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Triple-helix formation by oligonucleotides containing the three bases thymine, cytosine, and guanine

(triplex/psoralen/DNA sequence recognition/oligonucleotides)

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ABSTRACT **A homopurine·homopyrimidine sequence of human immunodeficiency virus (HIV) proviral DNA was chosen as a target for triple-helix-forming oligonucleotides. An oligonucleotide containing three bases (thymine, cytosine, and guanine) was shown to bind to its target sequence under physiological conditions. This oligonucleotide is bound in a parallel orientation with respect to the homopurine sequence. Thymines recognize A·T base pairs to form T·A·T base triplets and guanines recognize a run of G·C base pairs to form G·G·C base triplets. A single 5-methylcytosine was shown to stabilize the triple helix when incorporated in a stretch of thymines; it recognizes a single G·C base pair in a run of A·T base pairs. These results provide some of the rules required for choosing the more appropriate oligonucleotide sequence to form a triple helix at a homopurine·homopyrimidine sequence of duplex DNA. A psoralen derivative attached to the oligonucleotide containing thymine, 5-methylcytosine, and guanine was shown to photoinduce cross-linking of the two DNA strands at the target sequence in a plasmid containing part of the HIV proviral DNA sequence. Triplex formation and cross-linking were monitored by inhibition of *Dra* I restriction enzyme cleavage. The present results provide a rational basis for the development of triplex-forming oligonucleotides targeted to specific sequences of the HIV provirus integrated in its host genome.**

Short oligonucleotides can bind to the major groove of double-stranded DNA at homopurine·homopyrimidine sequences. This was first demonstrated by sequence-specific cleavage with azidoproflavine used to photoinduce cross-linking of the oligonucleotide followed by alkaline cleavage (1) or EDTA–Fe to induce cleavage under reducing conditions (2). Oligonucleotides containing thymine and cytosine bind in a pH-dependent manner with a parallel orientation with respect to the homopurine strand of homopurine·homopyrimidine sequences on double-stranded DNA (3–12). Cytosine methylation was shown to stabilize these triple helices (5, 6) as previously observed on polydeoxynucleotides with alternating sequences (13).

Purine-rich oligonucleotides can also bind to homopurine·homopyrimidine sequences (14, 15). The third strand binds in an antiparallel orientation with respect to the homopurine sequence in contrast to homopyrimidine oligonucleotides. This orientation was also found with oligonucleotides containing guanine and thymine or guanine, thymine, and adenine (14). However, energy minimization studies in our laboratory have suggested that third-strand orientation might depend on the number of ApG and GpA steps in the homopurine sequence (16, 17). Here we show that an oligonucleotide containing three bases (thymine, cytosine, and guanine) binds in a parallel orientation with respect to the homopurine sequence of a homopurine·homopyrimidine target of human immunodeficiency virus (HIV) proviral DNA (5′-$A_4GA_4G_6A$-3′ for the purine strand). Binding of this oligonucleotide to its target sequence depends very little on pH. Triple-helical complexes can therefore be formed under physiological conditions by oligonucleotides containing three bases (thymine, cytosine, and guanine) with cytosine (or 5-methylcytosine) to recognize guanine in adenine-rich tracts, guanine to recognize runs of guanine and thymine to recognize runs of adenine.

MATERIALS AND METHODS

Oligonucleotide Synthesis. The oligodeoxynucleotides used in this study were synthesized on a Pharmacia automatic synthesizer. The two complementary 29-nucleotide oligodeoxynucleotides were purified by reverse-phase chromatography and by gel electrophoresis. The unmodified 16-mers were obtained from the Pasteur Institute. The psoralen–oligonucleotide conjugates [Pso-16-mer T^mC(p) and Pso-16-mer T^mCG(p): see sequences in Fig. 2] were synthesized by treating 5-(ω-iodohexyloxy)psoralen with a 16-mer carrying a 5′-thiophosphate group as described (18).

Plasmid. The pLTR plasmid was constructed by insertion of HIVBRUCG provirus fragments (19) into pBR328 by standard procedures. The pLTR plasmid (a gift from H. Hirel from Rhône–Poulenc–Rorer) contained 1440 base pairs (bp) of the proviral HIV DNA.

