In vitro effect of antisense oligonucleotides on human immunodeficiency virus type 1 reverse transcription

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ABSTRACT
The molecular events involved in antisense-mediated inhibition of retroviral transcription were studied by analyzing the in vitro effect of antisense oligodeoxynucleotides on reverse transcription by Human Immunodeficiency Virus type 1 (HIV-1) reverse transcriptase (RT). Oligonucleotides have been designed to be complementary to three targets located in the 5' region of the HIV-1 RNA genome: the trans-activating response element (TAR), the U₅ region and a sequence contiguous to the primer binding site (PrePBS). Antisense oligodeoxynucleotides were used with their 3'-OH end either free or blocked by a dideoxynucleotide in order to avoid cDNA synthesis. Experiments with two recombinant forms of HIV RT, carrying or not RNase H activity, showed that antisense oligonucleotides can arrest reverse transcription by an RNase H-independent mechanism. The AntiTAR oligonucleotide did not affect reverse transcription. In contrast, the AntiU₅ and AntiPrePBS oligonucleotides led to an efficient inhibition of both forms of HIV RT. In the case of the AntiU₅, the inhibition obtained in the absence of the RNase H activity indicates that this effect can be related to features of the RNA secondary structure. The AntiPrePBS oligonucleotide did bind to its target only in the presence of PBS primer. Use of shifted oligonucleotides showed that the AntiPrePBS inhibitory effect depends on a cooperative annealing with the AntiPBS primer on the template.

INTRODUCTION
Use of antisense oligoribo- or oligodeoxynucleotides (RNA or DNA) has proven to be a powerful tool in the control of prokaryotic and eukaryotic gene expression. The purpose of this approach is to block the informational flow from DNA to protein via mRNA by introducing an oligonucleotide complementary to a region of the target mRNA (1–4). The use of antisense oligonucleotides can be extended to the replication of retroviruses which operate through reverse transcription, i.e. the copy of the retroviral RNA genome into a double-stranded proviral DNA which is integrated into the nuclear DNA of the transformed cell (5–8).

Retroviral reverse transcription is catalyzed by an RNA-dependent DNA polymerase coded for by the retroviral genome (9). This enzyme carries three enzymatic activities with essential functions in retrovirus replication: an RNA-dependent DNA polymerase, a DNA-dependent DNA polymerase activity which synthesizes the second strand of the proviral DNA and an RNase H activity which resides in the C-terminal of the catalytic subunit of the enzyme. As all DNA polymerases, reverse transcriptase needs a primer oligonucleotide carrying a free 3'-OH to start cDNA synthesis. The natural primer in the case of all retroviruses studied so far is a specific host tRNA whose 3' end is complementary to a region of 18-20 nucleotides. This region is located near the 5' end of the retroviral RNA (primer binding site or PBS).

Several approaches are being investigated to arrest the proliferation of human immunodeficiency virus (HIV), the causal agent of acquired immunodeficiency syndrome (AIDS). Reverse transcriptase is one of the most studied targets; thus azidothymidine (AZT), the therapeutic drug currently used against AIDS acts by specifically inhibiting chain elongation by this enzyme. The first suggestion for the therapeutic use of antisense oligonucleotides came from the pioneering studies on the inhibition of Rous sarcoma virus replication in cell cultures on the inhibition of Rous sarcoma virus replication in cell cultures with antisense oligodeoxynucleotides complementary to the 5'-end of the viral RNA (10). Similar approaches have been used to arrest the proliferation of vesicular stomatitis virus (11,12), herpes simplex virus (13), simian virus 40 (14), influenza virus (15) and HIV (16–18). An in vitro approach has been recently described, showing that antisense oligonucleotides can block reverse transcription of rabbit β-globin mRNA by avian or murine reverse transcriptases (7,19). The mechanisms by which antisense oligonucleotides directed against the HIV genome inhibit viral development have not been elucidated yet. The viral RNA itself, viral messenger RNAs,
genomic RNA splice sites, are potential sites where the antisense oligonucleotides could exert their effect. Here we have tested the possibility that reverse transcription could be arrested by an antisense oligonucleotide hybridized to the viral RNA. We have chosen three sites, between the 5' end and the PBS regions of HIV RNA, to test the effect of complementary oligodeoxyribonucleotides on cDNA synthesis reaction catalyzed by two recombinant forms of HIV reverse transcriptase as well as by avian myeloblastosis virus reverse transcriptase. The possibility to express in transformed yeast two forms of the HIV reverse transcriptase carrying (p66/p66) or not (p66*/p51) the RNase H activity (20-22) allowed us to study the involvement of this nuclease activity in the inhibitory effect of antisense oligodeoxyribonucleotides.

