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Blockage of AMV reverse transcriptase by antisense oligodeoxynucleotides

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Synthetic oligodeoxynucleotides, either unmodified or linked to an intercalating agent, have been used to prevent cDNA elongation by the AMV reverse transcriptase. Oligonucleotide/RNA hybrids specifically arrest primer extension. The blockage involves the degradation of the RNA part bound to the antisense oligonucleotide by the RNase-H activity associated with the retroviral polymerase.

Antisense oligonucleotide; Reverse transcriptase; Intercalating agent; Rabbit globin; *Trypanosoma brucei*

1. INTRODUCTION

Selective inhibition of gene expression can be achieved using complementary nucleic acid sequences, so-called antisense RNA and DNA. The expression of both endogenous and exogenous genes has been specifically turned off in cell-free systems as well as in micro-injected or in cultured cells [1,2]. Chemically modified oligodeoxynucleotides have been synthesized aimed at improving the efficacy of antisense inhibitors (increased resistance to DNases, improved uptake by intact cells, etc.) [3,4]. These compounds constitute powerful tools for molecular genetics, in particular in eukaryotic organisms. They are also receiving more and more attention as potential therapeutic agents [5].

Antisense oligonucleotides have been targeted to tRNA, rRNA and U snRNA but up to now biological studies have been mainly performed with oligonucleotides complementary to either pre-mRNA or mRNAs [6]. In most cases the oligonucleotide/mRNA hybrid prevented translation of the message. The inhibition of reverse transcriptase by antisense oligomers can also be envisaged. This can be achieved in 3 different ways: interaction with the enzyme, competition with the primer, or arrested elongation of the cDNA chain. We investigated the latter possibility.

The synthesis of cDNA by reverse transcriptase could be inhibited by an oligonucleotide complementary to a region downstream from the primer. In this case the stopper oligodeoxynucleotide should have as high an affinity as possible for its target sequence. Oligonucleotides covalently linked to an acridine derivative have been shown to exhibit a higher affinity than homologous unmodified oligomers for the complementary RNA sequence [7]. We report here some results which show that primer extension can be blocked by both unmodified and acridine-linked oligonucleotides.

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2. MATERIALS AND METHODS

2.1. *Oligonucleotides*

Unmodified oligodeoxyribonucleotides 5'd[CACCAACTTCTTCCACA], 5'd[TTGTGTCAAAAGCAAGT] and 5'd[CTGCTACTGGGAGCTTCTCA] were synthesized on a Biosearch synthesizer. They will be termed 17-SC, 17-Cap and 20-med, respectively. They were purified in one step by reverse phase HPLC on a Zorbax oligo column. A 12-mer, 5'(CTGTTCTAATAA), was synthesized either unmodified or linked at its 5' end to 2-methoxy, 6-chloro, 9-aminoacridine via pentamethylene linker arm, according to a previously published procedure [8]. These 12-mers were termed 12Tb and 12Tb(Acr). Oligomer purity was controlled by electrophoresis, on a 20% polyacrylamide/7 M urea gel, of 5' ^{32}P-end-labelled aliquots. (12Tb(Acr) was 3'-end-labelled with [^{32}P]α-ddATP.) Concentrations of oligonucleotide solutions were measured by UV absorbance. We used a molar extinction coefficient of 8850 $M^{-1} \cdot cm^{-1}$ at 425 nm for the acridine-linked oligomer.

2.2. *RNAs and enzymes*

T4 polynucleotide kinase was from Boehringer (Mannheim), AMV reverse transcriptase, *E. coli* RNase-H and RNasine were from Genofit (Geneva). Rabbit globin mRNA was purchased from Bethesda Research Laboratory and was used without treatment. A RNA fragment, about 150 nucleotides long, was obtained by oligonucleotide-directed cleavage of rabbit β-globin mRNA by *E. coli* RNase-H: 1.25 μg of globin mRNA were incubated, with 12 units of *E. coli* RNase-H, in the presence of 100 pmol of a 15-mer complementary to nucleotides 147-161 of the β-globin message. The reaction was performed for 30 min at 37°C, in 20 μl of a 20 mM Tris-HCl pH 7.5 buffer containing 10 mM $MgCl_2$, 100 mM KCl and 0.1 mM dithiothreitol. After incubation, RNA was phenol extracted, ethanol