Footprinting. The 29-mer duplex was 5′-labeled on its pyrimidine-containing strand by T4 polynucleotide kinase (New England Biolabs) using [γ-^{32}P]ATP. The 5′-labeled 29-mer duplex (containing a 1.3:1 excess of the unlabeled strand) was incubated in the presence of oligonucleotides at 4°C in a buffer containing 10 mM Tris·HCl (pH 7.0), 50 mM NaCl, 10 mM $MgCl_2$, and 0.5 mM spermine. For footprinting reactions 250 μM 1,10-phenanthroline, 60 μM $CuSO_4$, and 20 mM mercaptopropionic acid was added and incubated for 1 hr at 4°C. Samples were precipitated and washed with ethanol and then loaded on 20% polyacrylamide/7 M urea in a buffer containing 89 mM Tris borate and 12 mM EDTA (pH 8.3).

Cleavage by *Dra* I Enzyme. Enzymatic assays were performed at 30°C in a buffer containing 10 mM Tris·HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, and 0.5 mM spermine. After incubation, the enzymatic reaction was stopped by adding EDTA (25 mM). The restriction enzyme *Dra* I was purchased from Boehringer Mannheim. Experiments with *Dra* I restriction enzyme were carried out with the

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Abbreviations: HIV, human immunodeficiency virus; $Cu(OP)_2$, copper–1,10-phenanthroline complex.

