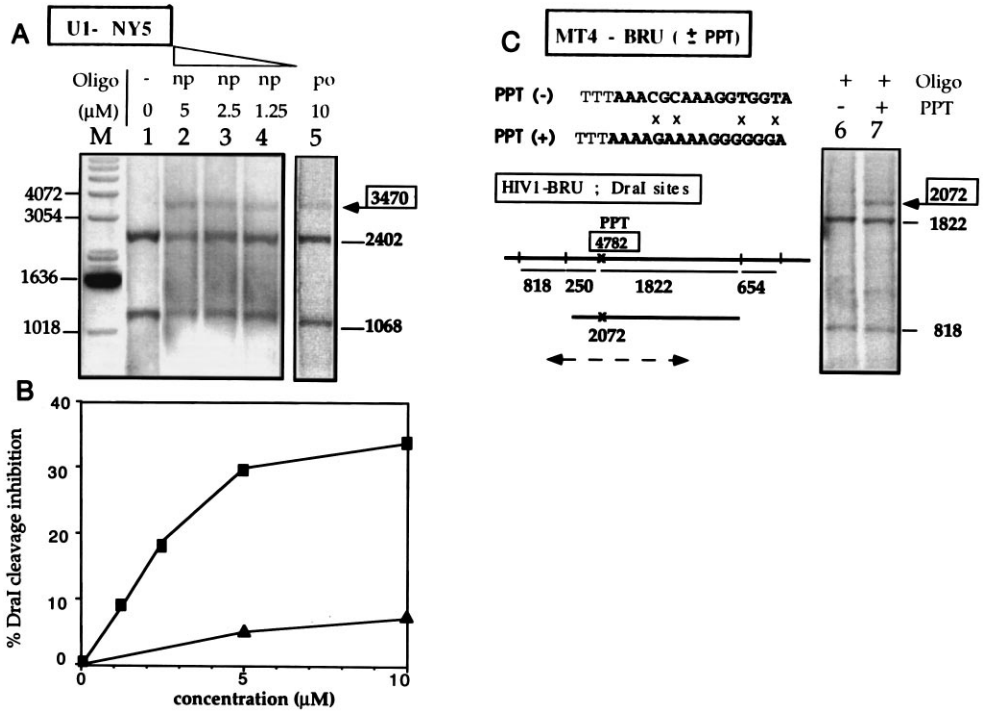

FIG. 2. Triplex formation in nuclei of permeabilized cells. Chronically infected cells were permeabilized with digitonin, incubated in the triplex-forming buffer in the absence or in the presence of Pso-15TCG, and irradiated. After cell lysis, followed by proteinase K and RNase A treatments, the genomic DNA was prepared and analyzed by *Dra*I protection assay. Chronically infected cells were used (U1–NY5, MT4–BRU). They were obtained by infection with different HIV-1 strains (called NY5 and BRU) and differed from each other by the HIV-1 sequence. However the PPT triplex site sequence was the same in all strains except in the MT4–BRU(−) cell line where the PPT sequence was mutated at four positions (see *C*). These sequence differences led to slightly different *Dra*I cleavage patterns, as indicated under the gels, but even though the fragment lengths were modified from one cell line to the other, specific triplex formation was always correlated with the appearance of a longer fragment of well-defined size; probe location is shown by a broken line for MT4-BRU (see Fig. 1 for U1-NY5). (*A*) U1 cells (HIV-1–NY5 sequence); the po or np oligomers and the concentration of the Pso-15TCG are shown by the gels. (*B*) Quantitation of *Dra*I cleavage inhibition at the PPT triplex site in nuclei of permeabilized U1 cells versus oligonucleotide concentration (np, ■; po, ▲). (*C*) MT4 cells [HIV-1–BRU(+), lane 7; or HIV-1-BRU(−), lane 6] treated with Pso-15TCG(po). The sequence of the mutated PPT [PPT(−)] is indicated and mismatches with the wild PPT [PPT(+)] are indicated by crosses (×).

transcription-enhancing activity and to contain several transcription factor binding sites (42, 43). Such a region associated with regulatory functions is generally distinguished from the bulk of chromatin by an increased accessibility of DNA to regulatory proteins and therefore appears also accessible to triplex-forming oligonucleotides.