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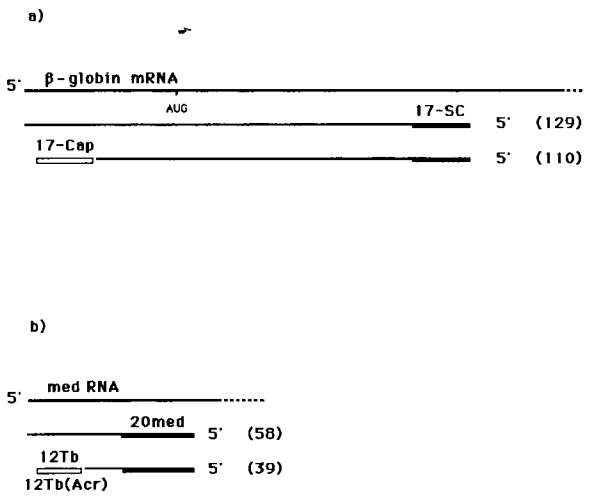

Fig. 1. Relative positions of primer and stopper oligonucleotides, (a) on the rabbit β-globin mRNA template, and (b) on the mini-exon derived RNA from *Trypanosoma brucei*. The full length cDNA products derived from 17-SC (a) and 20-med (b) are indicated below the RNAs. Bottom lines schematize truncated fragments resulting from the presence of stoppers 17-Cap (a) or 12Tb and 12Tb(Acr) (b). Bracketted numbers on the right indicate the expected sizes of DNA fragments.

precipitated and dissolved in 25 μl of sterile water. Mini-exon derived (med) RNA from cultured forms of *Trypanosoma brucei* was prepared according to a previously published procedure [9]. Med-RNA from trypanosomes and fragments of rabbit β-globin mRNA were analysed by electrophoresis on a 8% polyacrylamide/7 M urea gel followed by Northern blotting, using 20-med and a 17-mer complementary to nucleotides 51–67 of β-globin mRNA as probes, respectively.

2.3. *Primer extension*

For studies performed with the rabbit β-globin mRNA, the oligomer 17-SC, complementary to region 113–129 of the message was used as the primer. Unmodified 17-Cap, targeted to nucleotides 3–19, was used as stopper (Fig. 1a). 20-med was used as the primer and 12Tb and 12Tb(Acr) were used as stoppers for experiments with the mini-exon derived RNA of *T. brucei* (Fig. 1b), the precursor of the trans-spliced sequence present on every mRNA of this protozoan parasite [10]. For primer extension reactions, 0.9 pmol of RNA, 50 pmol of primer and the desired amount of stopper oligodeoxynucleotide were pre-heated for 2 min at 85°C and kept on ice for 30 min. After addition of 1 μl of 10×RT buffer (1 M Tris-HCl pH, 8.3, 500 mM KCl, 100 mM $MgCl_2$, 100 mM dithiothreitol) containing 8 U RNasine, 2 pmol of [^{32}P]α-dATP and 5 nmol of the four dXTPs, the volume of the samples was adjusted to 10 μl with sterile H_2O. AMV reverse transcriptase (14 U) was then added and the reaction incubated for 1 h at 39°C. Reaction was stopped by addition of 1 μl of 0.5 M EDTA.

3. RESULTS AND DISCUSSION

When reverse transcription of rabbit globin mRNA was primed by 17-SC a single band corresponding to the expected fragment about 130 nucleotides long was obtained (Fig. 2, lane 1). In contrast, when polymerisation was performed in the presence of 15 μM 17-Cap this band was hardly detectable. Instead, two higher mobility bands were seen, corresponding to DNA fragments

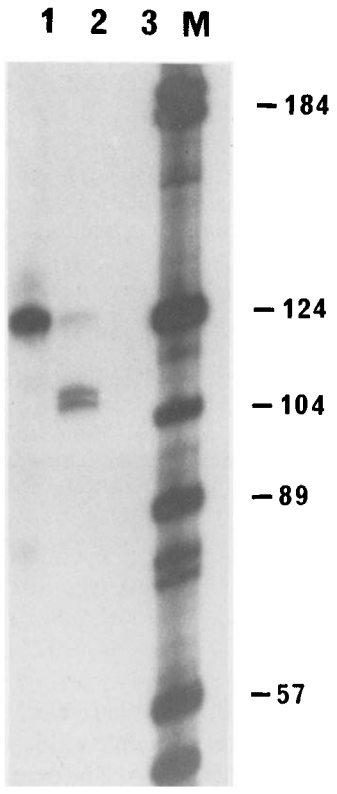

Fig. 2. Effect of 17-Cap on DNA synthesis by AMV reverse transcriptase. Primer extension from 17-SC on rabbit β-globin mRNA was performed in the absence (lane 1) or in the presence of 15 μM 17-Cap (lane 2). No 17-SC was added to the mixture in lane 3. Reaction was performed as indicated in section 2. DNA size markers were run in lane M.

slightly longer than 104 nucleotides (Fig. 2, lane 2). This is consistent with the synthesis of a cDNA fragment extending from the primer to the 5′ end of 17-Cap (Fig. 1a). Therefore the hybridization of this antisense oligonucleotide with the complementary sequence of the β-globin mRNA prevents transcription of this region. The extent of inhibition was dependent on 17-Cap concentration; 50% reduction of full length product was induced by about 0.5 μM of the stopper.