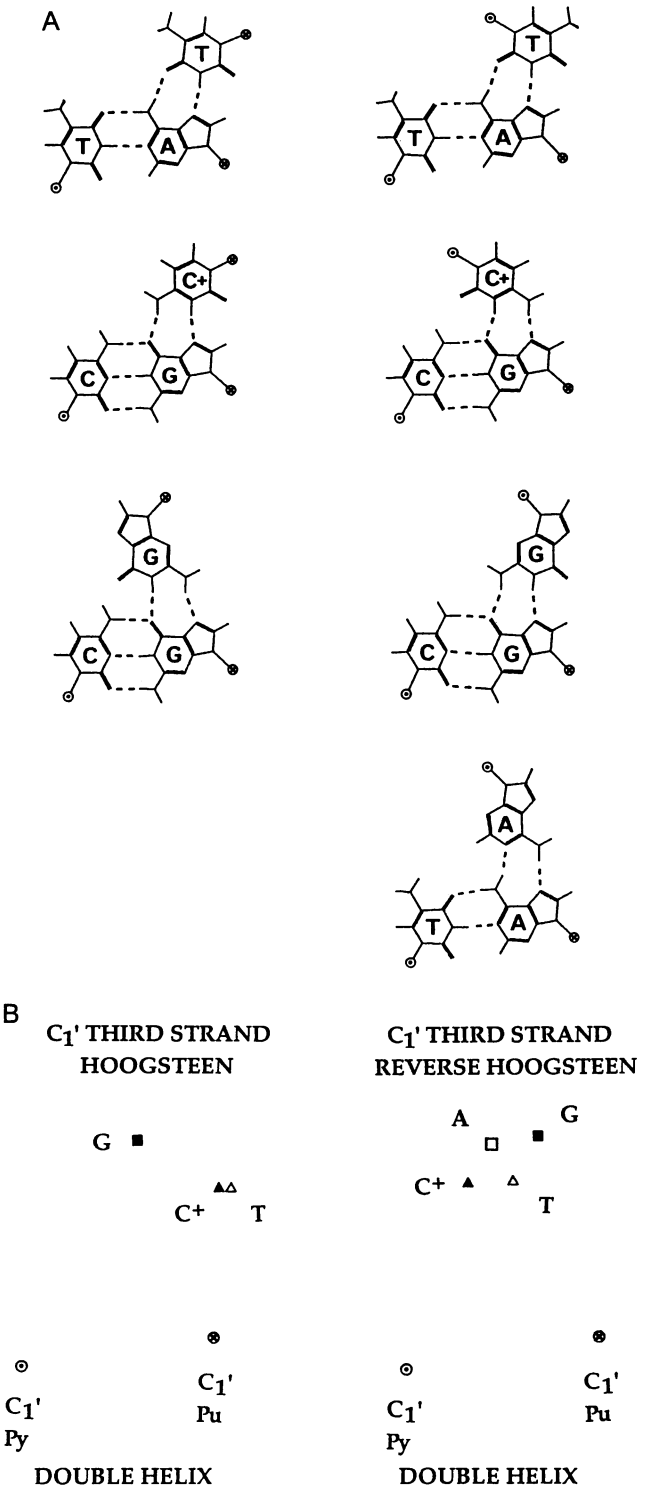

FIG. 1. (*A*) Models for T·A·T, C⁺·G·C, G·G·C, A·A·G base triplets within a triple-helix motif. The relative polarities of the phosphodiester deoxyribose backbones are indicated (⊙ and ⊗) with bases in the *anti* conformation. (*Left*) Third strand is oriented parallel to the homopurine sequence of the DNA target. (*Right*) Orientation is antiparallel. If bases in the third strand were in the *syn* conformation its orientation would be reversed. (*B*) Positions of $C_{1'}$ atoms in the plane of base triplets. The $C_{1'}$ atoms of the Watson–Crick strands are fixed to clearly demonstrate the variations in position of the $C_{1'}$ atom in the third strand. Hoogsteen and reverse Hoogsteen refer to the type of hydrogen bonds formed between the third base and the purine of the Watson–Crick base pair; they correspond, respectively, to *A* (*Left* and *Right*). Positions of the $C_{1'}$ atoms were obtained from energy-minimized structures (see ref. 17).

pLTR plasmid, which contained four *Dra* I cleavage sites (see Fig. 5).

Irradiation Conditions. The triple-helix-forming psoralen–oligonucleotide conjugates were tested on *Eco*RI-linearized pLTR plasmid. Irradiation of the triplex was performed with a xenon lamp (150 W) just before *Dra* I cleavage experiments. Light was filtered through a Pyrex filter in a water bath to remove radiations below 310 nm.

Spectroscopic Experiments. Absorbance of the oligonucleotide mixtures was measured at 258 nm as a function of temperature with a Uvikon 820 spectrophotometer. The rate of temperature variation was 0.15°C/min.

RESULTS AND DISCUSSION

Base Triplet Isomorphism and Third-Strand Orientation. The molecular recognition of purines already engaged in Watson–Crick base pairs is based on hydrogen bonding interactions with bases of the third strand. Thymine and adenine can form two hydrogen bonds with an adenine of a Watson–Crick A·T base pair (T·A·T and A·A·T base triplets). Guanine and protonated cytosine can form two hydrogen bonds with a guanine of a Watson–Crick G·C base pair (G·G·C and C⁺·G·C base triplets). These triplets are shown in Fig. 1*A*. It must be noted that two positions of the third strand are possible for three of these triplets (T·A·T, C⁺·G·C, and G·G·C), corresponding to the so-called "Hoogsteen" or "reverse Hoogsteen" hydrogen bonding pattern, by analogy with the model proposed by Hoogsteen (20) for hydrogen bonding between thymine and adenine. These two positions generate two triple-helical structures with the phosphodiester backbone located either proximal to the purine Watson–Crick strand (Hoogsteen bonds) or at nearly equal distances from the two Watson–Crick strands (reverse Hoogsteen bonds). The A·A·T triplet, which has a single orientation, belongs to the second category.

Fig. 1*B* shows the positions of the $C_{1'}$ atom of the nucleoside involved in the third strand. It can be seen that only Hoogsteen T·A·T and C⁺·G·C triplets are isomorphous. Even though it could be concluded that an oligonucleotide containing guanine and thymine would exhibit a less distorted backbone if G·G·C and T·A·T base triplets adopted the reverse Hoogsteen configuration (Fig. 1*B*), energy minimization studies show that Hoogsteen base triplets are energetically more favorable for runs of T·A·T or G·G·C base triplets (16, 17). Therefore, one might expect that the orientation of the third strand should depend on the number of GpT and TpG steps in the third strand. This has been experimentally verified in our laboratory and has led to the development of "switch" oligonucleotides that can recognize two consecutive homopurine sequences, each of them belonging to a different strand of the double helix (16).

