Chimeric alpha-beta oligonucleotides as antisense inhibitors of reverse transcription

Claudine Boiziau, Françoise Debart, Bernard Rayner, Jean-Louis Imbach, Jean-Jacques Toulimé

Abstract Alpha-beta chimeric 17-mer oligodeoxyribonucleotides containing either 5, 10 or 15 beta nucleotides were synthesized. The stability of the RNA/oligomer hybrids was only slightly affected by the alpha stretch and by the alpha-beta link, as was the affinity of the Moloney Murine Leukemia Virus reverse transcriptase for the duplexes. All chimeras inhibited in vitro cDNA synthesis in a cell-free system to various extents, via the degradation of the RNA target by RNase H.

Key words: RNase H; Antisense oligonucleotide; Moloney Murine Leukemia Virus

1. Introduction

One of the problems of using oligodeoxyribonucleotides in cultured cells or in vivo as gene expression inhibitors, is their sensitivity to nucleases. Most of chemical modifications introduced into the backbone of antisense oligonucleotides were aimed to make them resistant to these nucleases. As 3' exonucleases were shown to be mainly responsible for their degradation, oligonucleotides modified at their 3'-end only, were more resistant than the unmodified analogues; this led to a higher translation inhibition, due to an increased lifetime in the culture media and likely inside the cell [3,4].

The key role played by RNase H in the effect of antisense oligonucleotides on in vitro translation and reverse transcription was demonstrated [5-10]; either natural phosphodiester or phosphorothioate oligonucleotides inhibited protein or cDNA synthesis with a high efficiency through the cleavage of their RNA target. However, alpha oligonucleotides which could not elicit the action of RNase H were able to block gene expression in cell-free systems under certain conditions [8,11,12]. Taking this in mind, we thought that chimeric oligonucleotides made of a stretch of a units linked to an unmodified beta one could combine the properties of alpha and beta oligonucleotides. Such oligomers can be expected to (i) permit the action of RNase H at a stretch of alpha nucleotides linked to an unmodified stretch. (ii) be protected against the 3' exonucleases, and (iii) physically block translation or reverse transcriptase when the ,8 stretch is long enough.

2. Materials and methods

2.1. Oligonucleotides and RNA

Unmodified phosphodiester oligonucleotides were synthesized on a Milligen synthesizer. The fully modified alpha oligomer was a gift from N.T. Thuong (Orléans, France). Chimeric alpha/beta 17-mers (17αβ) were synthesized as previously described [13] on an ABI 381A DNA synthesizer via the phosphoramidite method and using the manufacturer's 1 pmol cycle. For each chimeric oligodeoxyribonucleotide, the allosteric effect was first assembled in the reverse (5' to 3') orientation with six deoxy-x-nucleoside 5'-cyanoethylphosphoramidites and 5'-linked deoxy-x-adenosine derivatized LCA-CPG. Then regular deoxy-β-nucleoside 3'-cyanoethylphosphoramidites (Millipore) were used to build up of the beta stretch in the classical 3' to 5' orientation. All other reagents for oligonucleotide synthesis were from Millipore. Standard protocols were applied for the deprotection and the reverse phase HPLC purification of chimeric oligonucleotides. The 21-mer RNA oligomer was enzymatically synthesized according to a published procedure [14]. All oligonucleotides used throughout this study are described (name and sequence) in Table 1; antisense oligonucleotides were checked for purity by 5'-end radiolabeling with T7 polynucleotide kinase and [α-32P]ATP (111 TBq/mmol, ICN).

2.2. Reverse transcription

0.25 pmol of rabbit β-globin mRNA (Gibco BRL) was preincubated for 15 min at 39°C, with 0.5 pmol of 5'-end radiolabeled primer (either 15PO(130) or 15PO(147)) and 1 to 5 pmol of one of the 17αβ oligonucleotides, in a total volume of 8 μl of 100 mM Tris-HCl, pH 8.3, containing 10 mM MgCl2 and 10 mM dithiothreitol (buffer A). After addition of 2 μl of 200 mM Tris-HCl pH 8.3 containing 10 mM MgCl2, 10 mM dithiothreitol, 360 μM each of the four dXTPs and 50 units MMLV RT (Gibco BRL), corresponding to 1.8 pmol cDNA synthesis was performed during one hour at 39°C. The transcripts were precipitated with ethanol and analysed on a 20% polyacrylamide/7 M urea gel. The yield of cDNA synthesis was quantified by counting the gel slices in a β-scintillation counter, and by comparing the full-length cDNA abundance in the absence and in the presence of antisense oligomer.