MATERIALS AND METHODS

 Materials

Unlabelled nucleotides, oligonucleotides or polynucleotides were obtained from Sigma or Pharmacia. Radioisotopes were purchased from Amersham and New England Nuclear Co. Calf intestinal alkaline phosphatase and polynucleotide kinase were from Boehringer Manheim. AMV reverse transcriptase was purchased from Genofit. HIV reverse transcriptase p66*/p51 and p66/p66 forms were isolated and purified as described before (22,23). T, RNA polymerase, RNasin, PstI and SphI restriction enzymes and terminal deoxynucleotidyl transferase were purchased from BRL, proteinase K from Boehringer and pancreatic ribonuclease A from Sigma. DNA molecular weight markers were from Boehringer-Manheim.

HIV reverse transcriptase purification

Recombinant HIV RT (p66*/p51 form; the p66* chain lacks 25 aminoacids at its carboxyl end) was purified from yeast transformed cells as described (20). The homodimeric p66/p66 form was purified essentially as in the case of the heterodimeric form except that a protease deficient yeast strain transformed with HTV sequences (a gift from Dr. J.L. Darlix, INSERM, Lyon), a 1100 base pairs DNA fragment, containing R, U and PBS 5' end (26). Sequences of the antisense oligonucleotides are given in B. In C is shown the linear representation of HIV RNA 5' end and lengths of blockage (bl: grey lines) and initiation (in: dark lines) products with 18-mer (in: 242 NT), AntiTAR (bl: 162 NT; in: 80 NT), AntiU (bl: 68 NT; in: 174 NT), AntiPrePBS (bl: 0 NT; in: 224 NT). After in vitro transcription, the construction gives a 1033 NT or a 341 NT-long RNA containing a 44 nucleotide extension at the 5' end of the synthesized RNA. These RNAs carry the sequences drawn in A and C. The position numbers indicated in the text correspond to the wild-type viral mRNA of HIV-1 (without the extension).

Figure 1. Antisense oligonucleotides. A, Hybridization sites of 18-mer primer and antisense oligonucleotides (AntiTAR, AntiU and AntiPrePBS) are located on a schematic representation of the secondary structure proposed for HIV RNA 5' end (26). Sequences of the antisense oligonucleotides are given in B. In C is shown the linear representation of HIV RNA 5' end and lengths of blockage (bl: grey lines) and initiation (in: dark lines) products with 18-mer (in: 242 NT), AntiTAR (bl: 162 NT; in: 80 NT), AntiU (bl: 68 NT; in: 174 NT), AntiPrePBS (bl: 0 NT; in: 224 NT). After in vitro transcription, the construction gives a 1033 NT or a 341 NT-long RNA containing a 44 nucleotide extension at the 5' end of the synthesized RNA. These RNAs carry the sequences drawn in A and C. The position numbers indicated in the text correspond to the wild-type viral mRNA of HIV-1 (without the extension).
Three sites within the 5' LTR of HIV RNA were chosen as targets for antisense oligodeoxynucleotides. Figure 1A shows the secondary structure proposed for the HTV RNA region involved for antisense oligodeoxynucleotides. Figure 1A shows the secondary structure predicted and rapid cooling, the solution was loaded on a 5.7 M cesium acetate. RNA/DNA pellet was then washed with 70% ethanol and resuspended in sterile water. After heating for 2 min at 85°C and rapid cooling, the solution was loaded on a 5.7 M cesium chloride mattress and centrifuged for 12 hours at 45000 rpm. RNA was recovered from the bottom of the tube, resuspended and ethanol precipitated.

