Oligodeoxynucleotide-directed photo-induced cross-linking of HIV proviral DNA via triple-helix formation

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ABSTRACT
The HIV proviral genome contains two copies of a 16 bp homopurine-homopyrimidine sequence which overlaps the recognition and cleavage site of the Dra I restriction enzyme. Psoralen was attached to the 16-mer homopyrimidine oligonucleotide, d9(TTTTCT-TTCCCCCCT)3, which forms a triple helix with this HIV proviral sequence. Two plasmids, containing part of the HIV proviral DNA, with either one (pLTR) or two (pBT1) copies of the 16-bp homopurine-homopyrimidine sequence and either 4 or 14 Dra I cleavage sites, respectively, were used as substrates for the psoralen-oligonucleotide conjugate. Following UV irradiation the two strands of the DNA targeted sequence were cross-linked at the triple-duplex junction. The psoralen-oligonucleotide conjugate selectively inhibited Dra I enzymatic cleavage at sites overlapping the two triple helix-forming sequences. A secondary triple-helix-forming site of 8 contiguous base pairs was observed on the pBT1 plasmid when binding of the 16 base-long oligonucleotide was allowed to take place at high oligonucleotide concentrations. Replacement of a stretch of six cytosines in the 16-mer oligomer by a stretch of six guanines increased binding to the primary sites and abolished binding to the secondary site under physiological conditions. These results demonstrate that oligonucleotides can be designed to selectively recognize and modify specific sequences in HIV proviral DNA.

INTRODUCTION
Homopyrimidine oligonucleotides bind to the major groove of duplex DNA at homopurine-homopyrimidine sequences, forming triple helical structures. They may be used to bring a reactive group to these specific sites in close proximity to the DNA base pairs. Psoralen has been previously attached to oligonucleotides targeted to single-stranded nucleic acid sequences (1–5). These psoralen-substituted oligonucleotides could be photo-cross-linked to a single-stranded target. Psoralens are bifunctional photoreagents which form covalent bonds with pyrimidine bases of nucleic acids (5,6). When the psoralen intercalates at TpA steps in the DNA double helix it can form a cross-link between the two strands of DNA. Recent work has shown that an 11-base homopyrimidine oligonucleotide covalently linked to a psoralen derivative via its C-5 position results in photo-induced cross-linking of the two strands of a 32 bp-long DNA fragment via triple-helix formation (7). Such a cross-link is expected to block replication and transcription by preventing opening of the double helix.

In this work we show that a psoralen moiety attached to a triple-helix-forming oligonucleotide intercalates at the duplex-triplex junction, improving oligonucleotide binding to the major groove and bringing the photoreactive agent into an appropriate position for photo-induced cross-linking of the two DNA strands. We have used plasmids containing part of the HIV proviral DNA to demonstrate the possibility of using such psoralen derivatives to photo-induce an irreversible cross-link at a specific sequence.

MATERIALS AND METHODS
Oligonucleotides and plasmids
The two complementary 29-mer oligodeoxynucleotides used in this study were synthesized on a Pharmacia automatic synthesizer. They were purified by reverse phase chromatography and gel electrophoresis. The unmodified homopyrimidine triple-helix-forming oligonucleotide (16-mer TC) (see sequence on figure 1) and the 16-mer TCCG containing T, C and G (see sequence on figure 8) were obtained from the Pasteur Institute. Cytosine was also replaced by 5-methylcytosine (9°C) in some of the oligonucleotides. Psoralen-substituted oligonucleotides (Pso-16-mer TC and Pso-16-mer TCCG) were synthesized by reacting 5-(α-iodohexyloxy) psoralen with a 16-mer carrying a 5'-thiophosphate group as previously described (7) (figure 1).

Two plasmids, pBT1 and pLTR (a gift from Dr H. Hirel, Rhône-Poulenc-Rorer), were used in these experiments. They were constructed by insertion of HIVBRUCG proviral fragments into pUC19 and pBR328, respectively, using standard procedures. The pLTR plasmid contained 1440 bp of the proviral HIV DNA with one 16-bp homopurine-homopyrimidine sequence overlapping a Dra I cleavage site. The pBT1 plasmid contained 8941 bp of HIV DNA with two copies of the 16-bp sequence.

