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Stable triple helices formed by oligonucleotide N3′ → P5′ phosphoramidates inhibit transcription elongation

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ABSTRACT **Oligonucleotide analogs with N3′ → P5′ phosphoramidate linkages bind to the major groove of double-helical DNA at specific oligopurine·oligopyrimidine sequences. These triple-helical complexes are much more stable than those formed by oligonucleotides with natural phosphodiester linkages. Oligonucleotide phosphoramidates containing thymine and cytosine or thymine, cytosine, and guanine bind strongly to the polypurine tract of human immunodeficiency virus proviral DNA under physiological conditions. Site-specific cleavage by the *Dra* I restriction enzyme at the 5′ end of the polypurine sequence was inhibited by triplex formation. A eukaryotic transcription assay was used to investigate the effect of oligophosphoramidate binding to the polypurine tract sequence on transcription of the type 1 human immunodeficiency virus *nef* gene under the control of a cytomegalovirus promoter. An efficient arrest of RNA polymerase II was observed at the specific triplex site at submicromolar concentrations.**

Oligonucleotides have been receiving increased attention over the past few years as a potential new class of pharmacologically active compounds. They can be used to control gene expression in a sequence-specific manner by targeting messenger RNAs (antisense oligos or ribozymes), chromosomal DNA via triple-helix formation (antigene oligos), or proteins (sense oligos and aptamers) (1–5). Because of these possible applications, a great deal of research effort has been devoted to the development of oligonucleotide analogs with increased resistance to nucleases and/or increased binding to nucleic acid targets (6).

In the antigene strategy, an oligonucleotide binds to the major groove of double-helical DNA (7, 8). A local triple helix that might inhibit transcription by competing with transcription factors or blocking elongation is formed (see ref. 3 for review). DNA target sequences are mostly restricted to oligopurine·oligopyrimidine tracts even though more complex heterogeneous sequences can be recognized by oligomers containing modified bases (9–11) or intercalators (12) that bind base pair inversions or by switching from one strand of DNA to the other when oligopurine sequences alternate on DNA strands (13–15). In most cases, the binding of triplex-forming oligonucleotides to target DNA sequences is not strong enough to expect the development of antigene oligonucleotides as therapeutically useful drugs. The binding strength can be increased by tethering intercalating agents to the ends of triplex-forming oligonucleotides (16, 17) or by inserting intercalating agents at internal sites to recognize base pair inversions in oligopurine·oligopyrimidine sequences (12).

Recently, the synthesis and some hybridization properties of a new family of oligonucleotide analogs containing N3′ → P5′ phosphoramidate linkages have been described (18) (Fig. 1).

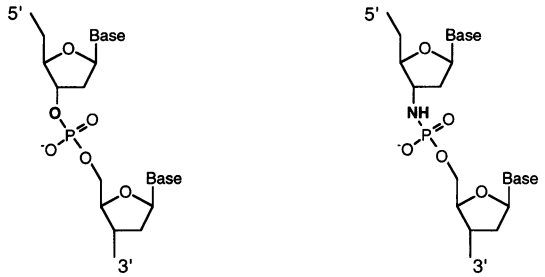

FIG. 1. Chemical structure of phosphodiester and N3′ → P5′ phosphoramidate linkages.

Here we show that antigene oligonucleotide phosphoramidates containing thymines and cytosines or guanosines bind very strongly to double-helical DNA at oligopurine·oligopyrimidine regions and inhibit *in vitro* transcription of the *nef* gene of the human immunodeficiency virus (HIV) at submicromolar concentrations.

MATERIALS AND METHODS

Thermal Denaturation Experiments. Melting profiles of mixtures of 1 μM double helix and 1.2 μM third strand were recorded in a pH 6.2 or pH 7.0 cacodylate buffer containing either 10 mM sodium cacodylate and 1 mM spermine or 10 mM sodium cacodylate, 100 mM NaCl, and 10 mM $MgCl_2$. The temperature was cooled from 60°C to 0°C at a rate of 0.15°C/min, let stand at 0°C for 30 min, then heated to 85°C to allow complete denaturation of the duplex. All the profiles were reversible. Absorption of the duplex was subtracted from that of the triplex, and the resulting profile showed one transition that was attributed to the melting of the triple helix into double helix and single strand. This curve allowed us to measure the half dissociation temperature of the triple helix.