Triple-Helix Formation by Thymine-Rich Oligonucleotides. The HIV genome contains two repeats of a 16-nucleotide polypurine sequence; one is present on the 5′ side of the U3 sequence in the *nef* gene (positions 8662–8677 in HIV-BRUCG) and the second copy is present in the *pol* gene (positions 4367–4382) (19). The first one is present in pLTR as a 16-bp homopurine·homopyrimidine sequence overlapping a *Dra* I cleavage site (see Fig. 5) and was chosen as a target for triple-helix formation.

We first studied the adenine-rich tract part of the targeted sequence. A 10-bp target (see sequence in Fig. 3*A*) was used to compare the binding of three decamers called 10-mer TC(p) (5′-T_4CT_4C-3′), 10-mer TG(p) (5′-T_4GT_4G-3′), and 10-mer TG(ap) (5′-GT_4GT_4-3′) where (p) and (ap) refer to parallel and antiparallel orientation with respect to the homopurine target strand, respectively (Fig. 2). Absorption melting experiments were performed at pH 6.8 (Fig. 3*A*). The same melting curves were obtained with both 10-mers TG(p)

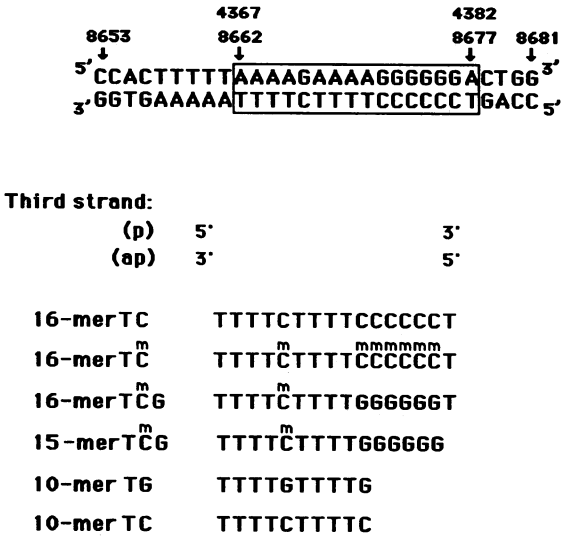

FIG. 2. Sequence of the double-stranded DNA target. The 16-bp homopurine·homopyrimidine sequence is boxed. Numbers above the sequence refer to position on the HIVBRUCG genome (19). Sequences of the third strands used in this work are shown below. The orientation of the third strand is given by reference to that of the purine stretch of the DNA target: parallel (p) or antiparallel (ap) orientation is with respect to the homopurine DNA sequence.

and TG(ap), whose sequences are almost symmetric. A transition was obtained when the 10-mer TC(p) was used as the third strand, whereas, with the TG 10-mers, we could only see the beginning of the transition above 0°C. The 10-bp targeted DNA fragment contained an $(A\cdot T)_n$ tract centrally interrupted by a G·C base pair. For the 10-mer TC(p) triple-helix-forming oligonucleotide, all triplets are isomorphous, whereas for the TG 10-mers the G·G·C base triplet is not isomorphous with the T·A·T triplet in both Hoogsteen and reverse Hoogsteen modes (Fig. 1*B*). Thus, at each of the two steps, TpG and GpT, present in the third strand a backbone distortion is expected and is associated with an energy penalty. This could explain part of the important decrease in the third strand half-dissociation temperature shown in Fig. 3*A*, together with the absence of positive charge on the G·G·C as compared to the $C^+\cdot G\cdot C$ triplet.

To analyze more precisely the mode of binding of the 10-base third strands, footprinting experiments were performed at 4°C, using the copper–phenanthroline complex $[Cu(OP)_2]$ as an artificial nuclease (21) and a 29-bp fragment as a target. Treatment of the 29-bp duplex with $Cu(OP)_2$ in the presence of mercaptopropionic acid led to cleavage reactions along the target strands with an efficiency depending on the local base sequence (Fig. 3*B*, lane 1). In the presence of the third strand, enhanced cleavage was observed at the duplex–triplex junction on the 3′ side of the labeled pyrimidine-containing strand. The triplex region was protected by the 10-mer TC(p), whereas 2 bases inside the triplex region (Fig. 3*B*, arrow) exhibited enhanced cleavage with the 10-mers TG(p) and TG(ap). These two cleavage sites corresponded to the 3′-TpC-5′ and 3′-CpT-5′ steps on the pyrimidine-rich labeled strand. This local absence of protection could clearly be attributed to the lack of binding of the central guanine of the third strand. This result is in agreement with the spectroscopic data. A central nonisomorphous G·G·C triplet is responsible for strong destabilization of the complex.

