Binding of oligopyrimidines to the RNA hairpin responsible for the ribosome *gag-pol* frameshift in HIV-1

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Received 12 January 1999; received in revised form 17 March 1999

Abstract The 12 bp stem of the RNA hairpin responsible for the *gag-pol* frameshifting of the ribosomes during translation of the polycistronic HIV-1 mRNA has a pyrimidine-rich 5′ strand and, consequently, a purine-rich 3′ strand. Electrophoretic mobility shift assays have shown that DNA oligopyrimidines, 12 and 20 nucleotides long (but not oligopurines or G,T-containing oligomers), designed to form triplexes actually bind to the double-stranded RNA target. RNase V1 footprinting studies have confirmed the interaction between the hairpin stem and the. RNA and 2′-O-methyl oligoribonucleotide analogues of the 12-mer oligodeoxypyrimidine as well as 5 propynyl,cytosine, containing the 12-mer oligodeoxypyrimidine, bind more strongly to the RNA target than the unmodified parent DNA oligomer. The complexes formed by the RNA hairpin and either the 12-mer oligodeoxypyrimidine or the 20-mer oligopyrimidine are stable at a neutral pH and in the absence of Mg^{2+} but blocked neither the reverse transcription nor cell-free translation of a RNA template in which the *gag-pol* frameshifting hairpin was inserted at the 5′ end of the luciferase open reading frame.

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Key words: RNA structure; Antisense oligonucleotide; Triple helix

1. Introduction

RNA molecules can adopt complex structural foldings which prevent the use of antisense oligonucleotides for the artificial regulation of gene expression [1]. Many of these structural elements are implicated in regulatory processes such as the iron responsive element (IRE), which regulates the stability and the translation efficiency of the transferrin receptor and ferritin messages, respectively [2], or the TAR element, which enhances the HIV-1 transcription upon selective binding to viral and host proteins [3]. Likewise, an RNA hairpin is part of the *gag-pol* frameshifting signal of HIV-1 and acts as a positive modulator of the Pol synthesis [4]. Indeed, the synthesis of non-structural viral proteins, required in lower amounts than structural ones, is dependent on a −1 slippage of the ribosome on the messenger. The ratio between these two types of proteins is highly critical for the viral development [5–7] as revealed by the occurrence of variants which present an increased frameshift frequency, under protease inhibitor pressure [8]. We hypothesized that the binding of an oligonucleotide to this target might pertub the frameshift efficiency or the synthesis of the downstream protein. The stem of this hairpin presents on the 3′ side a purine-rich strand appropriate for triple helix formation which constitutes one of the different approaches for designing oligonucleotides against folded RNA [1]. The triplex strategy, restricted to double-stranded oligopurine-oligopyrimidine sequences, consists in adapting an oligonucleotide third strand to the purine strand of the duplex. It has been widely used against DNA duplex [9]. Based on hydrogen-binding patterns, two types of triplexes have been described. In the first one, a purine third strand in an antiparallel orientation with respect to the purine target strand leads to the formation of T.A*A and C.G*G triplets. In the second one, a pyrimidine third strand parallel to the purine strand gives rise to T.A*T and $C.G^{*}C^{+}$ triplets. Limited information is available for the targeting of double-stranded RNA by a triplex-forming oligonucleotide. The overall stability of the triplex depends on the chemical nature of the three strands, the combination with three RNA strands being one of the most stable for the different sequences investigated so far [10–12].

We have investigated the binding of various purine and pyrimidine oligonucleotides designed to form triple helices with the *gag-pol* frameshifting RNA hairpin, by an electrophoretic mobility shift assay. Enzymatic and chemical footprints have been performed in order to map the interaction between the synthetic oligonucleotides and their RNA target. We report here that pyrimidine but not purine oligomers bind to this target in the range of a few nM, whatever the chemical nature of the sugar (deoxyribose, ribose or 2′-O-methyl,ribose). But none of the studied oligomers was able to prevent the use of the information borne by the target RNA, *i.e* to inhibit either the in vitro translation or reverse transcription.

