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

B. Bordier, C. Hélène, P. J. Barr, S. Litvak and L. Sarh-Cottin*
Institut de Biochimie Cellulaire du CNRS, 1 rue Camille Saint Saëns, 33077 Bordeaux cedex,
Laboratoire de Biophysique, INSERM U.201-CNRS UA.481, Muséum National d'Histoire Naturelle,
43 rue Cuvier, 75005 Paris, France and CHIRON Corp., 4560 Horton St Emeryville, CA 94608, USA

Received August 7, 1992; Revised and Accepted October 26, 1992

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 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,

* To whom correspondence should be addressed
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 oligodeoxynucleotides 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 oligodeoxynucleotides.

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). T₇ 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 the expression vector pBS24RT5 was used. (JSC 302, derivative of AB116 : MATa, leu-2, trp-1, ura 3-52, prBl-1122, pep 4-3, prc1-407 (cir²)) (20).

Reverse transcriptases assays

Incubation mixture without enzyme was preincubated at 37°C for 30 min, then incubation was carried out at 37°C for 30 min in a final volume of 0.05 ml with 50 mM Tris-HCl, pH 8.0, 6 mM MgCl₂, 2 mM dithiotreitol (DTT), 30 mM NaCl, 8 mM PBS RNA, 1.5 μM AntiPBS when used, 30 μM of [α-³²P]-dGTP (3000 Ci/mmol), 100 μM dXTP and : 100 nM of p66*/p51, 170 nM of p66/p66 or 1 unit of AMV RT. Antisense oligonucleotides were added to the preincubation mixture as indicated in legends of figures. Incubation conditions with rabbit β-globin mRNA were as for HIV RNA except that template was 20 nM and 17-mer primer 2 μM. To determine the exact location of the blocking sites, a sequence of HIV RNA was determined by adding 5 μM of ddCTP or ddTTP in the incubation mixture.

Purification of ‘PBS’ HIV RNA

A 1100 base pairs DNA fragment, containing R, U₅ and PBS HIV sequences (a gift from Dr. J.L. Darlix, INSERM, Lyon), was cloned in a blue-scribe plasmid PstI site. After vector linearization by SphI or by HphI, the insert was transcribed by T₇ RNA polymerase. After transcription, the construction gives a 1033 NT long (SphI) or a 341 NT long (HphI) 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). About 1 μg of SphI linearized DNA was incubated with T₇ RNA polymerase buffer, 50 mM DTT, 500 μM of each rXTP (rATP, rUTP, rGTP and rCTP), 35 units/μl of RNAsin and 50 units of T₇ RNA polymerase in a final volume of 0.1 ml for 2 hours at 37°C. Reaction was stopped by adding 100 μg/ml of proteinase K in 0.1% SDS for 15 min at 37°C. In the case of DNA linearized by HphI, 250 μCi of [α-³²P]-rCTP
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 T, and cobra venom or computer analysis studies (24,25) while the U, 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 AntiU, 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 Mg2+ (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 oligonucleotides 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 T, 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 HTV 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 enzyme.

Unmodified oligodeoxynucleotides

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

The AntiU, as shown in Figure 3A, the expected 174 nucleotide-long cDNA product was obtained, corresponding to reverse transcription initiation from AntiU, 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).](image-url)
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.

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
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-AntiU 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 222 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 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.

Figure 5. Effect of 3' modified AntiU3 (b-AntiU3) 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-AntiU3. 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 ddITTP (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 autoradiograms). 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 Anti PBS, 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

![Figure 6](image-url)  
**Figure 6.** Effect of 3' modified AntiPrePBS (b-AntiPrePBS). Reverse transcription assays were performed as described previously in the presence of p66*/p51 (□), p66/p66 (●) or AMV (■) reverse transcriptases. After gel electrophoresis analysis (see Materials and Methods), [³²P]-cDNA synthesis (242 NT fragment) was determined from densitometer tracings of the autoradiograms, relative to the synthesis observed in the absence of added oligodeoxynucleotide.

![Figure 7](image-url)  
**Figure 7.** Cooperativity of annealing between AntiPBS and AntiPrePBS. A 20-mer was targeted against PrePBS region in order to leave two nucleotides free between AntiPBS and this shifted AntiPrePBS. A. Nucleotide sequence of PBS RNA region. Overlapping by AntiPBS (dark line), AntiPrePBS (grey line) and shifted AntiPrePBS (light line) is indicated. B. Reverse transcriptase mixture was preincubated at 37°C with HIV RNA, with (+) or without (−) AntiPBS 18-mer primer and 5 μM of AntiPrePBS (lanes 1 and 2) or shifted AntiPrePBS (lanes 3 and 4). In lane (C), experiment was performed with HIV RNA and only 18-mer primer. After preincubation, HIV reverse transcriptase was added to the mixture for a 30 min incubation at 37°C.
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\(^{3'\text{ss}}\), 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 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\(_{5}\) 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\(^{-}\)-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 HTV 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

---

**Figure 8.** Viral RNA cleavage by the RNase H activity associated with the HIV RT. Reverse transcriptase was incubated with a \(^{32}\)P-labeled 341 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\(^{+}\)), B, p66/p66 HIV RT (RNase H\(^{-}\)), 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\(_{5}\) (lane 3), AntiPBS plus b-AntiU\(_{5}\) (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.
In a following step, we plan to assay in vitro an inhibitory effect through an RNase H-independent mechanism. As shown in the TAR pattern might represent such a potential target site, as of RNase H activity. The sequence adjacent to the TAR structure in HIV-1 infected cells. In reverse transcription initiation. Our results can provide a complexity of interactions between the different elements involved in block the initiation of reverse transcription.

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 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.

ACKNOWLEDGEMENTS

This work was supported in part by the Agence Nationale de Recherches sur le SIDA (ANRS), the Association de Recherches Contre le Cancer (ARC) and the University of Bordeaux II. We thank Dr Ester Saison-Behmoaras for stimulating discussions.

BB is supported by a fellowship from ANRS.

REFERENCES