Inhibition of *Dra* I Restriction Enzyme Cleavage by Oligonucleotides. A plasmid pLTR-HIV containing about half of the HIV proviral genome (19) was used as a substrate for the restriction enzyme *Dra* I. This plasmid contains four *Dra* I cleavage sites that generate DNA fragments of 19, 692, 1386, and 2403 bp (Fig. 2 *Left*). One of these four sites corresponds to the junction of the 16-bp polypurine·polypyrimidine sequence of the HIV provirus contained within the *nef* gene. Inhibition of *Dra* I cleavage was previously observed with oligonucleotides forming a triple helix on the 16-bp site (17). The cleavage reaction was performed at 37°C during 5 min with 10 units of *Dra* I in a pH 7.5 buffer containing 10 mM Tris·HCl, 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM dithiothreitol.

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Abbreviation: HIV, human immunodeficiency virus.

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***In Vitro* Transcription Assay.** The plasmid pSG-F47 (a gift of O. Schwartz, Institut Pasteur, Paris) contains a 780-bp fragment including the HIV-1 *nef* gene [from the pTG 1147 plasmid (20)] under the transcriptional control of the enhancer and promoter sequences of the major immediate early gene from human cytomegalovirus [a 619-bp segment from nucleotides −522 to +97 with respect to the cap site (21)]. The 16-bp polypurine tract motif is indicated in Fig. 3 (positions 8662–8677 in HIV-LAI) with the oligonucleotide sequences and their alignment with the duplex target. *In vitro* transcription was performed using a HeLa cell nuclear extract (Promega). The template DNA (≈1 μg of pSG-F47-digested with *Eco*RI and *Nhe* I) was incubated with varying concentrations of different oligonucleotides, and transcription was initiated by the addition of HeLa nuclear extracts (eight standardized transcription units) to a final volume of 25 μl containing 20 mM Hepes (pH 7.9); 100 mM KCl; 3 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM dithiothreitol; 20% glycerol; 400 μM ATP, CTP, and UTP; 20 μM GTP; and 0.4 μM [α-^{32}P]GTP. Transcription was allowed to proceed for 1 hr at 30°C and then stopped by a solution containing 0.3 M Tris·HCl (pH 7.4), 0.3 M NaOAc, 0.5% SDS, 2 mM EDTA, and 3 μg/ml tRNA. Transcripts were extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with ethanol. The transcription products were analyzed by electrophoresis on a 6% polyacrylamide sequencing gel.

RESULTS

Spectroscopic Studies of Triplex Formation. We have previously used a 23-bp DNA fragment from simian virus 40 DNA to investigate the stability of triple helices formed upon binding of 11-mer oligonucleotides containing phosphodiester linkages with deoxyribose, ribose, or 2′-O-methyl ribose as sugars (22), the α-anomers of nucleoside units (23), and 5′ conjugates with intercalating agents (16). Table 1 compares the thermal stability of triplexes formed by different DNA or RNA oligonucleotides with phosphodiester linkages to that of isosequential N3′ → P5′ phosphoramidates. It can be seen that the 11-mer phosphoramidate produced the most stable triplex, especially at pH 7.0, where the requirement for cytosine protonation leads to triplex dissociation with natural phosphodiester linkages. At pH 7, the 11-mer phosphoramidate gave a melting temperature of 45°C in the presence of 1 mM spermine, compared with 26°C for the RNA oligomer and a temperature lower than 10°C for the DNA oligomer. Substitution of 5-methylcytosine for cytosine in the 11-mer increased the melting temperature to 61°C.