2. Materials and methods

2.1. Oligonucleotides, chemicals and enzymes

Unmodified oligodeoxyribonucleotides were obtained from Genset. Oligonucleotides containing either 2 methoxy-6,chloro-9,aminoacridine or modified cytosines were from Eurogentec and peptide nucleic acid (PNA) from Perseptive Biosystem. Oligoribonucleotides and 2′-O-methyl, oligoribonucleotides were synthesized on a Millipore Expedite synthesizer. The 2′-O-methyl, oligoribonucleotides were purified by reverse phase (RP-) HPLC, using an acetonitrile gradient (0–48%) in a 100 mM ammonium acetate (pH 7.0) buffer, as previously described [13]. The purity was controlled by electrophoresis of 5′ ^{32}P-labelled products on a 20% denaturing polyacrylamide gel. Chemical products were from Sigma and enzymes were from Pharmacia Biotech unless otherwise indicated. Oligonucleotides used in this study are listed in Fig. 1.

2.2. Electrophoretic mobility shift assay

The oligonucleotides (5 pmol) were 5′ end-labelled by incorporating [γ^{32}P]ATP (37.5 MBq/mmol, ICN) with T4 polynucleotide kinase (Promega). The labelled oligonucleotides were separated from unincorporated ATP by electrophoresis on a denaturing 20% polyacrylamide gel. Unless otherwise indicated, the oligonucleotides were then mixed with the target RNA at different concentrations, at a maximal

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PII: S0014-5793(99)00427-5

final ratio of 1/10 in 50 mM Tris-acetate buffer (pH 7.0) containing 10 mM magnesium acetate. The mixture was heated for 30 min at 65°C and kept for 30 min at room temperature prior to a 6 h incubation at 4°C. The samples were then run overnight at 5 V/cm on a 12% non-denaturing polyacrylamide gel at 4°C. The activity of the bands corresponding to the free and bound oligonucleotide species was evaluated by Phosphorimager analysis.

For a kinetic association, the ^{32}P end-labelled oligonucleotide adjusted at a 2 nM final concentration was mixed with 1 μM RNA. For kinetic dissociation, the labelled oligonucleotide mixed with 5 μM RNA was incubated for 30 min at 65°C and 30 min at room temperature prior to a chase with 5 μM unlabelled oligonucleotide. Samples were then let at room temperature for various times prior to analysis by an electrophoretic mobility shift assay at 4°C. The band intensity was evaluated by a Phosphoimager.

2.3. Diethylpyrocarbonate (DEPC) interference mobility shift assay

5 nmol of 53FSHIV RNA was incubated in 250 mM Tris-acetate, pH 7.0, containing 50 mM magnesium acetate with 25% (v/v) DEPC, 25% (v/v) dimethylformamide (DMF) for 60 h at 4°C. The sample was then dried under vacuum and redissolved in 50 μl of water. Serial dilutions were performed with a solution of the desired oligonucleotide under the conditions described above. A control RNA sample was treated similarly except that DEPC was not added. The samples were then run on a 12% non-denaturing polyacrylamide gel.

2.4. Footprinting analysis

Footprinting analysis of RNA-oligonucleotide complexes was performed in a 50 mM Tris-acetate buffer (pH 7.0) containing 10 mM magnesium-acetate and 10 mM KCl with about either 0.02 pmol of 5′ end- or 3 pmol of 3′ end-labelled RNA target, for each point, mixed with the desired oligonucleotide at a final concentration of 10 μM. The RNA oligonucleotide mixture was heated for 10 min at 65°C and then let for 1.5 h at room temperature. The same conditions were used for the footprint performed on the oligonucleotide 12-mer oligodeoxypyrimidine (12Py) except that the labelled oligonucleotide was mixed with 10 μM 53FSHIV.

2.5. Enzymatic footprints

RNase V1 was added (7×10^{-3} U/μg RNA) with tRNA (500 μg/ml) to the solution containing 5′ end- or 3′ end-labelled RNA with or without oligonucleotide. The reaction was performed at room temperature and quenched on dry ice. The samples were then extracted with a phenol/chloroform/isoamyl alcohol (50:49:1) mix before ethanol precipitation. Samples were run at 50 V/cm on a 20% polyacrylamide gel containing 7 M urea in TBE buffer (89 mM Tris, 89 mM borate, 2 mM EDTA). For RNase T1 digestion under denaturing conditions, 20 000 cpm of end-labelled RNA was introduced in 25 mM sodium citrate pH 5.0 buffer containing 7 M urea, 1 mM EDTA and 0.25 mg/ml yeast tRNA. RNase T1 was then added at 0.65 U/μg of RNA and the reaction was allowed to proceed for 12 min at 55°C.