2.3. Analysis of RNA cleavage mediated by the RT RNase H activity

The activity of the RT RNase H on the 17αβ/RNA duplexes was checked either with a portion of the β-globin mRNA corresponding to nt 1-190(a), or with a 5'-end radiolabeled 21-mer RNA complementary to the 17αβ oligonucleotides (b), under conditions close to those used for cDNA synthesis (see above). (a) The 1-190 RNA fragment was generated by incubating 2.75 pmol of β-globin mRNA with 20 pmol of a 19-mer complementary to nt 172-190, in 20 ml of 20 mM Tris-HCl, pH 7.5, containing 10 mM MgCl2, 100 mM KCl, 0.1 mM dithiothreitol, and 2 units of E. coli RNase H (Promega). After 2 h at 37°C, the RNA was precipitated with ethanol and used without further purification. RT RNase H-mediated cleavage was analysed as follows: 0.25 pmol of the 1-190 RNA fragment was preincubated 15 min at 39°C, with 5 pmol of either 17α17β, 17α17β, or 17α17β, in the presence or the absence of 15PO(130), in 8 μl of buffer A. After addition of 2 ml of the reverse transcription buffer containing 1 nmol of each dXTP and 1.8 pmol whose efficiency was only slightly affected by the length of the unmodified stretch.
of MMLV RT, the incubation was performed at 39°C during 15 min only (to prevent the total digestion by the RT RNase H of the RNA hybridized to the neo-synthesized cDNA). The RNA fragments were extracted with chloroform, precipitated with ethanol, and loaded on a 10% polyacrylamide/7 M urea gel. Electrophoresis on a nylon membrane (Pall), they were probed with a 32P-radiolabeled 17-mer complementary to nt 2-19 of the 8-2 globin mRNA. (b) After 15 min preincubation at 39°C of 0.25 pmol of 21RNA (dedephosphorylated and 5'-end radiolabeled with J32P]ATP) with 5 pmol of one of the 172~215 in 8 μl of buffer A, 2 μl of the reverse transcription buffer B containing 1 nM of the four dXTPs and 1.8 pmol of MMLV RT were added. Incubated 15 min at 39°C, the digestion products were extracted with chloroform and precipitated with ethanol, before being loaded on a 20% polyacrylamide/7 M urea gel. A ladder of RNA fragments, prepared by incubating 0.5 pmol of 5'-end radiolabeled 21RNA in 10 μl of 0.15 M sodium bicarbonate (pH 9.5) for 10 min at 95°C, was loaded without any further treatment.

2.4. Duplex stability measurement
Melting temperatures (Tm) of oligonucleotide/RNA duplexes were measured by incubating 21RNA with the complementary oligonucleotide at equimolar concentration (0.5 μM), in a 10 mM sodium cacodylate buffer, pH 7.0, containing 50 mM sodium chloride and 1 mM magnesium acetate. Absorbance was monitored at 260 nm on a Uvikon 940 spectrophotometer, with temperature increased from 20 to 90°C, at 30°C/h. Melting temperature was calculated from the first order derived curve of the absorbance, versus temperature plot.

2.5. Quantitation of RNAloligonucleotide/MMLV RT complexes
2 pmol of 5'-end-radiolabeled 21RNA were incubated 5-10 min at 39°C with an equal quantity of one of the 172~215, in 100 mM Tris-HCl, pH 8.3, containing 10 mM MgCl2, 10 mM DTT and glycerol 15% (in these conditions, almost all the RNA was hybridized to the oligonucleotides, not shown). After addition of 2 to 6 pmol of MMLV RT, the samples (10 μl) were incubated 5 min at 39°C, and loaded on a 5% polyacrylamide, 0.13% bisacrylamide non-denaturing gel, buffered with 25 mM Tris-HCl pH 8 (at 4°C) containing 162 mM glycine. The migration was performed at 7.5 V/cm during 3 h, at 4°C. The quantitation of ternary complex was performed by counting, in a β-scintillation counter, the gel slices containing on the one hand, the remaining free RNA/oligonucleotide duplex, and on the other hand all the shifted species (ternary complex and smear). The affinity constant Kα was determined at the enzyme concentration (MMLV) leading to 50% conversion of the RNA/oligonucleotide duplex ([RNA/oligonucleotide]α) into a shifted species, according to the equation: $K_α = \frac{[MMLV]}{[RNA/oligonucleotide]α - \frac{1}{2}[RNA/oligonucleotide]α}$.