The restriction enzyme Dra I was purchased from Boehringer. Enzymatic assays were performed at 25°C in a pH 6.9 buffer containing 10 mM tris-HCl, 10 mM MgCl2, 50 mM NaCl, 1

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mM DTT and 0.5 mM spermine. After incubation, the enzymatic reaction was stopped by adding EDTA (25 mM). The pLTR plasmid contained 4 Dra I cleavage sites (TTTAAA) one of which overlapped three base pairs of the triple helix target site. The pBTl plasmid contained 14 Dra I cleavage sites, two of which overlapped the triple helix target site. The observed protection from Dra I cleavage by triplex-forming oligonucleotides allowed us to study the specificity of interaction between Pso-16-mers and the double-stranded DNA target. After incubation at the indicated temperature (25°C or 30°C), samples were analyzed by gel electrophoresis with horizontal slab gels (1.3% agarose). Densitometric analysis of photographic negatives of the gels stained with ethidium bromide were performed. Every quantitative analysis presented in this work assumed that fluorescence was proportional to the length of the DNA fragments; all densitometric data were subject to approximately 10 percent random deviation.

Irradiation conditions of the Pso-16-mers

Pso-16-mer TC oligonucleotide was tested on three targets in the same buffer that was used for Dra I cleavage as described above: the circular or EcoR I-linearized pLTR plasmid and the Apa I-linearized pBTl plasmid.

A xenon lamp (150W) in a Cunow housing system was used

Figure 1. Triple helix stability. The 16 bp homopurine.homopyrimidine sequence is boxed and the Pso-16-mer TC sequence is indicated below. Melting curves were obtained in a pH 6.0 buffer containing 10 mM Na cacodylate, 100 mM NaCl. Inset: Hyperchromism vs temperature for the triplex — duplex transition. Absorbance outside the melting region (A\textsubscript{i} before melting, A\textsubscript{f} after melting) were assumed to vary linearly with temperature and hyperchromism was calculated as (A\textsubscript{f}−A\textsubscript{i})/(A\textsubscript{f}−A\textsubscript{i}) with A = absorbance. The T\textsubscript{m} values (temperature of half dissociation of the third strand) were 29 and 20°C for the psoralen-substituted and underivatized 16-mers, respectively.
for experiments where the time dependence of the photoreaction was studied. In all other cases irradiation was performed with a xenon-mercury lamp (200 W) in an Oriel universal arc lamp housing system (model 660 57). Light was filtered through a pyrex filter in a water bath to remove radiation below 310 nm.

**Spectroscopic methods**

Absorption spectra were recorded on a Uvikon 820 spectrophotometer. Melting curves were obtained by increasing the temperature of 500 μl samples at a rate of 0. 15°C/ min.

**RESULTS**

**Thermal stability of the triple helix formed by the 16-mer TC oligopyrimidine covalently linked to psoralen**

Binding of the non-irradiated Pso-16-mer TC to its double-stranded DNA target was first studied by absorption spectroscopy. A double helical segment of 18 bp was formed by hybridizing an 18-mer containing 16 pyrimidine bases to the 29-mer single strand containing the complementary 16 purines. Then Pso-16-mer TC was added to this double helix (see figure 1 for the sequence). The absorbance at 258 nm was followed as a function of temperature. Two transitions were observed (figure 1). The first transition, at low temperature, existed only in the presence of the triple helix-forming oligopyrimidine while the second, at higher temperature, reflects the dissociation of the double-stranded target. Under our experimental conditions, the psoralen derivative attached to the 5'-end of the 16-mer TC increased the temperature of half-dissociation of the third strand (Tm) by about 9°C. Taken together with the cross-linking experiments described below and previous experiments on acridine-substituted oligonucleotides (8), these results suggest that the psoralen ring intercalates at the duplex-triplex junction and allows for stronger binding of the third strand to its target.

**Photo-induced cross-linking of triple helix-forming oligonucleotide-psoralen conjugates to double helical targets**

Photo-induced cross-linking of Pso-16-mer TC to a 29bp long duplex DNA. The photochemical reaction of Pso-16-mer TC was first studied on a 29-mer double-stranded DNA target (see sequence on figure 2). Upon irradiation with UV light of wavelengths longer than 310 nm, several slowly migrating species were observed after electrophoresis. These products were identified as the mono- and the bis-adduct of the 29 bp fragment with Pso-16-mer TC (see figure 7B, lane 5) as previously described on another system (7).