2.6. Chemical footprints

$KMnO_4$ was added at a final concentration of 0.5 mM in a solution containing 5′ end-labelled oligonucleotide with or without RNA target. The reaction was run at room temperature for 5 min, quenched on dry ice and samples were ethanol-precipitated. Pellets were then treated with hot piperidine (1 M, 30 min at 90°C). Samples were then reprecipitated with ethanol and analyzed by gel electrophoresis.

2.7. Biological assays (translation and reverse transcription)

Two plasmids were constructed to monitor the frameshift efficiency according to previous studies [4,14,15]. The frameshifting sequence including the hairpin structure preceeded by an initiation codon and a Kozak sequence was obtained by the controlled ligation of four different oligonucleotides (Eurobio). It was inserted, after digestion by appropriate restriction enzymes, in the *Nsi*I/*Bam*HI cloning site of plasmid PGEM luc (Promega), mutated at the first initiation codon of luciferase (AUG changed in UUG). In the first construct (PG FS(−1) HIV), the luciferase was in frame −1 with respect to the inserted initiation codon. In the second construct (PG FS(0) HIV), the insert obtained by PCR mutagenesis led to an in-frame luciferase gene. After plasmid linearisation at the *Sal*I site located downstream of the luciferase open reading frame, transcription was performed with the Sp6 RiboMAX large scale RNA production system (Promega) according to the manufacturer's instructions. Unincorporated NTPs were removed by gel filtration on Sephadex G75.

For translation, RNA was introduced either in wheat germ extract (WGE, Promega) or in rabbit reticulocyte lysate (RRL, Promega) at a final concentration of 5 nM in the presence of 0–5 μM oligonucleotides. This mix was incubated for 1 h at 4°C prior to translation which was allowed to proceed for 1 h at 25°C or at 30°C, in WGE and RRL, respectively.

For reverse transcription, RNA obtained from PG FS(−1) HIV, adjusted at a final concentration of 25 nM, was mixed with oligonucleotides (0–10 μM) and 500 nM of primer 5′-TCCAGCGGTTCCATCCTCTA, complementary to the 5′ end of *luc* in a 50mM Tris-HCl, pH 8.3, buffer containing 75 mM KCl, 3 mM MgCl and 10 mM DTT. The mix was incubated for 1 h at 4°C prior to the addition of 0.3 U/μl of HIV-1 reverse transcriptase (Amersham), 330 nM of [α-^{32}P]dCTP (111 Tbq/mmol, ICN) and 2.5 mM of each dNTP except dCTP wich was at 0.25 mM. After reaction (1 h at 30°C), the samples were extracted with a phenol/chloroform/isoamyl alcohol (50:49:1) mix before ethanol precipitation. Samples were then loaded on a 10% polyacrylamide gel containing 7 M urea in TBE buffer. The electrophoresis was run at 50 V/cm and the gel was analyzed by autoradiography.

3. Results

The *gag-pol* frameshifting signal of HIV-I contains a shifty heptanucleotide sequence (5′ UUU UUU A 3′), followed by a hairpin, seven bases downstream [16]. This hairpin, composed of a four base loop and a 12 bp stem whose 3′ strand contains 10 purines and two cytosines in the lower part, was used as a target for triplex-forming oligonucleotides. Two different RNAs were synthesized: 53FSHIV and 28FSHIV (Fig. 1), both of which contain the stem-loop structure targeted by synthetic oligonucleotides. As discussed below, the long RNA fragment can adopt an extended secondary structure which does not alter the stem of the hairpin.

Oligonucleotides susceptible to give rise to triple-stranded structures with the hairpin stem were prepared according to the rules leading to canonical triplets (Fig. 1). In the Py series, the oligopyrimidines 12Py and 20Py contained T and C residues able to form canonical U.A*T and C.G*C+ triplets whereas in the Pu series, oligomer 12gA could form U.A*A and C.G*G triplets. As GT triplex-forming oligonucleotides