Next, we investigated triplex formation with the HIV proviral DNA sequence corresponding to the polypurine tract of HIV RNA. This 16-bp fragment is repeated twice in the HIV proviral genome, and both regions are crucial to HIV gene expression (24). A 29-bp DNA duplex containing the 16-bp oligopurine·oligopyrimidine segment was used to investigate the binding of a 16-mer containing T and C nucleotides (5′-$T_4CT_4C_6T$-3′; Table 2, oligonucleotide 1) or a 15-mer containing T, C, and G nucleotides (5′-$T_4CT_4G_6$-3′; Table 2, oligonucleotide 5). These two oligonucleotides with phosphodiester linkages were previously shown to bind to their 16-bp target (17). Binding of the (T,C,G)-containing oligonucleotide 5 was only slightly dependent on pH (because of the presence of a single cytosine), much less than the (T,C)-containing oligonucleotide 1, whose binding to double-stranded DNA was not detected at pH 7.0. When the phosphodiester linkages in the oligonucleotides were replaced with N3′ → P5′ phosphoramidate ones, the triplex-forming ability of the oligomers was strongly enhanced (Fig. 4). The melting temperature of the triplex formed by the (T,C)-containing phosphoramidate 2 was increased by 43°C at pH 6.2, and a stable triplex was still observed at pH 7.0 (t_m = 40°C) in contrast to the isosequential phosphodiester oligomer 1 (Table 2). The binding of the (T,C,G)-containing phosphoramidate 6 (Table 2) was increased by 32°C at pH 7.0. Replacement of cytosine with 5-methylcytosine in (T,C)-containing phosphoramidate oligonucleotides increased the stability of triple-helical complexes as was previously observed with phosphodiesters. For the phosphodiester (T,C,G) 15-mer, which contains a single cytosine residue, a small increase in stability was observed, whereas no increase in stability could be detected for the phosphoramidate. However, in the latter case, the melting of the triplex containing 15 base triplets was very close to that of the duplex containing 29 bp. This made it difficult to determine melting temperatures with high accuracy. In a buffer containing 150 mM NaCl and 10 mM $MgCl_2$ at pH 7.2, the melting of the triplex formed by the 15-mer (T,C,G)-containing phosphoramidate was increased by ≈3°C, when the single cytosine was replaced by 5-methylcytosine (55.5–58.5°C).

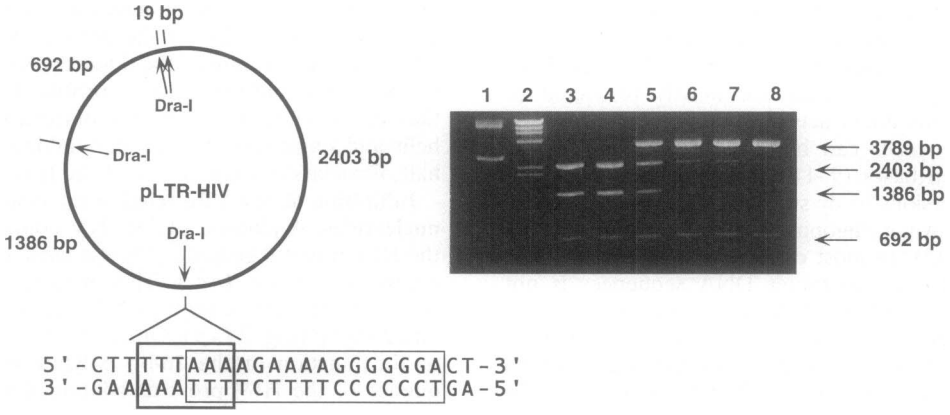

FIG. 2. Inhibition of *Dra* I restriction enzyme cleavage by oligonucleotides. A plasmid pLTR-HIV containing four *Dra* I cleavage sites that generate DNA fragments of 19, 692, 1386, and 2403 bp was used as a substrate for the restriction enzyme *Dra* I (*Left*). One of these four sites corresponds to the junction of the 16-bp polypurine·polypyrimidine sequence of the HIV provirus contained within the *nef* gene. Agarose gels (*Right*) reveal the extent of *Dra* I inhibition at this site from the decrease of the 1386- and 2403-bp fragments and the presence of a 3789-bp fragment. Lane 1: plasmid pLTR alone; lane 2: length markers obtained from λ DNA cleaved with *Hin*dIII; lane 3: *Dra* I cleavage in the absence of oligonucleotide [the same result was obtained in the presence of 200 nM 15 TCG (PO), line 7 in Table 2]; lane 4: in the presence of 200 nM Acr-15 TCG; lane 5: in the presence of 200 nM 16 TC (PN), line 2 in Table 2; lane 6: in the presence of 200 nM 16 TC (PN), line 4 in Table 2; lane 7: in the presence of 200 nM 15 TCG (PN), line 6 in Table 2; lane 8: in the presence of 200 nM 15 TCG (PN), line 8 in Table 2. *C* indicates 5-methylcytosine; PN refers to N3′ → P5′ phosphoramidate linkages, and PO refers to natural phosphodiesters.