**Inhibition of Dra I cleavage of the pLTR plasmid.** We first used the pLTR plasmid as a target for Pso-16-mer TC. Cleavage by Dral restriction enzyme was used to detect triple helix formation. The pLTR plasmid contains 4 Dra I cleavage sites (TTTAAA). The cleavage reaction products have lengths of 1386, 692, 19 and 2403 bp for the supercoiled plasmid and 412, 692, 19, 2403 and 974 bp for the plasmid linearized at the EcoRI site (position 0). All these fragments were observed on agarose gels, except for the short 19 bp fragment. The Dra I recognition sequence overlaps the 16-mer oligonucleotide binding site by three base pairs at only one of these sites (position 3526). If triple helix formation inhibits binding and/or cleavage by Dra I at this site, the lengths of the generated fragments should be 2403 + 1386 = 3789, 692, 19 for the supercoiled and 2403 + 974 = 3377, 412, 692 and 19 for the linear plasmid.

The linearized pLTR plasmid was incubated in the presence of 8 μM Pso-16-mer TC in a pH 6.9 buffer at 25°C (see conditions in Materials and Methods) and the irradiation time was varied from 0 to 330 sec (figure 2). After 9 min of enzymatic cleavage time in the presence of the non-irradiated Pso-16-mer TC (8 μM), we obtained fragments of 3377, 2403, 974, 692 and 412bp (lane 1/0 sec. in figure 2). This result indicates that the Pso-16-mer TC with no irradiation was able to decrease the efficiency of Dra I cleavage at position 3526 without any effect at positions 412, 1104 and 1123. After a longer cleavage time (20 min), the enzyme cleaved 100% of the TTTAAA site overlapping the 16 bp homopurine homopyrimidine sequence (position 3526) and converted the 3377 bp fragment into two fragments of 2403 and 974 bp (lane 2/0 sec. in figure 2). The transient inhibition could easily be explained by the fact that the interaction of the non-irradiated Pso-16-mer TC with its double-stranded target is reversible, since the duplex and triplex forms are in equilibrium with one another. In contrast, after irradiation for 20, 45 and 330 seconds in the presence of Pso-16-mer TC the inhibition was irreversible and we obtained only the 3377, 692 and 412 bp fragments, even after 20 minutes of incubation with Dra I enzyme. The 2403 and 974 bp long fragments were not observed (figure 2, lanes 2/20, 45, 330 sec.). A similar result was obtained for the supercoiled plasmid.

In order to quantitate the efficiency of the cross-linking reaction, the linear and supercoiled pLTR plasmids were
In the presence of increasing Pso-16-mer TC concentrations, the fraction of the 3789 bp and 2403 bp fragments for the supercoiled plasmid was plotted as a function of Pso-16-mer TC concentration (figure 3). Fifty percent inhibition of cleavage was obtained at a Pso-16-mer TC concentration of approximately 1 nM. A non-specific 16-mer with the same base composition (TCTCTCTCTCTCTCTT) and a Pso-13-mer (Pso-CTTTTTCCTTCTC) were used as controls at 10 nM concentrations and no inhibition of Dra I cleavage was observed in these cases.

Inhibition of Dra I cleavage of the pBTI plasmid. The Pso-16-mer TC was targeted to the pBTI plasmid, which contains two copies of the 16-bp-long homopurine, homopyrimidine sequence. The restriction enzyme Dra I has 14 cleavage sites on this plasmid (figure 4A). For the plasmid linearized at the Apa I site (position 1336), the cleavage reaction products have lengths of 1822, 1819, 1683, 1397, 1043, 818, 692, 654, 571, 450, 250, 217, 129, and 19 bp. On a non-denaturing 1.3% agarose gel, fragments longer than 500 bp were easily visualized (figure 4B). The presence and the length of the shorter fragments were verified on a 2% agarose gel (results not shown).