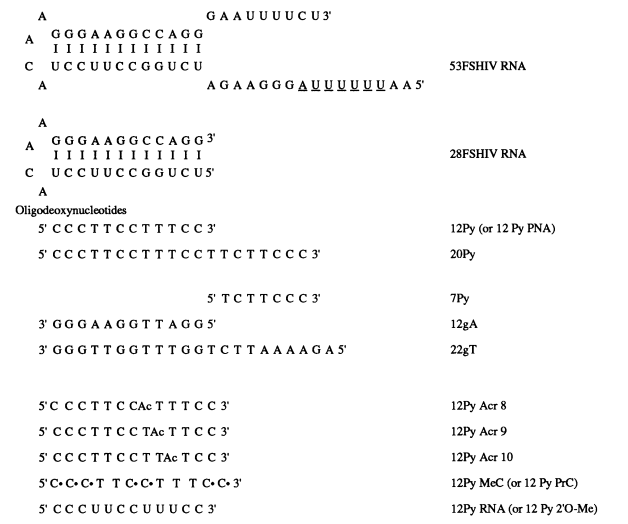


Fig. 1. RNA and oligonucleotides sequences. Ac (acridine) indicates the position at which this residue has been inserted as a phosphoramidite synthon. MeC: 5 methyl,cytosine; PrC 5 propynyl,cytosine, introduced at the positions indicated by 'C•'. PNA, peptide nucleic acid; 2′-O-Me, 2′-O-methyl oligoribonucleotide

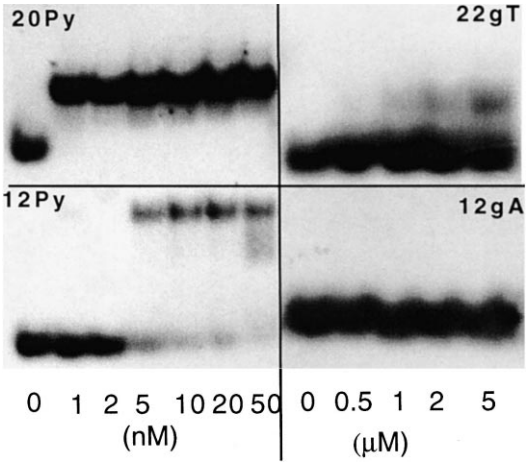


Fig. 2. Analysis of oligonucleotide-RNA complexes by an electrophoretic mobility shift assay. Oligonucleotides were ^{32}P 5′ end-labelled and mixed with 53 FS-HIV at the concentration given at the bottom of the lanes at pH 7.0 in 50 mM Tris-acetate buffer, containing 10 mM magnesium-acetate. Note that the RNA concentrations are in the nM range for oligopyrimidines and in the μM range for GT- and GA-containing oligonucleotides.

have also been described [9,17], 22gT was also assayed. Thymine was introduced in the third strand to read the inverted G.C pairs leading to G.C*T triplets which were described to be the less destabilizing combination [18–20] for DNA triple helices. Moreover, in 20Py and 22gT, an anchor leading potentially to additional Watson/Crick base pairs with the single-stranded RNA region at the bottom of the hairpin was linked by a T residue to the putative Hoogsteen sequence, to help binding to the target.

3.1. Binding of pyrimidine oligonucleotides to the FS HIV target

The binding of the different oligodeoxyribonucleotides to the RNA HIV-1 frameshift hairpin 53FSHIV was first assessed by an electrophoretic mobility shift assay. Complexes formed by oligo-pyrimidines 20Py and 12Py were detected in the low nM concentration range (Fig. 2 and Table 1). We also investigated the behavior of 12Py analogs containing modifications known to strengthen the third strand interaction with a DNA duplex (Table 1). Chemically-modified derivatives of 12Py with modified cytosines (methyl, MeC or propinyl cytosine, PrC) led to a decreased K_d, even though the high affinity of these oligomers and the limited sensitivity of the method (detection of radiolabelled oligomers) did not allow the accurate evaluation of the K_ds. A similar effect was observed with 12Py RNA or its 2′-O-methyl derivative. In contrast, acridine incorporation in 12Py at positions facing the cytosines in the purine-rich strand of the FS HIV RNA hairpin did not result in an increased affinity. Such a modification was previously demonstrated to stabilize mismatched DNA triple helices but the stability of the resulting complex was strongly dependent on the mismatched base in the third strand and on the surrounding sequence [21]. More surprising were the results obtained with the PNA analog of 12Py which presented a more than 50-fold increased K_d, compared to the parent oligonucleotide. These derivatives are known to give rise to extremely stable complexes with DNA duplexes [22].