Binding of a 16-Mer Containing Thymine, Cytosine, and Guanine. The studies reported above show that a higher stability is obtained for the adenine-rich tract of the 16-bp HIV sequence if a cytosine rather than a guanine is incorporated in the thymine-containing third strand. We then

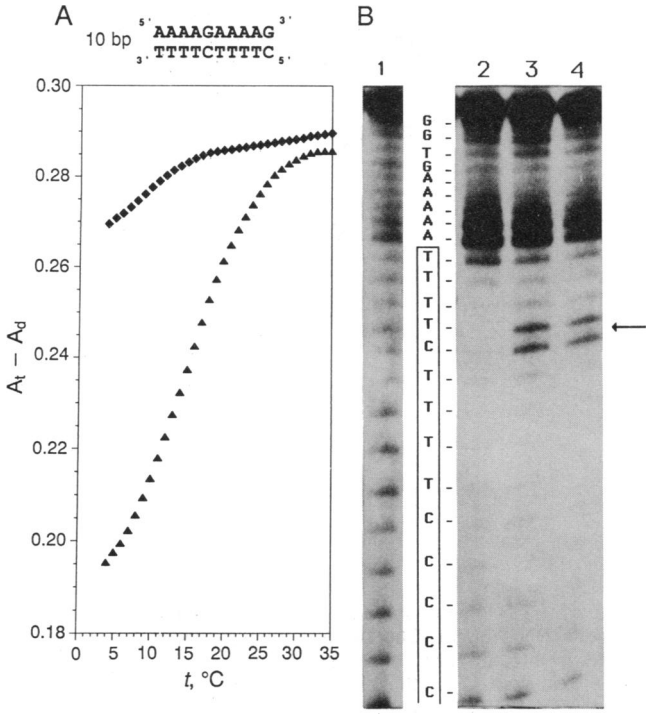

FIG. 3. Triple-helix formation by thymine-rich oligonucleotides. (*A*) (*Upper*) Sequence of the double-stranded DNA target (10 bp), which is the A·T part of the complete 16-bp homopurine·homopyrimidine sequence of Fig. 2. (*Lower*) Transition curves obtained by measuring absorbance of triplex mixture at 258 nm as a function of temperature. To make the figure clearer, the duplex absorbance (A_d) was subtracted from the triplex absorbance (A_t) and ($A_t - A_d$) was plotted versus temperature. A duplex solution at 3 μM was used for triplex formation with different 10-mers (3.2 μM): ▲, 10-mer TC(p); ◆, 10-mer TG(p) and TG(ap). (The same melting curves were obtained independently of the orientation of the 10-mer TG, whose sequence is almost symmetrical.) The buffer contained 10 mM sodium cacodylate (pH 6.8), 100 mM NaCl, and 10 mM $MgCl_2$. (*B*) Footprint of the 10-mers bound to the 29-mer duplex (29 bp) (see sequence in Fig. 2). The 29-mer duplex was 5′-labeled on its pyrimidine-containing strand. Digestion by $Cu(OP)_2$ was performed in the absence (lane 1) or in the presence of 80 μM 10-mer [lane 2, 10-mer TC(p); lane 3, 10-mer TG(ap); lane 4, 10-mer TG(p)].

proceeded to compare cytosine and guanine in the third strand for the recognition of the six adjacent G·C base pairs. Since protonation of cytosine is required to form two hydrogen bonds with a G·C base pair, it was expected that contiguous cytosines would not be favorable to triple-helix formation due to negative cooperativity in proton binding. Substitution of 5-methylcytosine to cytosine is known to enhance triplex stability, especially when cytosines are not contiguous (5, 6, 13). However, this does not hold for a stretch of cytosines as in the C_6 sequence of 16-mer TC where only a modest increase in stability was detected (Fig. 4). Therefore, we synthesized 16-mers containing G_6 instead of C_6 but keeping a 5-methylcytosine in the thymine-rich tract. Two 16-mers were synthesized with opposite orientations. These two 16-mers (16-mer T^mCG) were compared to a 16-mer containing only thymine and cytosine (16-mer TC) or thymine and 5-methylcytosine (16-mer T^mC) synthesized in a parallel orientation with respect to the homopurine strand (see Fig. 2).