Oligonucleotides synthesis

Oligodeoxynucleotides were synthesized on an Applied Biosystems automatic synthesizer following the manufacturer's instructions.

Antisense modification

Oligodeoxynucleotides (150 μM) were incubated in a final volume of 0.2 ml in presence of 5x tailing buffer (0.5 M potassium cacodylate (pH 7.2), 10 mM CoCl₂, 1 mM DTT), 1 mM of the ddiXTP complementary to the neighbouring nucleotide on the viral sequence and 2x56 units of terminal transferase for 2x4 hours. Reaction products were purified on Sep-pak minicolumns and ethanol precipitated.

Urea-PAGE analysis

Samples were phenol extracted, ethanol precipitated, then dried and resuspended in sample buffer (50% sucrose, 0.2% bromophenol blue, 0.2% xylene cyanol, 8 M urea) and migration buffer TBE 1x(450 mM Tris, 450 mM borate, 10 mM EDTA, pH 8.0). 10%-8 M urea polyacrylamide gels were prerun at 1000 V for 1 h. Migration occured 3-4 hours at 15000 V. Gels were subjected to autoradiography.

RESULTS

Target Sites

Three sites within the 5' LTR of HIV RNA were chosen as targets for antisense oligodeoxynucleotides. Figure 1A shows the secondary structure proposed for the HIV RNA region involved in the studies described in this work. The highly structured R region was deduced from nuclease T₁ and cobra venom or computer analysis studies (24,25) while the U₅ leader stem structure around the PBS site is a computer analysis prediction (26).

The AntiTAR oligonucleotide (position : +12/+36) has been designed to overlap the TAR (transactive responsive) region, a sequence necessary for transactivation of HIV by the Tat protein. Several experiments suggest that a major effect of Tat is to increase the rate of transcription and translational efficiency. TAR RNA forms a stable loop structure and maintaining this structure is important for the Tat response (27,28). The AntiTAR oligonucleotide could act at the reverse transcription level as well as at the transcriptional level, by inhibiting Tat protein-TAR RNA interaction, though it seems that Tat transactivates HIV transcription via a nascent RNA target (29). The AntiU₅ oligonucleotide (position : +105/+130) has been directed against an expected single-stranded RNA region, in such a way that addition of a single nucleotide at its 3' end would lead to a pseudo three-way junction with the other two stems of the R region. Such junctions have been shown in DNA to form asymmetric structures in presence of Mg^{2+} (30). Therefore, stacking of two of the junction arms together could physically block reverse transcription. The third antisense oligodeoxynucleotide, AntiPrePBS (position : +161/+180), is complementary to the upstream region adjacent to the PBS. The three antisense oligodeoxynucleotide sequences are shown in Figure 1B. In Figure 1C are shown the lengths of cDNA corresponding to blockage by the antisense oligodeoxynucleotides or to products of reverse transcription expected with the different oligonucleotides used as primers when their 3'-OH end is available.

The two recombinant forms of HIV reverse transcriptase used in this work were purified from transformed yeast cells. The p66*/p51 enzyme corresponds to a protein from which the last 25 amino acids at the carboxy terminus of p66 have been deleted leading to a reverse transcriptase completely lacking RNase H activity (22). The p66/p66 form is produced in a protease deficient yeast strain and possesses both DNA polymerase and RNase H activity (23). Also used in this study is a commercial preparation of avian myeloblastosis virus (AMV) reverse transcriptase purified to apparent homogeneity. As seen in Figure 2, when the HIV RNA fragment, prepared by the T₇ directed transcription system as described in Methods, was used as template, and an 18-mer synthetic oligonucleotide complementary to PBS used as primer (AntiPBS ; position : +181/+198), the three reverse transcriptases gave the expected 242 nucleotide-long cDNA product although with different kinetics. In the case of AMV reverse transcriptase, a noticeable product was obtained after 5 min of incubation (results not shown) while longer times of incubation were needed for HIV reverse transcriptase. As shown with other template-primer systems, the p66/p66 form has a lower specific activity than the p66*/p51 form (compare figure 2 and figure 5 and 6), probably due to template cleavage by RNase H activity associated with p66*/p51.