Assays for cleavage protection by the Pso-16-mer TC were performed under the conditions described above for the pLTR plasmid. Inhibition of Dra I cleavage by the triple helix-forming oligopyrimidine (see figure 4A) at position 4149 should lead to the disappearance of the 1822 and 250 bp fragments, and the appearance of a longer fragment of 1822 + 250 = 2072 bp. The fragments of 1819 and 1397 bp should disappear due to protection by Pso-16-mer TC bound to the second triple helix site (position 8444), and a 1819 + 1397 = 3216 bp fragment should be observed in their place. The two fragments of 2072 and 3216 bp whose detection would demonstrate the specificity of the observed cleavage inhibition are both longer than those generated by Dra I cleavage of pBTI in the absence of the oligonucleotide (all smaller than 1822 bp).

To test the efficacy of the Pso-16-mer TC on the Apa I-linearized pBTI plasmid, increasing concentrations of Pso-16-mer TC were incubated and irradiated in the presence of the plasmid in standard Dra I cleavage buffer (see Materials and Methods). The above predictions were confirmed when low concentrations (<1 nM) of Pso-16-mer TC were irradiated in the presence of the pBTI plasmid prior to Dra I cleavage. Two new bands were observed on the resulting gels corresponding to DNA fragments with the expected lengths of 2072 and 3216 bp (figure 4B, lane 2 + ).

At high concentrations of Pso-16-mer TC (5 μM), a new phenomenon was observed (figure 4B, lane 1 + ). The expected 3216 bp fragment was observed while the 2072 bp fragment was present in small amounts. Furthermore, an unexpected fragment of approximately 2890 bp was observed. The 1819 and 1397 bp fragments should disappear due to
Figure 5. Sequences around the 14 Dra I cleavage sites in pBT1. The observed triple helix binding sites of the Pso-16-mer TC (see figure 4) are underlined.

Figure 6. Time course of photo-induced reaction of Pso-16-mer TC (4.5 μM) with the Apa I linearized pBT1 plasmid. Irradiation was performed at 30°C with the 150 W xenon lamp for different time periods and then Dra I restriction enzyme was added as described in figure 4B. Three different DNA fragments were analyzed after electrophoresis on native agarose gels. They correspond to cleavage inhibition at the three triple helix sites: n: 3216 bp (site position 8444); δ: 2072 bp (site position 4149); m: 2890 bp (site positions 4149 and 3899). For each lane fragment intensity was compared to that of the 1683 bp long fragment (which remained unaffected) and then normalized.

fragments disappeared as expected if cleavage was inhibited at position 8444 to generate the 3216 bp fragment. The 1822 bp fragment disappeared, but so did the 818 bp fragment. As depicted in figure 4A, this result might be explained if Pso-16-mer TC had been cross-linked not only at the expected triplex sites at positions 8444 and 4149 but also at position 3899. If this were the case, a fragment should be observed with length 1822 + 250 + 818 = 2890 bp, as experimentally observed (figure 4B), rather than the 1822 + 250 = 2072 bp fragment expected if cleavage inhibition had occurred only at the specific site at position 4149.

Figure 5 shows the sequences of the 14 Dra I cleavage sites. The two sites at 4149 and 8444 have the appropriate sequence to form 16 base triplets with the Pso-16-mer TC. The Dra I cleavage site at position 3899 can form only 8 contiguous triplets with Pso-16-mer TC, with Pso intercalated at the 5' TpA sequence where it can be photo-cross-linked. The others sites can form less than 5 base triplets. At short irradiation times or at low oligonucleotide concentrations only the two specific sites formed the cross-link. In figure 6, an experiment is shown in which Pso-16-mer TC was incubated and irradiated for increasing periods of time at 30°C. The concentration of Pso-16-mer TC (4.5 μM) was chosen so as to ensure a total inhibition of the three triple helix sites under our standard experimental conditions for long irradiation times. Figure 6 clearly shows the formation and disappearance of the 2072 bp fragment and the appearance of the 2890 bp fragment with increasing irradiation times. We obtained 50% inhibition of cleavage at the 16 bp-long triple helix site at position 8444 with an irradiation time of around 10 seconds, whereas 40s were necessary for an equivalent level of cleavage protection at the 8 bp long triple helix site at position 3899.