Absorption melting experiments were performed to follow the binding of the guanine-rich third strand to an 18-bp double-stranded target (see sequences in Fig. 4). No well defined transition was observed with the 16-mer T^mCG(ap), whereas the 16-mer T^mCG(p) gave rise to a transition as did the homopyrimidine oligonucleotides. The 16-mer T^mCG(p)

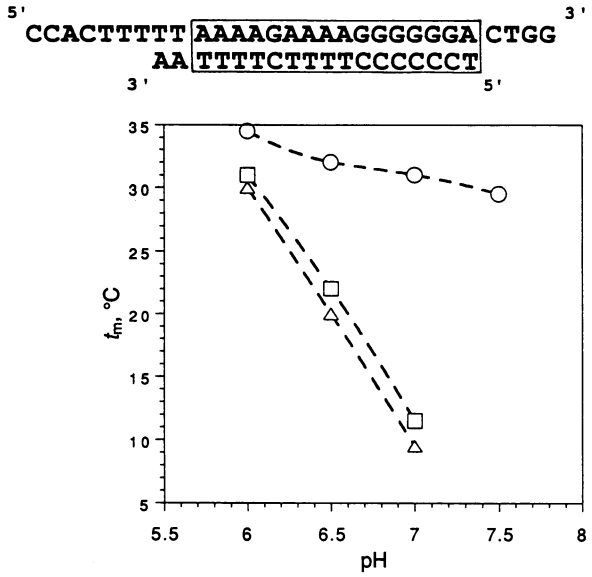

FIG. 4. Triple-helix stability. (*Upper*) Sequence of the double-stranded DNA target. The 16-bp homopurine·homopyrimidine sequence is boxed. (*Lower*) pH dependence of the temperature of half-dissociation of the third strand (t_m). Melting curves were measured in a pH 6.5 buffer containing 10 mM sodium cacodylate and 10 mM $MgCl_2$. Triplex mixtures were obtained by mixing equimolar amounts of the three strands (1.2 μM). △, 16-mer TC(p); □, 16-mer T^{m}C(p); ○, 16-mer T^{m}CG(p).

gave more stable complexes than did the 16-mers TC(p) and T^{m}C(p). Triple-helix stability was only slightly dependent on pH for the 16-mer T^{m}CG(p), whereas the 16-mers TC(p) and T^{m}C(p) bound in a strongly pH-dependent manner due to the requirement for protonation of cytosines to form two hydrogen bonds with G·C base pairs. The same results were obtained by using *Dra* I restriction enzyme cleavage of the 29-bp fragment as a probe of triplex stability (data not shown). Multivalent cations such as spermine or Mg^{2+} were necessary for binding of this guanine-rich oligonucleotide.

The 16-mer T^{m}CG(p) contains two steps associated with an energy cost due to the lack of isomorphism of base triplets: the central TpG step and the 3′-terminal GpT step. It was expected that the central 5′-TpG-3′ step would be easily accommodated due to the stability of the triple helices formed by $T_4{}^mCT_4$ and G_6 on its 5′ and 3′ sides, respectively. On the contrary, the terminal GpT step should not be favorable to the formation of the last T·A·T triplet. Footprinting studies using $Cu(OP)_2$ as a cleaving agent were in agreement with these conclusions (data not shown). In some further experiments, we therefore used a 15-mer T^{m}CG(p) lacking the terminal thymine. This 15-mer has the same t_m as the 16-mer T^{m}CG(p).

Specificity of Binding of the 15-Mer and 16-Mer Containing Thymine, Cytosine, and Guanine. To illustrate the specificity and the stability of the triplex formed by 15-mer T^{m}CG(p) at physiological pH (pH 7.5), *Dra* I cleavage inhibition assays were performed by using as a substrate the pLTR plasmid containing part of the HIV genome with the 16-bp homopurine·homopyrimidine sequence (positions 3526–3541 in pLTR), which overlaps one of the four *Dra* I cleavage sites (5′-TTTAAA-3′) (Fig. 5).