Unmodified oligodeoxynucleotides

Because of their free 3'-OH, unmodified antisense oligodeoxynucleotides may serve as primers for retroviral reverse transcriptase. In their presence, it is consequently expected to visualize both primed synthesis and blocked products primed from the AntiPBS oligonucleotide.

AntiU₅. As shown in Figure 3 A, the expected 174 nucleotide-long cDNA product was obtained, corresponding to reverse transcription initiation from AntiU₅. In the presence of AntiPBS, the full-length 242 NT band is weaker than in control experiments. A few bands of about 70-80 NT, not present in

Figure 2. Kinetics of HIV cDNA synthesis by both reverse transcriptases. Reverse transcription assays were performed as described in Material and Methods except for the times of incubation: 0 (1), 5 (2), 10 (3), 15 (4) or 30 min (5).
the control lane, appeared with longer exposures (not shown). The decrease of the 242 NT band radioactivity was observed with both forms of HIV reverse transcriptase. In the case of AMV reverse transcriptase (Figure 3B), no inhibition of full length cDNA synthesis was observed, though initiation from AntiU5 was clearly seen.

**AntiTAR.** Annealing of this oligonucleotide in order to form a preinitiation complex with HIV RNA fragment (template) seemed very poor. The expected 80 NT-long cDNA that should be produced if the AntiTAR were used as primer, was never observed. In presence of AntiPBS, the full length 242 NT cDNA shows a quantitative radioactivity equivalent to the control one, indicating that no blocking activity with AntiTAR was detected neither with HIV nor AMV reverse transcriptase.

**AntiPrePBS.** In the absence of AntiPBS, no specific priming activity by the AntiPrePBS oligonucleotide was observed. However, if AntiPBS is added, the products synthesized are quantitatively different depending on the reverse transcriptase used. When both oligonucleotides are present, the two expected products, 242 and 224 NT long, were observed. In the case of p66*/p51 enzyme, devoid of RNase H, the full length product (242 NT) seemed predominant while the 224 NT cDNA was the major product when p66/p66 (Figure 3A) and AMV reverse transcriptase (Figure 3B) were used.

### 3' end blocked oligodeoxynucleotides

In order to avoid cDNA synthesis initiation from antisense oligonucleotides, the 3'-OH end of AntiTAR, AntiU5 and AntiPrePBS was modified by a dideoxynucleoside residue, complementary to the viral sequence, incorporated with terminal transferase as described in the Methods section. These oligonucleotides will be designated by b- preceding the target site. As shown in Figure 4, the absence of synthesis when AntiPBS was omitted indicated that 3'-end substitution had been highly efficient. Thus the 174 NT product observed with unmodified AntiU5 was absent with the 3'-substituted oligonucleotide. In the presence of AntiPBS, the 224 NT AntiPrePBS initiated cDNA product was also absent.

**b-AntiU5.** The inhibitory effect of this oligodeoxynucleotide was considerably increased by a factor of about 50–100, when compared with the unmodified one (as measured by densitometer tracings of autoradiograms). In this case, bands corresponding to an arrest of cDNA synthesis (a weak band of 68 NT, position of arrest : +131; four stronger bands of 73, 74, 76 and 77 NT, positions : +126, +125, +123 and +122) can be seen clearly in Figure 4 and 5 in the p66*/p51 enzyme lane and with a longer exposure time for p66/p66.