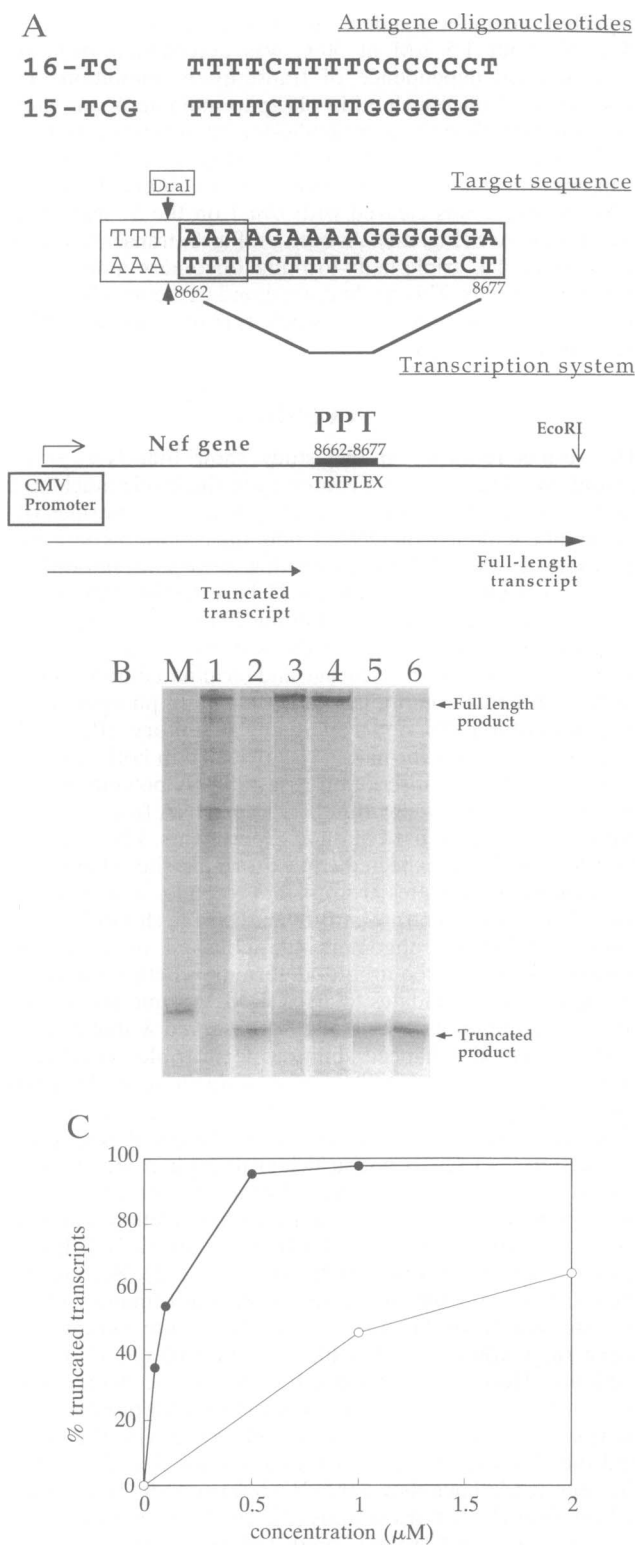

FIG. 3. Inhibition of transcription elongation by oligonucleotides. (*A*) Description of transcription system. The plasmid pSG-F47 contains a 780-bp fragment of the HIV-1 *nef* gene under the transcriptional control of the cytomegalovirus promoter. The 16-bp purine–pyrimidine polypurine tract motif is indicated (positions 8662–8677 in HIV-1) with the oligonucleotide sequences and their alignment with the duplex target. (*B*) *In vitro* transcription assay. Lanes 1–6: transcripts obtained in the absence (lane 1) or in the presence of different oligonucleotides [lane 2: Acr-15TCG(PO), 2 μM; lanes 3–6: 15-TCG (PN) line 6 in Table 2, 0.02, 0.05, 0.5, and 1 μM, respectively]. Lane M: Run-off transcript obtained with the pSG-F47 plasmid digested by *Dra* I (see *A*). The transcription products were analyzed by electrophoresis on a 6% polyacrylamide sequencing gel. (*C*) Dose–response of transcription inhibition by oligonucleotides. The percentage of truncated transcripts is reported as a function of the oligonucleotide concentration [○, Acr-15 TCG (PO); ●, 15-TCG (PN)]. Quantitation was obtained by phosphorimager analysis (±10%) and the reported percentages were corrected for length effects, taking into account the fact that the transcripts are uniformly radiolabeled.

Table 1. Oligonucleotides and t_m values of the triplexes formed with a 23-bp double-stranded DNA target

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3′-GGACTA  TTTCCTCCTCT  ACTTCT-5′
5′-CCTGAT  AAAGGAGGAGA  TGAAGA-3′
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Oligonucleotide	Backbone	t_m, °C* pH 6.2	t_m, °C* pH 7.0
TTTCCTCCTCT	PO/DNA	30	<10
TTT*C*CT*C*CT*C*T	PO/DNA	35	<10
Acr-TTTCCTCCTCT†	PO/DNA	ND	18
TTTCCTCCTCT	PN	68	45
TTT*C*CT*C*CT*C*T	PN	ND	61
UUUCCUCCUCU	PO/RNA	54	26
UUUCCUCCUCU	PO/2′-OMe	56	26

PO and PN are abbreviations for the phosphodiester and the N3′ → P5′ phosphoramidate linkage, respectively; DNA, RNA, and 2′-OMe indicate that the sugars are deoxyriboses, riboses, or 2′-O-methyl riboses, respectively; *C* represents 5-methylcytidine. ND, not determined; <10 indicates that the transition was not completed at 0°C, which did not allow us to determine a t_m value accurately.