Finally, a 1000-fold increased K_d was observed with 12Py when the target RNA, previously modified by DEPC, a reagent which carbethoxylates the N-7 of adenines, was used (result not shown). This position is involved in Hoogsteen interactions between the third strand and the targeted duplex.

In order to verify that the interaction of 12Py was restricted to the hairpin stem and did not involve the flanking sequences, the binding of this oligomer and its propynyl-C-containing derivative to the shortened target 28 FS HIV was also monitored by an electrophoretic mobility shift assay. Increased K_ds (Table 1), compared to those obtained for these oligomers with the full length target, indicated that sequences at the bottom of the stem contributed to the stability of the complex, as confirmed below. Assays in which the target and oligonucleotides 12Py or 20Py were heated and allowed to renature separately led to similar results.

The association between the target and the pyrimidine oligodeoxynucleotides was a fast process: the free pyrimidine oligonucleotide was no longer detected after 30 s at room temperature in the presence of a 500-fold excess of RNA target (not shown). A pulse chase experiment in which unlabelled oligodeoxynucleotides were added to a 5′ end-labelled oligonucleotide RNA mixture revealed that complexes made with 20Py were stable for more than 1 h whereas those with 12Py were much shorter-lived. Indeed, about 40% of the 12Pys were freed after 30 s (Fig. 3)

3.2. Influence of the pH and salt on complex formation

Pyrimidine triplexes involving T.A*T and C.G*C$^+$ triplets

Table 1
Binding properties of different oligonucleotides monitored by an electrophoretic mobility shift assay in a 50 mM Tris-acetate buffer, pH 7.0, containing 10 mM potassium-acetate, at 4°C

Oligonucleotides	53FSHIV	28FSHIV
20 Py	+++	
12 Py	++	+
7 Py	0	
12 Py MeC	+++	
12 Py PrC	+++	++
12 Py Acr8	++	
12 Py Acr9	++	
12 Py Acr10	++	
12 Py RNA	+++	
12 Py 2′-O Me	+++	
12 Py PNA	+	

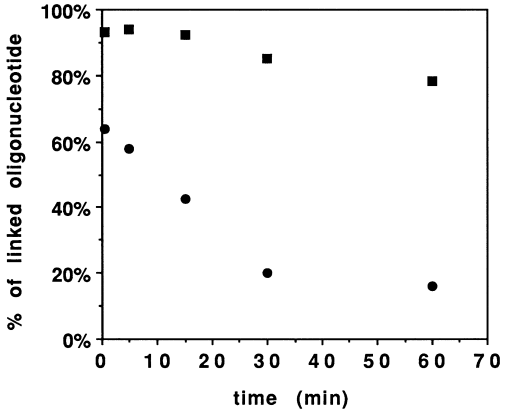


Fig. 3. Kinetics of the oligopyrimidine-RNA complex dissociation. ^{32}P 5′end-labelled 12Py (●) or 20Py (■) were mixed with 53FSHIV, chased by unlabelled oligonucleotide and analyzed by an electrophoretic mobility shift assay as described in section 2.

are pH-dependent [23]. Gel mobility shift assays with 12Py and 20Py were thus performed under acidic conditions (pH 5.0). Unexpectedly, complexes between oligonucleotides 12Py or 20Py and 53FSHIV were more stable at a neutral than at an acidic pH (Table 2). Although the formation of triplexes is strongly dependent on Mg^{2+}, complexes were still observed when magnesium was substituted by potassium although a higher K_d was obtained (Table 2). Addition of NaCl up to a 500 mM final concentration in the presence of 10 mM magnesium did not lead to a significant change in K_d (data not shown).

3.3. Footprinting experiments

In order to identify the interactions between the target hairpin and the pyrimidine oligonucleotides, footprinting studies were performed. Digestion patterns of 53FSHIV RNA with V1 endonuclease, which cleaves double-stranded RNA, are presented in Fig. 4. The cleaved region extends both in the 5′ (Fig. 4A and 3′parts (results not shown) at the bottom of the 12 bp long predicted stem. This might correspond to six additional base pairs (including a GU pair) from G11-U53 to U17-A47 (Fig. 4B). Therefore, the 53FSHIV RNA target might present a stem longer than that previously described [4,15,16,24]. The addition of 20Py greatly enhanced the RNase V1 cleavage of the stem (from G11 to G20). Curiously, addition of 12Py also exacerbated the cleavage in the lower part of the extended stem (from G12 to A14) whereas protection from the enzyme activity was detected in the upper part of the stem (from C18 to U25). A footprint performed with 3′ end-labelled RNA target also led to an increased digestion in the presence of 20Py (from C41 to C52) whereas a slightly decreased digestion was observed from C40 to C52 in the presence of 12Py (Fig. 4B).