In order to determine the minimal concentration of b-AntiU5 to obtain blockage bands, we performed experiments shown in Figure 5 where increasing concentrations of the 3'-end blocked AntiU5 were added to the reaction mixture. Both forms of HIV reverse transcriptase were sensitive to concentrations as low as 0.1 mM while AMV reverse transcriptase seemed unaffected by the oligonucleotide up to concentrations of 5 mM (Results not shown).

It should be noted that with HIV reverse transcriptase p66*/p51 devoid of RNase H activity, as well as with p66/p66, an increasing accumulation of the 70–80 NT arrested products synthesized from the PBS primer was observed. The observation

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**Figure 3.** Effect of unmodified antisense oligonucleotides (n-ODN) on initiation of reverse transcription. cDNA synthesis assays were performed as described in Material and Methods. Mixture was preincubated at 37°C with HIV RNA, with (+) or without (-) AntiPBS 18-mer primer and 10 μM of AntiU5, AntiTAR or AntiPrePBS as indicated. In lane (C), experiment was performed with HIV RNA and only 18-mer primer. After preincubation, p66*/p51, p66/p66 (A) or AMV RT (B) were added to the mixture for a 30 min incubation at 37°C. Lengths of synthesized products observed are indicated by an arrow.
Figure 4. Effect of 3' modified antisense oligonucleotides (b-ODN) on initiation of retroviral cDNA synthesis. A dideoxynucleotide complementary to the viral sequence was added with terminaldeoxynucleotidyl transferase as described in Material and Methods. Assays were performed as in Figure 3 except that 1 μM of b-ODNs was used. After preincubation, p66*/p51 or p66/p66 were added to the mixture for a 30 min incubation at 37°C. Lengths of synthesis products observed are indicated by an arrow.

that the most intense arrest sites corresponded to DNA fragments of 73–74 NT (Figure 5) may correspond to special features in the 5' end hybridization of b-AntiU5 to the stem region (U5-Leader stem) as schematically shown in Figure 1A.

b-AntiTAR. Use of 3' blocked AntiTAR (b-AntiTAR) confirmed the inefficiency of this antisense molecule (Figure 4). Even at high concentrations of oligonucleotide, total length cDNA products are observed at the same level as in control with all enzymatic forms.

b-AntiPrePBS. As shown in Figure 6, an inhibitory effect of this 3' modified oligonucleotide complementary to the region immediately contiguous to the PBS sequence was found. Both forms of HIV RT and AMV reverse transcriptase were affected by the blocked oligonucleotide at increasing concentrations.

Shifted AntiPrePBS
As shown in Figure 3, unmodified AntiPrePBS was not used as a primer in the absence of AntiPBS, suggesting that under these conditions AntiPrePBS did not anneal to the complementary template sequence. AntiPBS annealing led to subsequent positioning of AntiPrePBS which could then be used as a primer. In order to determine if the effect of AntiPrePBS was dependent on the strict vicinity of these two oligonucleotides (AntiPBS and AntiPrePBS), we performed the experiment shown in Figure 7. A shifted AntiPrePBS was designed to leave two nucleotides free in HIV RNA between AntiPBS and AntiPrePBS (Figure 7A). As seen in Figure 7B, in absence of AntiPBS, neither AntiPrePBS (lane 1) nor shifted AntiPrePBS (lane 3) could be used as primer by reverse transcriptase. In the presence of AntiPBS, the two expected bands of 242 and 224 NT were obtained with AntiPrePBS (lane 2), as in Figure 3. With shifted AntiPrePBS (lane 4), no 222 NT product (shifted AntiPrePBS initiated cDNA) could be seen, suggesting a cooperative effect of the strictly contiguous oligodeoxynucleotides. Similar results were obtained with both forms of HIV reverse transcriptase.