3. Results and discussion

3.1. Cleavage of an RNA target by the RT RNase H
Antisense sequences, 17 nt long, were synthesized as αβ chimeras: three different oligomers (17α,21β,17α,21β0 and 17α,β1) were prepared, whose length of the β region was 5, 10 or 15 nucleotides, respectively (Table 1). As a oligomers hybridize in a parallel orientation to their complementary sequence, these chimeric oligomers had a 3'→3' phosphodiester junction between the α and β blocks and positioned two 5' ends. In addition, we prepared the unmodified 17-mer (17β1) and the fully modified derivative 17α,7β. We first assayed the RNase H sensitivity of hybrids formed by these chimeras with a 21 nt-long complementary RNA. As shown in Fig. 1, 17α,21β,17α,21β0 and 17α,7β induced cleavage at two sites, one at the end of the RNA/oligonucleotide duplex, the second one at the next position, i.e. one nucleotide inside the hybrid. Cleaved products represented 20 and 60% of the total RNA for 17α,21β, 17α,21β0, respectively, whereas no cleavage was detected in the presence of 17α,7β (Fig. 1). Therefore, sensitivity to RNase H was more important with the oligomer containing a 10 nt stretch of β residues than with the one containing 15 β nucleotides. These differences were not due to a certain variation of the hybridization efficiency of oligonucleotides to the RNA: the melting temperatures ranged from 55 to 61°C for the three 17β oligonucleotides and their unmodified (17β1) and fully modified alpha analogues (17α,7β), in agreement with previous results [15,16]. Furthermore, the 3'-3' link between the alpha and the beta portions did not significantly destabilize the hybrids [13]. Therefore, under our conditions (10 mM Mg2+), every RNA target should be involved in a duplex, as the antisense oligonucleotide was in a 20-fold excess compared to the target.

The unexpected increased cleavage observed with 17α,7β0 compared to 17α,21β, could be due either to various modes of binding of the RT on the two hybrids or to a different structure of the heteroduplexes formed by the two chimeras, which leads to a different susceptibility of the bound RNA to RNase H.

So, ten contiguous β-anomeric nucleotides in the antisense oligomer induced the RT RNase H-mediated cleavage of a short RNA template. However, the situation could be different when the target is embedded into a long RNA strand. An RNA fragment corresponding to nt 1-190 of the rabbit β-globin mRNA was generated from the intact message (see section 2), which contained the binding site (nt 113-129) for the 17β oligonucleotides. In agreement with the results obtained with the 21-mer RNA, fragments (representing 60% of the initial RNA target) were detected in the presence of 17α,7β, resulting...
from cleavage probably generated at the level of the hybrid extremity (Fig. 2). In contrast, 17αβ15 led to two main degradation products (corresponding to 50% of the RNA), the shorter fragment resulting from cleavage inside the RNA/17αβ15 hybrid, at about ten nucleotides from the extremity of the RNA/β-portion, and consequently at about five nucleotides from the 3'-5' junction (Fig. 2); such cleavages occurring opposite from two to nine nucleotides of the 3'-OH extremity of an unmodified oligonucleotide were previously described with MMLV RT [17]. In addition, 20% of the RNA hybridized with 17αβ15 was a substrate for the RT RNase H; a band of cleavage was detected at the level of the duplex (Fig. 2). No cleavage of this RNA was detected in the absence of RT (not shown). This was at variance with the results obtained with the short RNA, as well for the rate as for the cleavage pattern. It is striking to note that 17αβ15 is silent as far as cleavage was concerned with the short target, whereas it elicited cleavage of the large target. This suggests that RT enzyme engaged some interaction with the RNA template outside the heteroduplex. This is in agreement with the work of Ben-Artzi et al. [18]: the distance between the 3'-OH extremity of the unmodified oligonucleotide and the cleavage sites generated by MMLV RT is larger when the 5' end of the targeted RNA corresponds to the 3'-OH extremity of the oligonucleotide (distance = 22–24 nt), than when a long overhanging RNA is targeted (distance = 18 nt). Such interactions could: (i) stabilize the duplex/RT complex, which was not the case (see below), or (ii) allow the positioning of the RNase H catalytic site in front of the phosphodiester window of the RNA/17αβ15 duplex.