To analyse more precisely the photo-induced reaction at the different sites, we used two synthetic DNA fragments, 23 and 29 bp, containing the homopurine.homopyrimidine target...
sequences of 8 and 16 bp, respectively (for sequences see figure 7B). At 25°C, these two labeled double-stranded DNA fragments were incubated and irradiated in the presence of increasing concentrations of Pso-16-mer TC. The photo-products were analyzed on a 10% denaturing polyacrylamide gel (figure 7). As expected, two series of adducts were obtained: those of Pso-16-mer TC adducted to both the 23 bp fragment and 29 bp targets. As observed for the pBT1 plasmid (figure 4B), with decreasing concentrations of Pso-16-mer TC, the photo-adducts on the 8 bp-long triple helix site decreased more rapidly than those on the 16 bp-long site. The same trend was observed when the temperature was raised (results not shown).

The behaviour of Pso-16-mer TC at the two triple helix binding sites was not as different as we might expect for the formation of either 16 or 8-base triplets. Even though the stability of the shorter triple helix was expected to be low at 25 or 30°C and pH 6.9, the experimentally observed cross-linking demonstrated that at least the first photochemical event (mono-adduct formation) was rapid compared to the complex lifetime. The absence of cross-linking at the other Dra I cleavage sites (5 or less base triplets, see figure 5) indicates that, under the experimental conditions used, eight contiguous base triplets were required to confer a sufficient lifetime on the triple helical complex for the photochemical reaction to occur at high oligonucleotide concentrations.

**Sequence specificity of a triplex-forming oligonucleotide containing guanines**

Triple helix formation by homopyrimidine oligonucleotides requires protonation of cytosines to form C.G×C+ base triplets which are isomorphous to T.A×T base triplets (9). The ability of cytosines in the third strand to protonate strongly depends on their distribution within the homopyrimidine sequence. The 16-mer TC has six contiguous cytosines which is unfavorable for protonation. Therefore, at the pH of our photochemical experiments (6.9) it is likely that the stretch of six cytosines did not contribute substantially to triplex stability.

This hypothesis was tested by using a triple helix-forming oligonucleotide conjugated to psoralen (Pso-16-mer T+CG) where the stretch of six cytosines was replaced by guanines, and the cytosine interrupting the stretch of adenines was methylated at its C-5 position (see sequence on figure 7A). The 16-mer T+CG oligonucleotide was shown to bind more strongly to the 16-bp homopurine homopyrimidine sequence than the 16-mer TC at neutral pH (10). This result is due to the formation of C.G×G base triplets which do not require protonation of any bases (9). It should be noted that the 16-mer T+CG binds in a parallel orientation with respect to the homopurine sequence as does the 16-mer TC. The behaviour of the two psoralen derivatives was compared under the conditions previously described at 25°C and pH 6.9. At a 2.5 μM concentration of Pso-16-mer T+CG an almost complete inhibition of Dra I cleavage at the two 16 bp long sites was observed (see intensities of 1822 / 1819 bp long fragments in lane 1, figure 8). Under the same conditions, Pso-16-mer TC resulted in less inhibition at these two sites but cleavage inhibition was observed at the secondary 8 bp-long site, as revealed by the presence of the 2890 bp long fragment (lane 2, figure 8), which did not appear when Pso-16-mer T+CG was used (lane 1, figure 8).

**DISCUSSION**

A 16-nt homopyrimidine oligonucleotide d3(T4C3T4C3T) was covalently linked via its 5′-phosphate to a psoralen derivative and used to cross-link the two strands of HIV proviral DNA at specific sequences upon UV irradiation. The reaction takes place on supercoiled plasmids, as well as on linear DNA fragments, containing the target sequences. Photo-induced cross-linking requires the consecutive absorbance of two photons by a psoralen molecule intercalated at the 5′TpA3′ junction of the duplex and triplex structures. As previously reported, the majority of psoralen molecules first react by forming a cyclobutane ring between the 4′, 5′-double bond of the furane ring and the 5′, 6′-double bond of the thymine base located on the 5′ side of the homopurine target sequence (7). The second photochemical reaction involves cyclobutane addition of the 3′, 4′-double bond of the pyrone ring with the 3′-terminal thymine of the homopyrimidine target sequence. This orientation of the psoralen ring with respect to the 5′TpA3′/5′ApT3′ sequence at the duplex-triplex junction is more favorable to cyclobutane ring formation than that obtained by 180° rotation of the psoralen derivative around the C(5)-O bond (see figure 1) as shown by molecular modeling studies (J.S. Sun, unpublished results).