Figure 5. Effect of 3' modified AntiU5 (b-AntiU5) on initiation of reverse transcription. Reverse transcription assays were performed as described in Material and Methods. Preincubation mixture contained 0 μM (lane C), 0.1 μM (1), 1 μM (2) or 10 μM (3) of b-AntiU5. After preincubation, p66*/p51 or p66/p66 were added to the mixture for a 30 min incubation at 37°C. The exact location of blocking sites was determined by addition of 5 μM of ddTTP (lane A) or ddCTP (lane G) in the incubation mixture to establish the HIV RNA sequence. Lengths of synthesis products and antisense aborted cDNAs observed are indicated by an arrow.
Antisense oligonucleotides induce HIV RNA cleavage by the RNase H activity associated with HIV RT.

In order to see if the retroviral RNase H activity was involved in the inhibition of the two recombinant forms of HIV RT by antisense oligonucleotides, we performed the experiment described in Figure 8. A 341 nucleotide long RNA, carrying the PBS site, was synthesized with T7 RNA polymerase. The labeled template was incubated in the reverse transcriptase assay under the conditions described in Methods, except that no labelled dXTP was added to the reaction mixture. Similar experiments were performed with the p66*/p51 (RNase H⁺) and p66/p66 (RNase H⁻) forms of HIV RT as well as with E.coli RNase H as a control.

When the AntiPBS primer (lane 2 of Figure 8A) or AntiU₅ (lane 3) or both of them (lane 4) were used to block reverse transcription of HIV RNA by the p66*/p51 form of the enzyme, the RNA template remained intact. The same pattern was observed when the AntiPrePBS oligonucleotide was present (results not shown). These results confirm that no cryptic RNase H is associated with this recombinant form of HIV RT which has been deleted of a few aminoacids from its C-end.

In contrast, the template was clearly cleaved in the presence of the p66/p66 recombinant form of HIV RT, when antisense oligonucleotides can anneal to the viral RNA (Figure 8B). In the presence of AntiPBS (lane 2), p66/p66 cleaved the template to about 50% level (as evidenced by densitometer tracings of autoradiographs). When b-AntiU₅ was added to the viral RNA (lane 3) the amount of cleaved RNA was less important and the yield of cleaved RNA was lower than with AntiPBS. This result suggests that annealing of the latter oligonucleotide to the PBS is more efficient than annealing of b-AntiU₅ to its target. Indeed, when AntiPBS and b-AntiU₅ were used together with the p66/p66 HIV RT (lane 4), the amount of full length RNA remaining intact (about 85% of the initial input) was very similar to that observed with AntiPBS alone (lane 2), showing the limited involvement of RNase H activity in the inhibition of p66/p66 under these conditions. In the case of the b-AntiPrePBS oligonucleotide (lane 5), no cleavage was observed, indicating that this antisense cannot bind to its target in the absence of annealing of the neighbouring primer. These results are in perfect agreement with the experiments described in Figure 3, showing that no priming of cDNA synthesis from AntiPrePBS occurred in the absence of AntiPBS. Addition of the primer allowed AntiPrePBS to bind its target as evidenced by the emergence of the expected cleavage products (lane 6). In Figure 8C we have performed similar experiments with E.coli RNase H. Results obtained with HIV RT p66/p66 and E.coli RNase H were similar, except for some minor differences which may be accounted for by the cleavage specificity of each enzyme as well as by the different specific activity of both nucleases. It is interesting to point out that the lack of AntiPrePBS effect, in the absence of AntiPBS, was also observed with the E.coli nuclease (Figure 8C, lane 5) while the addition of AntiPBS led to efficient cleavage of full length HIV RNA (lane 6). However, it should be pointed out that, with E.coli RNase H, the two bands corresponding to the longest cleavage products were of similar intensity and that about 90% of full-length RNA was cleaved. In contrast, with p66/p66, the shortest of the two bands was predominant and only 50% of the 341 nucleotide long HIV RNA was cut.