*Using 10 mM sodium cacodylate buffer containing 1 mM spermine.
†11-mer was substituted at its 5′ end with an acridine derivative (see ref. 16).

Inhibition of Restriction Enzyme Cleavage. The sequence of HIV-1 proviral DNA on the 5′ side of the polypurine tract of both 16-bp oligopurine–oligopyrimidine regions provides a cleavage site for the restriction enzyme *Dra* I. This enzyme cleaves exactly at the junction of the triple helix site (5′-TTT↓AAA-3′) and triple-helix formation inhibits DNA cleavage (17). A plasmid (pLTR) carrying one copy of the 16-bp polypurine·polypyrimidine sequence was used as a substrate for the *Dra* I enzyme. The plasmid contains four *Dra* I sites and a complete cleavage of the plasmid gave four fragments. One cleavage fragment was too short (19 bp) to be observed on nondenaturing agarose gels (Fig. 2). Inhibition of cleavage by triple-helix formation at the 16-bp sequence should lead to the disappearance of the 1386- and 2403-bp cleavage products and to the appearance of a 3789-bp fragment. The (C,T)-containing phosphodiester *1* had no inhibitory activity on *Dra* I cleavage at pH 7.5, even after 5′ conjugation to an acridine derivative. In contrast, the 16-mer phosphoramidate *2* inhibited DNA cleavage with an IC_{50} of 200 nM at 37°C. The phosphodiester (T,*C*,G)-containing 15-mer (Table 2, oligonucleotide 7) did not inhibit cleavage at 37°C up to a concentration of 20 μM. Attachment of an intercalating acridine derivative to the 5′ end conferred upon the oligonucleotide an inhibitory activity above 1 μM. Under the same conditions the isosequential oligonucleotide phosphoramidate *6* proved to be much more efficient with an IC_{50} around 60 nM. Methylation of the single cytosine did not markedly change this activity. These results are consistent with the strong increase of triplex stability for phosphoramidates measured by thermal dissociation experiments (Table 2).