Potassium permanganate, which leads to the oxydation of the 5-6 double bond of thymine, revealed a reduced modification at every T position of 12Py in the presence of the RNA target (Fig. 5), confirming that the entire oligonucleotide is involved in interactions with the target.

3.4. Biological assays

We investigated the effect of the different oligopyrimidines targeted to the hairpin stem on in vitro translation and reverse transcription. For cell-free translation assays, we used a construct in which the stem-loop structure was placed at the beginning of the luciferase encoding sequence either in frame −1 or in frame 0 (see section 2). None of the oligomers described above, at a concentration up to 5 μM, induced any effect on the luciferase synthesis either in the rabbit reticulocyte lysate or in wheat germ extract (not shown).

We also monitored the effect of oligopyrimidines on the in vitro reverse transcription using the above RNA construct as a template. The cDNA synthesis of a 175 nucleotides long fragment, primed downstream of the hairpin, by the HIV-1 reverse transcriptase was not affected by any of the oligomers studied (20Py, 12Py or its analogues) at concentrations up to 5 μM (not shown).

Table 2
Binding properties of oligopyrimidines determined by an electrophoretic mobility shift assay

Oligonucleotides	pH 7.0	pH 5.0	pH 7.0, KCl*
12 Py	++	+	+
20 Py	+++	+	++

*Experiment performed at pH 7.0 in a 50 mM Tris-acetate buffer containing 10 mM potassium acetate, at 4°C. See legend of Table 1 for the meaning of the symbols.

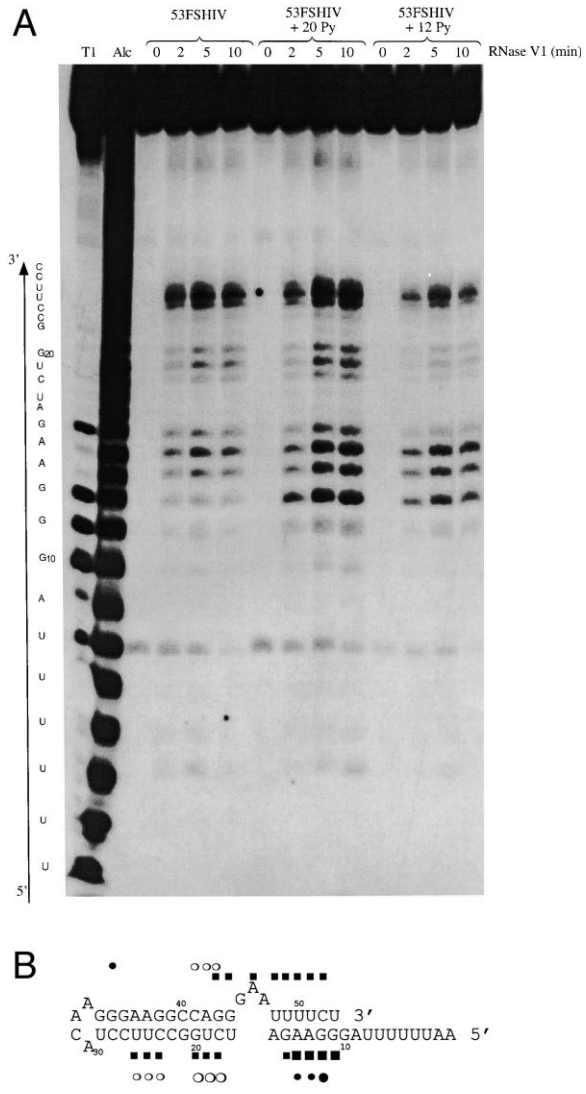


Fig. 4. RNase V1 footprinting of oligopyrimidine-RNA complexes. ^{32}P 5′ end-labelled (a) mixed with 10 μM of either 20Py or 12Py and incubated with RNase V1 as described in section 2 for the time indicated at the top of each lane. Alc and T1: alcaline and RNase T1 digestions performed under denaturing conditions of 53FSHIV is indicated on the left side. (b) The secondary structure of 53FSHIV and RNAse V1 footprint of 53FSHIV RNA. Digestion pattern modifications, induced by 20Py (■) or 12Py (●), were shown with large and small symbols to indicate a strong and weak change, respectively. Major cleavage sites are indicated with filled symbols whereas protected positions correspond to open symbols.