Effect of AntiU₅, AntiTAR and AntiPrePBS in an heterologous system

As the direct effect of some oligonucleotides on HIV reverse transcriptase had been described previously (31,23), we investigated the non-specific (sequence-independent) effect of...
these antisense molecules on cDNA synthesis by HIV and AMV reverse transcriptases. For that purpose, we used rabbit β-globin mRNA system primed with a 17-mer oligonucleotide complementary to the coding region of this mRNA. At the highest concentrations used in the previous experiments, anti-HIV oligonucleotides did not affect β-globin cDNA synthesis, pointing to the fact that inhibition of cDNA synthesis described in this work can be ascribed to an ‘antisense’ effect and not to direct interaction with the enzyme (Results not shown).

DISCUSSION

The antisense strategy is based on the possibility of targeting oligonucleotides to a complementary sequence on an RNA molecule in order to arrest gene expression. Until now, single stranded RNA has been widely chosen as a target for this strategy. Antisense oligonucleotides used to block retroviral proliferation, as observed in infected cell cultures (22), may act at several steps: retroviral regulatory pathways that involve a protein interaction with viral genome, transcription, processing and translation of viral mRNAs as well as at the reverse transcription level, a crucial step in retrovirus RNA replication.

The present studies were aimed at analyzing the effect of some antisense oligodeoxynucleotides on an in vitro HIV reverse transcription system. We have chosen the 5’ end region of the HIV RNA genome, since this region is crucial in reverse transcription initiation, circularization of the retroviral replication intermediates (‘first jump’) (5), transactivation during viral transcription, splicing and encapsidation (32-35). Three synthetic antisense oligonucleotides were assayed to block reverse transcription in the presence of an 18-mer primer complementary to HIV PBS region. Full reconstitution of the initiation complex with the natural primer, tRNA<sub>3</sub>Lys, has not been achieved yet (23,36). Therefore, in the present study, we have used a synthetic 18-mer oligodeoxynucleotide complementary to PBS (AntiPBS) as a primer.

Oligodeoxynucleotides used in these studies are either unmodified or 3’-end-terminated by a dideoxynucleotide, the latter in order to avoid further elongation by retroviral DNA polymerases. An antisense molecule targeted against the TAR DNA sequence, in the R region of HIV RNA (AntiTAR), had no effect, probably because of difficulties to form a preinitiation complex with this highly structured region of HIV RNA (24). This region could be involved in tertiary interactions with another part of the HIV RNA, explaining why transactivation of transcription by Tat occurs at the level of a nascent RNA (29). No blocking effect was observed either with modified or unmodified AntiTAR as shown in Figures 3 and 4. However, this antisense might be active in vivo, since virions contain many copies of a HIV NC (nucleocapsid) protein, which could allow AntiTAR to alter reverse transcription and transactivation.

An interesting result was obtained with an oligonucleotide targeted against the U<sub>5</sub> region of HIV. When its 3’ end was modified, this antisense exhibited a strong arrest with both HIV reverse transcriptases. As the p66*/p51 form is completely devoid of RNase H activity (21), we can conclude that this effect is essentially mediated by an RNase H<sup>-</sup> independent mechanism as confirmed by template cleavage experiments (Figure 8). In order to test what mechanism is operating, we are presently studying the effect of oligonucleotides shortened at their 5’ or 3’ end.

The third antisense oligodeoxynucleotide we assayed was complementary to the region adjacent to PBS (AntiPrePBS). Although the effect of this oligonucleotide was higher with the RNase H-containing enzymes (AMV and p66/p66), antisense blocking by an RNase H-independent mechanism is clearly operating with the p66*/p51 HIV RT.

No hybridization of the AntiPrePBS to HIV RNA was observed in vitro. As shown in our experimental conditions, specific priming did not occur in the absence of 18mer AntiPBS, suggesting that the prePBS region must be highly structured (26) and inaccessible to AntiPrePBS oligonucleotide for preinitiation.