The HIV proviral DNA contains two target sequences for the 16-nt homopyrimidine oligonucleotide. Both can be cross-linked with high efficiency. However, we observed that cross-linking also occurred at a site allowing formation of only eight consecutive base triplets. The reaction at this secondary site was dependent on both the oligonucleotide concentration and the duration of irradiation (figures 6 and 7). The psoralen-carrying homopyrimidine oligonucleotide has a stretch of six cytosine bases which should be protonated to form six C.G×C+ base triplets. This protonation is very unlikely at the pH of the experiments (6.9), due to the negative cooperativity of protonation for contiguous cytosines. Therefore, the six cytosines of the 16-nt
homopurine oligonucleotide are not expected to contribute substantially to the stability of the triplexes at neutral pH. Consequently, triplex stability is due for the most part to the thymine-rich portion (T<sub>4</sub>C<sub>4</sub>) of the oligonucleotide.

The secondary site observed on the pBT1 plasmid (see figure 5 for the sequences) has a 5<sup>e</sup>TpA<sup>3</sup> sequence at the duplex-triplex junction where psoralen cross-linking can take place. One of the important parameters determining the formation of this cross-link is the lifetime of the triplex structure compared to the time needed for an excited psoralen to react with a thymine. If the first photochemical reaction involves the first excited singlet state of psoralen the reaction might take place in the nanosecond time range. If structural rearrangements are necessary to bring the reactive bonds in the appropriate orientation and distance and/or if the reaction occurs from the psoralen triplet state, cyclobutane ring formation might occur in the microsecond time range (11). Therefore, the photochemical reaction can ‘trap’ rather short-lived complexes provided irradiation takes place over a sufficiently long time period. From a comparison of the sequences that are cleaved by Dra I (figure 5), it can be concluded that five contiguous triplets are not sufficient (no cross-linking was observed at sites 1119, 9841 or 10552), while eight triplets are sufficient for photo-induced cross-linking (site 3899) to take place. In addition, the cross-linking requires that a 5<sup>e</sup>TpA<sup>3</sup> sequence is present at the triplex-duplex junction.

Replacement of the six contiguous cytosines in the 16-nt oligonucleotide by six guanines results in the formation of a stable triple helix. The difference in stability increased when the pH was raised, since guanines (in contrast to cytosines) do not need to be protonated in order to form base triplets with C.G Watson – Crick base pairs (9). The 16-mer T<sup>e</sup>CG, covalently linked to psoralen at its 5'-end, formed cross-linked products with only the two specific homopurine.homopyrimidine target sequences on the pBT1 plasmid. The secondary site observed with the 16-mer TC did not exhibit any reaction with the 16-mer T<sup>e</sup>CG. A comparison of the reactivity under the same experimental conditions (see figure 8) revealed that the presence of guanines in the 16-nt oligomer not only stabilized the complexes with the 16-bp sites but apparently destabilized its complex with the secondary binding site. This observation might have several origins: i) the 16-nt homopyrimidine oligonucleotide may form hydrogen bonds with bases outside the triplex forming region of the secondary target site (i.e., among the 8 base pairs on its 3'-side), ii) the presence of the six guanine residues in the 16-mer T<sup>e</sup>CG may introduce repulsive forces within this same region, iii) the presence of a stretch of six guanines in the 16-mer T<sup>e</sup>CG leads to the formation of multimeric species, such as four-stranded helices which has been observed in other G-rich oligonucleotides (12).

The results presented in this paper demonstrate that it is possible to design oligonucleotide-psoralen conjugates that promote photo-induced cross-linking of the two DNA strands at specific target sequences on the HIV proviral genome. The 16-bp sequence that we have chosen as a target is not present in the available sequence of the human genome. Therefore it might be possible to induce specific cross-linking of the HIV genome inserted within the DNA of infected cells without any effect on the host genome. Such a reaction might be of interest as a new approach to treat HIV-infected cells and induce specific and irreversible damage in the proviral genetic information.

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