The cleavage reaction products have lengths of 412, 692, 19, 2403, and 974 bp for the plasmid linearized at the *Eco*RI site (position 0). For one of these sites (position 3526), the *Dra* I recognition sequence overlaps by 3 bp the 16-bp oligonucleotide binding site. If *Dra* I could no longer recognize and cleave this site in the presence of a triplex-forming

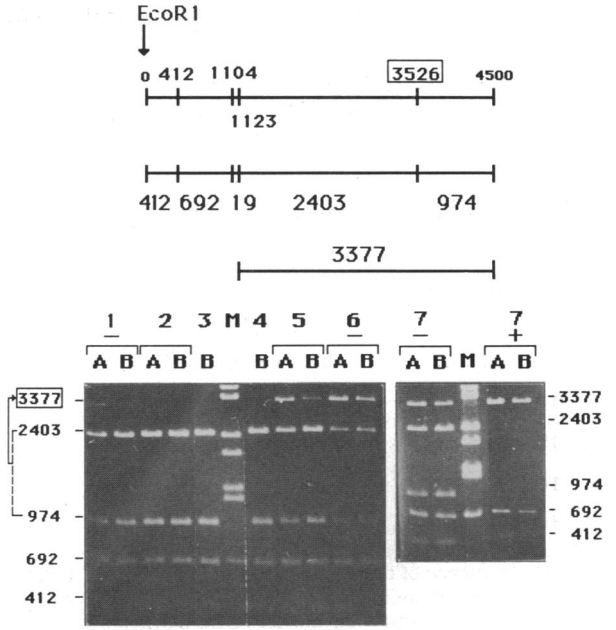

FIG. 5. (*Upper*) Schematic representation of pLTR plasmid linearized at its *Eco*RI site. Positions of *Dra* I cleavage on *Eco*RI-linearized plasmid are indicated together with the lengths of cleavage products. *Dra* I enzyme cuts in the center of the sequence 5′-TTTAAA-3′. The triple-helix site is boxed. (*Lower*) Specific inhibition of *Dra* I cleavage on the pLTR linear plasmid. The plasmid (10 nM) was incubated in the absence of any third strand (lane 4) or in the presence of Pso-16-mer T^{m}C without irradiation (lanes 1−), 16-mer-T^{m}C(p) (lanes 2), 16-mer T^{m}CG(ap) (lane 3), 15-mer T^{m}CG(p) (lanes 5), Pso-16-mer T^{m}CG(p) without irradiation (lanes 6− and 7−), or with irradiation (lanes 7+). Enzymatic assays were performed at 30°C in a pH 7.5 buffer containing 10 mM Tris·HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, and 0.5 mM spermine. After incubation of each third strand at 30 μM (lanes 1–6) or 3 μM (lanes 7) with its double-stranded target, *Dra* I (1.4 units/μl) was added and the enzymatic reaction was stopped by adding EDTA (25 mM) after 15 or 30 min (lanes A and B, respectively). Samples were then loaded on a 1.2% nondenaturing agarose gel. Irradiation was performed (lanes 7+) just before *Dra* I cleavage experiments at 30°C for 5 min to ensure that the photoreaction had reached its plateau. Lane M, λDNA/*Bst*EII digest.

oligonucleotide, the lengths of the generated fragments should be 412, 692, 19, and 3377 bp (2403 + 974).

The plasmid was incubated at pH 7.5 and 30°C in the absence of the third strand (Fig. 5, lane 4) or in the presence of 30 μM 16-mer T^{m}C(p) (lanes 2), 16-mer T^{m}CG(ap) (lane 3), or 15-mer T^{m}CG(p) (lanes 5) and then a *Dra* I cleavage reaction was performed for 15 (lanes A) or 30 (lanes B) min. The 3377-bp fragment was obtained only for the triplex formed with the 15-mer T^{m}CG(p) (lanes 5). After longer incubation times of *Dra* I (lane 5B) the intensity of the 3377-bp fragment decreased, whereas that of the 2403- and 974-bp fragments increased. With the duplex–triplex equilibrium being dynamic, the enzyme eventually cleaved when incubated for a long period of time; only a transient inhibition was observed. Under our experimental conditions (pH 7.5 and 30°C), the complex formed by the 16-mer T^{m}C(p) with its 16-bp target sequence was too weak to allow any *Dra* I cleavage inhibition (lanes 2); the *Dra* I cleavage reaction was as complete as in the control (lane 4).