Inhibition of *in Vitro* Transcription by Oligonucleotide Phosphoramidates. An *in vitro* transcription assay with nuclear HeLa cell extracts was used to determine the efficiency of oligonucleotide phosphoramidates to arrest transcription. A plasmid containing the *nef* gene of HIV under the control of the cytomegalovirus promoter was transcribed *in vitro* after cleavage with *Eco*RI and *Nhe* I restriction enzymes. *Eco*RI

Table 2. Oligonucleotides and t_m values of the triplexes formed with double-stranded DNA target

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3'-GGTGAAAAA | TTTTCTTTTCCCCCCT | GACC-5'
5'-CCACTTTTT | AAAAGAAAAGGGGGGA | CTGG-3'
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Oligonucleotide			t_m, °C*	
No.	Structure	Backbone	pH 6.2	pH 7.0
1	TTTTCTTTTCCCCCCT	PO	23	<10
2	TTTTCTTTTCCCCCCT	PN	66	40
3	TTTTC*T*TTTC*CCCCC*T	PO	25	<10
4	TTTTC*T*TTTC*CCCCC*T	PN	ND	47
5	TTTTCTTTTGGGGGG	PO	32	30
6	TTTTCTTTTGGGGGG	PN	62†	62†
7	TTTTC*T*TTTGGGGGG	PO	34	32
8	TTTTC*T*TTTGGGGGG	PN	ND	62†

*Melting temperature, t_m °C (±1°C), was determined at two pH values in 10 mM sodium cacodylate buffer, containing 100 mM NaCl and 10 mM $MgCl_2$; other abbreviations are as detailed in Table 1.

†The melting temperature of the 15 base-triplets triple helix occurred very close to that of the 29-bp duplex. The t_m values were obtained after substraction of the melting curves of the duplex from that of the mixture of all three strands. *C*, 5-methylcytidine.

cleaved upstream of the cytomegalovirus promoter and downstream of the *nef* gene. A single RNA transcript of ≈900 nt was obtained from the *nef* gene. *Nhe* I cleaved a murine long terminal repeat promoter contained in the retroviral vector and prevented formation of a second transcript. An arrest of transcription at the triplex-forming site should lead to synthesis of a truncated transcript of ≈400 nt (Fig. 3).

Addition of the (T,*C*,G)-containing 15-mer phosphodiester oligonucleotide 7 (Table 2) to the transcription medium did not inhibit transcription up to a concentration of 10 μM. Covalent attachment of an intercalating acridine derivative to the 5′ end of 15-mer 7 (Table 2) enhanced the binding affinity. The melting temperature of the triple-helical complex increased from 34 to 52°C at pH 6.2. The oligonucleotide-intercalator conjugate was able to arrest transcription at the expected site on the 5′ side of the polypurine tract (Fig. 3). An IC_{50} of about 1.5 μM at 30°C was determined from the concentration dependence of transcription inhibition. The unsubstituted oligonucleotide phosphoramidate *6* was much more efficient than the phosphodiester-intercalator conjugate, with an IC_{50} of ≤0.1 μM at 30°C (Fig. 3*C*). The site of transcription arrest was compared with that obtained when the DNA template was cleaved with *Dra* I on the 5′ side of the target sequence (Fig. 3*A*). As seen on Fig. 3, transcription was arrested by the triple-helix-forming oligomers a few bases before the triplex. This might correspond to the position of the active site of RNA polymerase when its front is arrested by the triple-helical complex.