Upon cDNA synthesis from a remote primer, a regular RNA/DNA duplex can form in the vicinity of the antisense/RNA hybrid. Therefore, it was of interest to check if an oligonucleotide bound immediately upstream of the antisense sequence could modify the pattern and efficiency of cleavage.

### Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Target</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17α0β13</td>
<td>5'-ACACCTTCTTCAACCCAC3'</td>
<td>113-129</td>
<td>58</td>
</tr>
<tr>
<td>17α0β10</td>
<td>5'-ACACCTTCTTCAACCCAC3'</td>
<td>113-129</td>
<td>58</td>
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<tr>
<td>17α0β45</td>
<td>5'-ACACCTTCTTCAACCCAC3'</td>
<td>113-129</td>
<td>55</td>
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<tr>
<td>17α0β32</td>
<td>5'-ACACCTTCTTCAACCCAC3'</td>
<td>113-129</td>
<td>55</td>
</tr>
<tr>
<td>17α0β17</td>
<td>5'-ACACCTTCTTCAACCCAC3'</td>
<td>113-129</td>
<td>61</td>
</tr>
<tr>
<td>1PO(3)</td>
<td>5'-TTGTGTCAAAAGCAAGT3'</td>
<td>3-19</td>
<td>-</td>
</tr>
<tr>
<td>15PO(130)</td>
<td>5'-TGCCCCAGGCCCTCAG3'</td>
<td>130-144</td>
<td>-</td>
</tr>
<tr>
<td>15PO(147)</td>
<td>5'-GTAGACAACAGCAGG3'</td>
<td>147-161</td>
<td>-</td>
</tr>
<tr>
<td>15PO(172)</td>
<td>5'-AAGGACTCGAAGAACCCTCT3'</td>
<td>172-190</td>
<td>-</td>
</tr>
</tbody>
</table>

The upper part contains the oligodeoxyribonucleotides, used as antisense, primer or probe; at the bottom the sense 21-mer RNA, corresponding to nt 112-130 of the β-globin mRNA is given. The name of oligonucleotides is composed of their length, their chemical structure (PO: natural phosphodiester, and the distinction between alpha and beta configurations for the antisense chimeras 17αβ), and, in parenthesis, the position of the first targeted nucleotide of the β-globin mRNA, except for short, for the five antisense oligomers (17αβ10, 17αβ15, 17βα), which have all the same target. The alpha stretches are underlined. In the fourth column, the melting temperatures determined for the antisense oligomers hybridized with 21RNA, under the conditions indicated in materials and methods, are given.

A 15-mer (15PO(130)) bound adjacent to antisense chimeras will extend significantly the portion recognized as a substrate by RNase H. Surprisingly, the degradation products induced by either 17αβ15, 17αβ10 or 17αβ45 chimeric oligonucleotides were identical in the presence and in the absence of 15PO(130) (not shown). As reverse transcription could be primed by 15PO(130), this indicated that cleavage of the RNA/chimeric hybrids by RNase H was a fast reaction, degrading the RNA moiety before a polymerizing RT had a chance to unwind the RNA/antisense duplex. Therefore, these chimeric oligonucleotides could constitute potential inhibitors of reverse transcription.

### 3.2. 17αβ chimeras blocked reverse transcription

Reverse transcription was primed with 5'-end radiolabeled 15PO(130). Addition of one of the 17αβ led to a decrease of the synthesis of the full-length cDNA 144 nt long (Figs. 3 and 4a), correlated with the appearance of shorter fragments (Fig. 3). Either 17αβ10 or 17αβ15 blocked elongation of cDNA after the introduction of a single dXTP to the primer; in contrast, 17αβ15 induced abortive cDNA synthesis after addition of one to eleven dXTPs (Fig. 3). Arrest profiles were correlated with the cleavage of the RNA template (fragment 1–190) by RNase H for all the 17αβ chimeras. Furthermore, the inhibition of cDNA synthesis induced by 500 nM chimera was identical to the RNase H cleavage rate for 17αβ15 (50%) and 17αβ45 (20%) (Figs. 2 and 4a), whereas for 17αβ10 it was 35% (Fig. 4b), which was significantly less than the cleavage rate (60%).