Irreversible Sequence-Specific Cross-Linking of a Psoralen-Substituted Oligonucleotide Containing Thymine, Cytosine, and Guanine. To obtain an irreversible triple-helical complex, the third strand was attached to a photoactivatable reagent. We previously showed that a psoralen-substituted oligonucleotide containing thymine and 5-methylcytosine could be

cross-linked to both DNA strands under UV irradiation of a triple-helical complex (18). This reaction required attachment of the psoralen via its C-5 position in order to bring psoralen in the appropriate orientation to form cyclobutane rings with two thymines, one on each strand. Conjugation of the same psoralen derivative was carried out with both 16-mer T^{m}C(p) and 16-mer T^{m}CG(p) (see *Materials and Methods*).

In the absence of irradiation, covalent linkage of the psoralen derivative to the 5′ end of the third strand led to an enhancement in triplex stability due to intercalation of the psoralen at the triplex–duplex junction (Fig. 5: compare lanes 1 to 2 and lanes 6 to 5). However, even if the complex formed between the Pso-16-mer T^{m}CG(p) and the 16-bp homopurine·homopyrimidine target in the pLTR plasmid was strong enough to inhibit *Dra* I cleavage at pH 7.5 and 30°C in the micromolar range (lanes 7−), *Dra* I eventually cleaved the TTTAAA sequence overlapping the homopurine stretch of the pLTR plasmid after long incubation times. But upon irradiation above 310 nm, the triplex became irreversibly trapped (compare lanes 7+ and 7−). Consequently, an almost complete inhibition of *Dra* I cleavage was obtained (lanes 7+).

CONCLUSION

We have shown that a triplex-forming oligonucleotide containing the three bases thymine, methylcytosine, and guanine (5′-T$_4$^{m}CT$_4$G$_6$-3′) binds to duplex DNA at a specific homopurine·homopyrimidine sequence. The orientation of the third strand is parallel with respect to the homopurine strand involved in Hoogsteen hydrogen bonding.

The actual orientation of a third strand containing thymine and guanine is dependent on the number of GpA and ApG steps present in the homopurine sequence; each such step is associated to a backbone distortion since nonisomorphous base triplets are involved (Fig. 1). It appears that the antiparallel orientation is favored when there are many GpA and ApG steps in the homopurine-targeted sequence, whereas a parallel orientation should be obtained with few ApG and GpA steps. The orientation giving the highest affinity (parallel or antiparallel) of oligonucleotides containing thymine and guanine remains to be elucidated for each triple-helix motif (16).

The results presented in this study provide some rules for the choice of the sequence of the third strand. An isolated guanine in stretches of adenine is better recognized by 5-methylcytosine than by guanine. A stretch of guanines in the homopurine sequence forms a stronger complex under physiological conditions if the third strand contains guanines rather than cytosine (or 5-methylcytosine). A stretch of adenines followed by a stretch of guanines is recognized by an oligonucleotide containing thymine (to form T·A·T triplets) and guanine (to form G·G·C triplets) with the orientation of the third strand parallel to the homopurine sequence.

The results presented in this study show that an oligonucleotide containing thymine, 5-methylcytosine, and guanine provides a good stability of triple-helical complexes on a proviral HIV sequence. This oligonucleotide binds under physiological conditions to its target sequence. Its binding can be strongly enhanced by attaching an intercalating agent to the 5′ end of the third strand (5, 22). Attachment of a psoralen derivative leads to efficient photoinduced cross-linking of the oligonucleotide to its target sequence at the 5′-TpA-3′ step that flanks the homopurine sequence on the 5′ side. These results provide a rational basis for exploring the possibility of targeting proviral HIV DNA site specifically in HIV-infected cells.

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