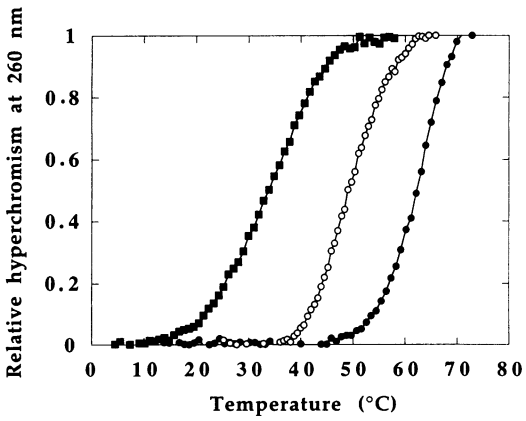

FIG. 4. Normalized melting curves for dissociation of the third strand from the double helix (see Table 2 for sequences). The double helix and third-strand concentrations were 1 and 1.2 μM, respectively. Melting curves were recorded in a pH 7.0 buffer containing 10 mM sodium cacodylate, 100 mM NaCl, and 10 mM $MgCl_2$. The following oligonucleotides were used: 15-mer (T,*C*,G)-phosphodiester (Table 2, line 7) (■); 16-mer (T,*C*)-phosphoramidate containing 5-methylcytosines (Table 2, line 4) (○); 15-mer (T,C,G)-phosphoramidate (Table 2, line 6) (●). The normalized melting curves were obtained after subtraction of the melting curve obtained with the duplex only from that obtained with the mixture of duplexes with the third strand. Under the same conditions, the 16-mer (T,C)-phosphodiester gave a melting temperature below 10°C, even when substituted with 5-methylcytosine (see Table 2).

DISCUSSION

The results reported in this study show that N3′ → P5′ phosphoramidate linkages confer upon these oligonucleotide analogs a strong binding affinity toward polypurine·polypyrimidine sequences in DNA. Under the conditions described in Table 2, the (T,C,G)-containing oligophosphoramidate formed a triple helix containing 15 base triplets that was as stable as the target duplex containing 29 bp (including 13 G·C base pairs). Oligophosphoramidates protected duplex DNA from restriction enzyme cleavage and terminated transcription elongation under conditions where the parent phosphodiester oligonucleotides did not exhibit any inhibitory effect. The specificity of triplex formation was revealed in both the *Dra* I cleavage and the transcription assays. RNA polymerase was blocked at a single site within the 900-bp DNA fragment from the *nef* gene of HIV used in these experiments. The arrest site coincided with the expected triplex-forming site. Oligonucleotide phosphoramidates are resistant to endo- and exonucleases (18). They penetrate into living cells as shown by using fluorescein-labeled oligomers (unpublished observations). Further studies are required to determine whether the ability of oligophosphoramidates to arrest RNA polymerase in an *in vitro* transcription assay will also be observed within cells.

The observed enhanced ability to form triple-helical complexes by the phosphoramidates may be due to several reasons. First, the apparent pK of cytosine might be increased in oligonucleotide phosphoramidates as compared with phosphodiesters, but this is not sufficient to explain the observed stability of triplexes; the triplex formed by the (T,C,G)-15-mer-oligonucleotide phosphoramidate was strongly stabilized when compared with the (T,C)-16-mer even though it contained a single cytosine residue (see Table 2). Second, the phosphoramidate linkage favors a BI conformation of the oligonucleotide backbone over the BII conformation, and sugar rings adopt an RNA-like N-conformation (25). This facilitates Hoogsteen hydrogen bonding to the duplex when the third strand is in a parallel orientation with respect to the polypurine target sequence, as was observed with the (T,C)- and the (T,C,G)-containing oligomers studied in this report. Previous works have shown that RNA forms more stable triple helices than DNA third strands (22, 26–28) (see Table 1). In contrast, a destabilization is predicted for reverse Hoogsteen hydrogen bonding as observed with (G,A)-containing oligonucleotides, which bind antiparallel to the polypurine sequence (28). Preliminary experiments with (G,A)-containing oligonucleotide phosphoramidates seem to confirm this view. They indicate a poor binding of the phosphoramidates to a polypurine sequence with antiparallel orientation. Third, the presence of the NH groups in the backbone is expected to markedly change the hydration properties of the triple helix, and this might contribute to the additional stabilization. More physicochemical data are needed to explain the remarkable stability of triple helices formed by oligophosphoramidate third strands. NMR, infrared, and Raman spectroscopic stud-

ies are under way to gain more insight into the physicochemical nature of the interactions involved in triple-helix formation.

The results presented above show that oligonucleotide N3′ → P5′ phosphoramidates with (T,C) and (T,C,G) sequences that bind parallel to a polypurine target sequence of DNA provide a new class of antigene agents that are expected to block transcriptional processes, even when they are targeted to the transcribed portion of a gene, downstream of the transcription initiation site.

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