Cellular environments. To examine triplex formation in different cellular environments other cell lines were used (MT4–BRU, Fig. 2*C*; H9–BRU and U937-NL43, data not shown). Unlike the cloned U1 cells that contain two copies of the HIV genome per cell (NY5 strain) at defined integration sites, MT4–BRU, H9–BRU, and U937–NL43 are populations of chronically infected cells: they contain from 1 to 5 copies of the HIV-1 genome (with the same PPT triplex site) randomly integrated at different locations (44–46). They produce virus constitutively, indicating that these cells are persistently transcribing the viral genes. Triplex formation was examined in these cells following permeabilization, and analyzed as described above. As observed in the U1 cell system, the triplex site was also accessible in the nuclei of all these cells, but the triplex formation efficiency was slightly higher (compare lane 5 of Fig. 2*A* to lane 7 of Fig. 2*C*). Such a difference could certainly be explained by a positional effect of proviral integration in the cellular genome.

Mutated target and sequence specificity. Two MT4 chronically infected cells [MT4–BRU(+) and MT4–BRU(−)] were used to illustrate specificity of triplex formation by oligonucleotide–psoralen conjugates. MT4–BRU(+) cells contain the HIV-1–BRU wild-type sequence. MT4-BRU(−) cells are chronically infected MT4 cells generated by infection with a viral strain containing a mutated PPT sequence in the *pol* gene (Fig. 2*C*; ref. 47). The experiments show that presence of four mismatches within the PPT triplex region completely abolished the binding of Pso-15TCG(po) even though the 5′-TpA-3′ sequence allowing photoaddition of the psoralen moiety was present in both targets (Fig. 2*C*, lanes 6 and 7).

Quantitation of Triplex-Induced Cross-Links by Competitive PCR Assays. The samples of genomic DNA which were analyzed using the *Dra*I protection assay were also characterized using a PCR-based assay. Two primer sets were designed and their locations are schematically shown in Fig. 3*A*. One is located within the HIV *gag* gene, in a region which does not contain a binding site for 15TCG. The other is located around the PPT triplex site. For each primer set a competitor DNA fragment was constructed that contained the sequence of the target genomic DNA segment of interest but lacking several base pairs at one extremity (see *Materials and Methods* and Fig. 3*A*). This technique allowed us to coamplify the two fragments in the same test tube using the same set of primers and to resolve the genomic and competitor amplification products by PAGE. A fixed amount of genomic DNA of interest was mixed with increasing amounts of competitor for each primer set and amplified (Fig. 3*A*). According to the competitive PCR principle, the ratio between the two molecular species remains unchanged during the amplification process (26–28). The number of added competitor molecules is known (see *Materials and Methods*) and the ratio between the PCR products is measured, and consequently the initial amount of target DNA can be calculated. Cross-linked DNA molecules are not substrates for PCR amplification, because presence of a cross-link