These results demonstrate that the complexes described above were not stable enough to compete with either the translation machinery or the reverse transcriptase. The conditions (temperature and ionic concentration) were different in the electrophoretic mobility shift assay and in the biological experiments. The highest temperature, 30 or 25°C compared to 4°C, will have decreased the affinity. But on the contrary, the higher monovalent ion concentration in biological experiments will have the opposite effect on the complex stability. It is difficult to conclude whether our failure was related to a low

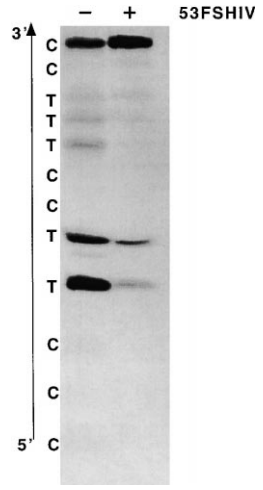


Fig. 5. KMnO4 footprint of 12Py-53FS HIV RNA complexes. ^{32}P 5′end-labelled 12Py was mixed with 53FSHIV and reacted with KMnO4 as described in section 2. The oligonucleotide sequence is given on the left.

affinity of the oligomers for their target under the conditions used for the biological assays.

It should be noted that the target sequence was located about 20 nucleotides downstream of the initiator AUG of the reporter gene. It is known that antisense targeted to the encoding region of the message does not arrest physically the translating ribosome. In addition, this indicated that the complexes formed by 12Py or 20Py are not recognized as substrates by RNases H which have been shown to play a key role in the inhibition of reverse transcription and in the cell-free translation in wheat germ extracts.

4. Discussion

We investigated the ability of either oligodeoxyribopurines or oligodeoxyribopyrimidines to bind to the stem region of the RNA responsible for the *gag-pol* frameshifting in the HIV-1 mRNA translation. This study constitutes one of the first examples of the selective targeting of a double-stranded RNA region by synthetic oligonucleotides. Pyrimidine, but not purine, oligonucleotides were shown by a band shift assay to form complexes, characterized by a K_d in the nM range at 4°C, in the presence of 10 mM Mg^{2+}.

The oligomer sequences were originally designed to allow the formation of triple-stranded structures, taking into account the recognition of two inverted GC pairs by a T residue. Nothing is known about the relative stability of RNA.RNA*DNA triplet mismatches, but triplets formed in inverted purine.pyrimidine pairs have been shown to weaken DNA triple helices [25,26] and G.C*T is the least destabilizing mismatched triplet [18–20]. Moreover, independent studies performed previously on three different sequences have found that the combination of an oligopyrimidine DNA third strand with an RNA duplex did not yield detectable triplexes [10–12]. It is therefore of interest that complexes were observed despite these important drawbacks.

The oligomer 20Py was designed to form 7 bp with the 5′ region at the bottom of the hairpin and 12 base triplets with the double-stranded stem. The Watson-Crick base pairing of the seven base anchor cannot account for the binding of 20Py. Firstly, the 7-mer had a very low affinity for the target RNA 53FSHIV (Table 1). Secondly, 12Py (i.e. the isolated potential triplex-forming part of 20Py) binds to the target RNA. Moreover, this 12-mer binds also to the RNA stem-loop 28FSHIV. The reduced affinity observed in this latter case, compared to the 53 nucleotides long RNA, could be either due to a second type of complex with the wings at the bottom of the hairpin or to different structures of the RNA-DNA bimolecular complexes. Indeed, it was demonstrated in a previous study that the binding of a triplex-forming oligomer to a DNA hairpin gave rise to intramolecular rearrangement of the DNA target which ultimately affected the structure and the stability of the complex [27].