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**Figure 8.** Viral RNA cleavage by the RNase H activity associated with the HIV RT. Reverse transcriptase was incubated with a <sup>32</sup>P-labeled 340 NT long HIV RNA as template, under the conditions described in Materials and Methods except that no labelled dXTP was added to the reaction mixture. In order to test RNA cleavage, three enzymes were used: A, p66*/p51 HIV RT (RNase H<sup>-</sup>), B, p66/p66 HIV RT (RNase H<sup>+</sup>), C, E. coli RNase H. RNA template was preincubated at 37°C without oligonucleotide (lane 1), or with AntiPBS 18-mer primer (lane 2), 1 μM of b-AntiU<sub>5</sub> (lane 3), AntiPBS plus b-AntiU<sub>5</sub> (lane 4), 1 μM of b-AntiPrePBS (lane 5) or AntiPBS primer plus b-AntiPrePBS (lane 6). Full length RNA is indicated by an arrow.
complex formation. A cooperative effect is observed when the 18-mer primer is present in the reaction mixture. The hypothetical PBS loop may favor AntiPrePBS binding through cooperativity between the two contiguous oligonucleotides (Figure 7A). Therefore, AntiPrePBS oligonucleotide might be able to hybridize directly in vivo because the complex between natural primer and viral PBS RNA is already formed. When AntiPrePBS was shifted two nucleotides upstream from the 5' end of the PBS sequence, the antisense-primed band disappeared and cDNA synthesis pattern was unaffected. Shifted oligonucleotides fail to form a preinitiation complex when they are not strictly contiguous to AntiPBS. Thus, cooperative binding of neighbouring oligonucleotides is required for the inhibitory effect.

Moreover, when AntiPrePBS was added to HIV RNA, no cleavage products were observed in the presence of E. coli RNase H and p66/p66 HIV RT (RNase H*), showing the inability of this oligonucleotide to bind its target in the absence of the contiguous primer. The expected cleavage products were observed, both with E. coli RNase H and HIV RT, when the primer AntiPBS was added. These results show that there is a cooperative annealing effect of adjacent oligonucleotides. When AntiPBS and AntiPrePBS were both present in the incubation with E. coli RNase H, two bands, whose size difference corresponded to the size of the PBS region, were produced to a similar extent, while the shortest band was predominantly observed, (> 80%), with the p66/p66 RT form.

Synthetic oligodeoxynucleotides described above do not exert significant effect with an heterologous β-globin mRNA system showing that the inhibitory effects on reverse transcription are thus target specific.

Recent reports show that cDNA synthesis by avian and murine reverse transcriptases, using globin mRNA as template, can be prevented by antisense oligonucleotides (7,19). In most cases the RNase H activity of these reverse transcriptases seemed to play an important role in the inhibition of DNA synthesis. However, results obtained with an α-oligonucleotide, resistant to RNase H, showed that DNA polymerization could be arrested via an RNase H-independent mechanism by oligonucleotides targeted immediately upstream of the PBS. With HIV RT and HIV RNA, we observed an RNase H-independent mechanism of antisense blockade by both the AntiPrePBS at the initiation step and the AntiU3 oligonucleotide during cDNA elongation. The difference between the two sets of results may be ascribed to the different structures of both templates (β-globin mRNA and HIV RNA) as well as to the different kinetic behaviour and pausing of reverse transcriptases from different origins.

We have identified target sites on the viral RNA where oligonucleotides could block reverse transcription in the absence of RNase H activity. The sequence adjacent to the TAR structure on HIV-RNA might represent such a potential target site, as shown with the blocked AntiU3 oligodeoxynucleotide, which act through an RNase H-independent mechanism. As shown in the present study, an oligonucleotide adjacent to the PBS can also block the initiation of reverse transcription.

Molecular analysis of an in vitro HIV system shows the complexity of interactions between the different elements involved in reverse transcription initiation. Our results can provide a rational basis to choose antisense oligonucleotides which exert an inhibitory effect in vitro. In a following step, we plan to assay these oligonucleotides, with some chemical modifications, on HIV-1 infected cells.

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