The dependence on RNase H cleavage for cDNA synthesis arrest led us to think that chimeric αβ oligomers could interfere with cDNA synthesis primed by a remote oligonucleotide, in contrast to the fully modified oligonucleotide 17αβ, which blocked reverse transcription only when adjacent to the primer [8]. Indeed, synthesis of the 161 nt-long cDNA primed by 15PO(147), was decreased in the presence of any 17αβ (Fig. 4b). In addition, the patterns of inhibition were similar – with a shift of the 17 nt – to those observed with the adjacent primer 15PO(130) (not shown). This suggested that antisense chimeric oligomers
inhibited reverse transcription via the same mechanism whatever the primer position. This was in agreement with the capacity of any 17αβ to induce digestion of the long RNA target by RNase H. However, the efficiency of inhibition of reverse transcription by 17αβ chimeric oligomers is rather low compared to other derivatives. An unmodified beta oligonucleotide targeted to the 5' end of the RNA template, its phosphorothioate and 2'-O-allyl analogues led to 50% cDNA synthesis arrest at 60 nM, 100 nM and 100 nM, respectively [9,19], i.e. at concentrations five to ten times lower than 17αβ oligomers. In the first two cases, oligonucleotides acted via RNase H cleavage of the template, whereas the 2'-O-allyl oligoribonucleotide physically blocked the scanning RT. In this latter case, the antisense oligomer displayed a much higher affinity for its target than any other derivative.

3.3. Affinity of reverse transcriptase for the RNA/17αβ oligonucleotide heteroduplexes

Above results indicated that the RNA/17αβ hybrids were substrates for the RT RNase H, whatever the size of the β stretch, at least for long RNA templates. The affinity of MMLV RT for RNA/oligonucleotide hybrids was evaluated by band-shift assay: the addition of MMLV RT to a radiolabeled RNA/17αβ duplex led to the appearance of a slow migrating species in the presence of either heteroduplexes (Fig. 5a). From the amount of the shifted species, we evaluated that the enzyme had approximately the same affinity for the three heteroduplexes (Fig. 5b): $K_a = 2.5 \times 10^6$ M$^{-1}$. In a previous work, we reported that MMLV RT had a similar affinity for another RNA/β and RNA/α duplex [19]. Here, it seemed that the 3'-3' alpha-beta junction was not a major hindrance to the binding of MMLV RT to an RNA/17αβ duplex.

How does RT bind to such non-natural RNA/17αβ duplexes? It could be proposed that this was determined by the 3' extremity of the β stretch interacting with the DNA polymerase site of the enzyme, as in the case of unmodified oligonucleotides [20–23]; consequently, the RNase H cleavage site should have been shifted by five nucleotides when extending the alpha stretch from two to seven and to twelve nucleotides. This was clearly not the case, as well with the short 21-mer RNA as with the 190 nt long RNA. Therefore, this suggested that the position of RT on the RNA/17αβ duplexes was roughly the
same whatever the size of $\alpha$ and $\beta$ stretches, leading to RNase H cleavages located close to the 5' end of the beta stretch. This indicates that the 3'-3' internal junction of the chimera did not determine the positioning of the MMLV RT. It is also likely that RT is interacting with the alpha moiety of the chimeras [16]. Together this means that this enzyme is not able to determine the relative orientation of the RNA strand (template) and of the complementary one (antisense sequence) as the alpha strand runs parallel to the template, i.e. in the opposite orientation of natural double-stranded nucleic acids.

4. Conclusion

We demonstrated that chimeric $\alpha/\beta$ oligonucleotides can inhibit reverse transcription. First, as these oligomers have only 5' ends, they will not be substrates for 3' exonucleases which have been shown to be the predominant activity in serum. Moreover, the beta stretch, which is the Achilles' heel of the molecule as it is sensitive to nucleases, does not need to be very large: a block of five nucleotides in the beta configuration was sufficient to induce both cleavage of the template and arrest of cDNA synthesis; however, the efficiency of inhibition was significantly less than with a longer $\beta$ region. More interesting, an oligonucleotide containing a 10 nt unmodified block was a reverse transcription inhibitor as efficient as its analogue with a 15 nt beta stretch.

References