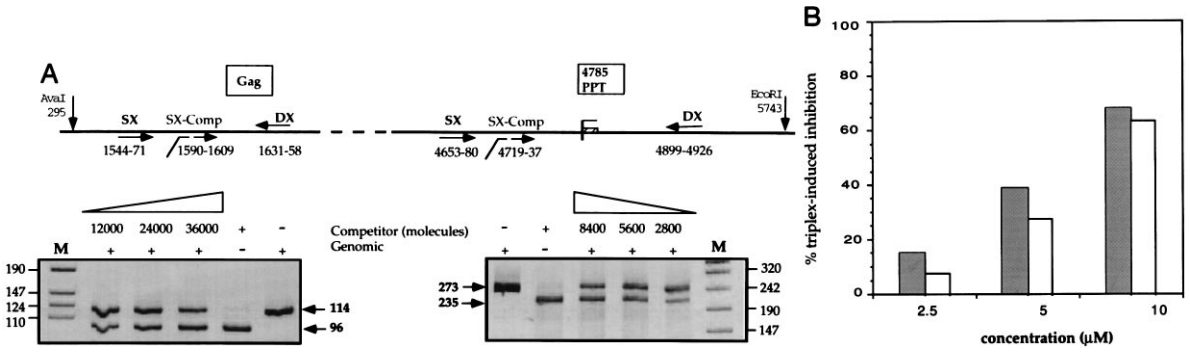

FIG. 3. Triplex-induced cross-link detection on genomic DNA by a competitive PCR-based assay. (*A Upper*) Schematic representation of the genomic HIV region, where the two primer pairs (Gag–SX, 5′-ATAATCCACCTATCCCAGTAGGAGAAAT-3′; and Gag–DX, 5′-TTTGGTCCTTGTCTTATGTCCAGAATGC-3′; PPT–SX, 5′-CCCTACAATCCCCAAAGTCAAGGAGTAA-3′; and PPT-DX, 5′-GATCTCTGCTGTCCCTGTAATAAACCCG-3′) are located (converging arrows). For each primer pair, a competitor DNA fragment for quantitative PCR was constructed using DX primer and the internal SX–Comp primer [Gag–SX–Comp, 5′(Gag–SX)-CCTGGGATTAAATA-AAATAG-3′; PPT–SX–Comp, 5′(PPT–SX)-CAGGTAAGAGATCAGGCTGA-3′, broken arrows] and quantitated. Before PCR amplification, genomic DNA was digested by *Eco*RI and *Ava*I which cut outside of the amplified regions as indicated. Nucleotide numbers refer to positions in HIV-1–NY5 (GenBank). (*A Lower*) Competitive PCR experiment for quantitation of the amount of cross-linked DNA molecules. Genomic DNA irradiated with 10 μM of Pso-15TCG(po) (see Fig. 1*B*, lane 2, which corresponds to the same sample analyzed by *Dra*I protection assay) was quantitated by coamplification of a fixed amount of sample with increasing concentrations of competitor, as indicated near the gels. For each gel the genomic and competitor products are indicated. The results of quantitation obtained for these samples (20000 Gag molecules and 7400 PPT molecules) indicate that 63% of the DNA molecules are cross-linked. Lane M, DNA marker (VIII, Boehringer Mannheim). (*B*) Comparison of quantitations of the same sample after Pso-15TCG(po) treatment obtained by *Dra*I protection assay (grey bars, corresponding to the samples of the experiment reported on Fig. 1) and by competitive PCR-based assay (white bars).

prevents strand separation and thus no amplification can take place. The PPT primer set allowed us to determine the number of initial DNA molecules that were not cross-linked with Pso-15TCG. The *gag* primer set allowed us to determine the number of all initial DNA molecules, both cross-linked and not. The fraction of DNA molecules cross-linked by Pso-15TCG could then be calculated. The quantitation of triplex-derived adducts on genomic DNA obtained by competitive PCR assay and *Dra*I protection assay were compared (Fig. 3*B*). The PCR assay showed systematically slightly lower percentages. This is consistent with the fact that the PCR-based assay measures the amount of triplex-induced cross-links whereas the *Dra*I cleavage assay reflects the amount of cross-links and mono-adducts (5–10% under our conditions) which both are able to inhibit *Dra*I cleavage.

The quantitative PCR-based assay can be used to analyze triplex formation at target sites that do not contain a restriction site overlapping the triplex region. Therefore it could be used as a general approach to measure site-specific cross-linking for genomic DNA. In addition this assay is less time and material consuming, and the unique requirement is to have a site that will allow DNA strands cross-linking with the psoralen or otherwise modified oligonucleotide.

CONCLUSIONS

It has been shown in model systems using transiently transfected plasmidic targets that triplex-forming oligonucleotides could be used for site-directed mutagenesis (10, 11) or for specific transcriptional inhibition of a gene of interest (3–9). The next step was to extend these data to experiments with chromosomally located genes in mammalian cells. There have been several reports describing inhibition of gene expression in eukaryotic cells using triplex-forming oligonucleotides to target endogenous genes (12–17). However no direct evidence was provided that this inhibition was due to triplex formation rather than any other oligonucleotide-directed mechanism. The only demonstration of intracellular transcriptional inhibition by triplex formation made use of a plasmid target that has been mutated within the target sequence to provide the appropriate control system without changing the oligonucleotide sequence (3, 9). When research and therapeutic applications of the triplex strategy are contemplated, it is important to demonstrate unambiguously that the oligonucleotide binds to the expected DNA target. This type of demonstration is clearly required to provide a "proof-of-concept" for the antigene strategy, especially in light of the recently described effects non-antisense of antisense oligonucleotides which are due to binding to other targets than mRNA but are still sequence-specific ones (48–51). The accessibility of the targeted site is a necessary condition for research and therapeutic applications of triplex-forming oligonucleotides as specific ligands for double-stranded DNA in cells.

In the present study we have used HIV-1 chronically infected cells to examine the accessibility of the proviral genome to a triplex-forming oligonucleotide. In permeabilized mammalian cells, we have shown that the PPT sequence of HIV-1 is cross-linked after UV-irradiation to an oligonucleotide–psoralen conjugate in a sequence-specific way.

The accessibility of a DNA sequence within the chromatin and the supranucleosomal structure is expected to depend on its transcriptional state and on the cellular environment, and it cannot be predicted *a priori*. Therefore it was important to develop techniques that may be used to identify and analyze target accessibility in cells. We have established quantitative methods (based on the use of oligonucleotide–psoralen conjugates and permeabilized cells with nuclear integrity) which can be used to answer the question of accessibility. In particular the described PCR-based assay can be applied to a variety of triplex forming sites.

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