The pH and ionic dependences of the oligopyrimidine-hairpin RNA complexes are unusual with respect to DNA triple helices, whereas Mg^{2+} is required for third strand binding [28], 12Py and 20Py were still able to bind to the RNA target in the presence of 10 mM K^+ and in the absence of magnesium ions. As the formation of C.G*C^+ triplets requires the protonation of the cytosine residues in the third strand, C-containing oligopyrimidine DNA generally binds more strongly to the double-stranded DNA at an acidic than at neutral pH [29–31]. In contrast, we got more stable oligodeoxypyrimidine-hairpin RNA complexes at pH 7.0 than at pH 5.0 (Table 2). However, it has been reported that cytosine can remain protonated at a neutral pH [32,33] and experiments made on triplex with circular duplex RNA showed that a pH transition induced the smallest variation of complex formation free energy compared to the DNA triplex [31]. Moreover, adjacent protonated cytosines lead to electrostatic repulsions which result in an adverse effect on the triplex stability [34]. The presence of three blocks of adjacent cytosines in 12Py might have shifted the optimal value for binding towards a neutral pH value. Alternatively, three strand helices have been described with C.G.*C triplets which involve only one hydrogen bond between the duplex and the Hoogsteen strand [35,36].

Our preliminary kinetic data on 12Py-RNA hairpin complexes have shown that complexes between pyrimidine oligonucleotides and 53FSHIV are generated in a few seconds, at room temperature, but 12Py dissociation is a fast process (Fig. 3), since about 40% of the complex is dissociated within 30 s. The longer lifetime of the complex observed with 20Py probably results from the contribution of its Watson Crick anchor although such a binding was not clearly established. Alternatively, structures might also be responsible for this different behavior as suggested by the RNase V1 footprints (see below). A previous kinetic analysis performed by a gel electrophoretic mobility shift assay [37] or by protection against DNase I digestion [38,39] with duplex DNA indicated that triplex formation with a DNA pyrimidine third strand is a slower process than duplex association. No kinetic data on triplexes made with an RNA duplex are available.

The above points argue against triple helix formation between 12Py (or 20Py) and the *gag-pol* frameshifting hairpin RNA. In contrast, several features are in agreement with a triple-stranded structure. The ribooligonucleotide 12Py exhibited a higher affinity for the hairpin RNA 53FSHIV than the DNA parent oligomer, in agreement with previous studies which indeed demonstrated that RNA.RNA*RNAs are the most stable triple helices [10–12]. The propynylation or the methylation of the fifth position of C residues led to an in-

creased affinity for the RNA hairpin in fair agreement with previous results on DNA triple helices [40]. The extensive chemical modification of 53FSHIV by diethylpyrocarbonate resulted in a 1000-fold decreased K_d, suggesting that the N(7) position of A residues is important for 12Py-binding. In triple helices, N(7) is involved in Hoogsteen hydrogen-binding between the purine strand of the duplex and the pyrimidine third strand. In addition, the RNase V1 footprint clearly demonstrates that 12Py interacts with the hairpin stem.

The RNase V1 footprint of the 20Py 53FSHIV RNA complex is far less clear, although a complex is formed as shown by a band shift assay, no protection of the stem was detected, suggesting either an alternative complex or an oligomer-induced conformation change of the target. It was reported that RNase V1 was able to cut a RNA/DNA heteroduplex, although with a low efficiency compared to double-stranded RNA [41]. However, it is unlikely that regular Watson-Crick RNA-DNA heteroduplexes are formed due to partial complementarity between 53FSHIV and 20Py as the oligodeoxynucleotides could not prime reverse transcription nor could it induce RNase H degradation of the target in the wheat germ extract.

It is also unlikely that 12Py gives rise to C-C^+-mediated complexes. Such an 'i-form' has been demonstrated to take place with DNA [42] but not with RNA sequences [43]. In addition, such complexes are also expected to be stabilized at an acidic pH.

In conclusion, we have demonstrated that an oligopyrimidine can bind to an RNA double strand although we are not presently able to specify the structure of the oligonucleotide-RNA complex. Our study extends the repertoire of antisense sequences. Beyond the complementary sequences successfully used in numerous studies [44], one can now consider the selective targeting of RNA structures.t

Acknowledgements: We are grateful to J. Michel (INSERM U 386) for the oligonucleotide synthesis and purification. This work was supported by The Agence Nationale de la Recherche sur le SIDA (ANRS). K.A. was the recipient of ANRS and FRM fellowships.

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