In addition to the appearance of these short fragments, it should be noted that the yield of cDNA synthesis was lower in the presence of 17-Cap (lanes 1 and 2, Fig. 2). This resulted from a competition between the primer and the stopper. In the case shown in Fig. 2, there was three times more 17-Cap than 17-SC. Indeed, the yield of cDNA synthesis varied with the overall oligonucleotide concentration. As the lenghtened 17-Cap escaped detection on our gel this resulted in an apparent decrease of reverse transcription.

The use of oligonucleotides to block reverse transcriptase in intact cells will necessitate the penetration of the plasmic membrane and a tight binding to the target sequence. Additional energy of interaction can be provided by covalent linking of an intercalating agent

to the end of the oligonucleotide: stacking interactions between this agent and base pairs of the hybrid have been shown to result in increased stability compared to the unmodified oligomer [7,11]. It has also been shown that an acridine derivative linked to the end of an oligonucleotide promoted its uptake by trypanosomes in culture [6].

We investigated the effect of acridine-linked oligomers on the synthesis of cDNA, by AMV reverse transcriptase, from the mini-exon derived RNA of *T. brucei* taken as a model. The mini-exon sequence present at the 5′ end of every mRNA of the parasite was previously chosen as a target for antisense oligonucleotide [12–15]. We transcribed the *T. brucei* med-RNA from a primer located immediately downstream of the mini-exon sequence (Fig. 1b). The synthesis of the full length cDNA fragment (58 nucleotides) occurred in a very low yield due to the presence of a strong pause site at position 27–28, corresponding to the bottom of a strong hairpin structure [15]. As shown in Fig. 3, addition of 12Tb(Acr) resulted in the appearance of a shorter mini-exon cDNA. The length of this DNA fragment (about 40 nucleotides) is consistent with a block occurring at the binding site of 12Tb(Acr). However, the level of inhibition was not different from that obtained with the homologous unmodified oligomer 12Tb. It should be pointed out that these oligomers had a 3′-OH terminus and could therefore be lengthened by the polymerase. Indeed, we observed on the gel a band indicating that both 12-mers were converted into longer oligomers (about 15 nucleotides long for the unmodified 12Tb; Fig. 3, lane 1). The relative contribution of stacking interactions, between the acridine residue and base pairs of the hybrid, to the overall binding decreases as the oligonucleotide length increases [7]. With respect to reverse transcriptase inhibition, this very likely accounts for the similar results obtained with the unmodified and acridine-linked 15-mers synthesized from 12Tb and 12Tb(Acr). The full length and the truncated cDNAs contained 14 and 9 adenine residues, respectively. Therefore, the actual yield of inhibition was close to 50% as determined from densitometer tracing of lane 1 (Fig. 3).

The hybrids formed by primer and stopper oligonucleotides with the template RNA are substrates for the RNase-H activity associated with the AMV polymerase. Of course, cleavage of the RNA region bound to the stopper will preclude cDNA synthesis beyond the hybridization site. In order to shed some light on the mechanism by which antisense oligonucleotides block the AMV polymerase, we analysed the template RNA after the polymerisation reaction. As shown in Fig. 4, incubation of β-globin RNA in the presence of AMV reverse transcriptase, 17-SC and 17-Cap, led to the appearance of a cleavage product whose length was consistent with the attack of

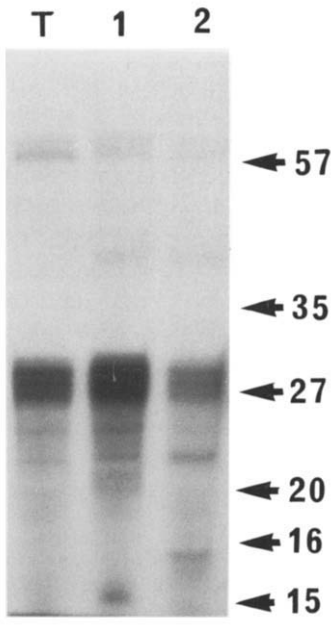

Fig. 3. Effect of 12Tb on DNA synthesis by AMV reverse transcriptase. Primer extension from 20-med was performed in the absence of any other complementary oligonucleotide (lane T) or in the presence of 60 μM 12Tb (lane 1) or 12Tb(Acr) (lane 2). The incubation was performed as indicated in section 2.

the RNA strand, by the polymerase-RNase-H associated activity, at the level of the 17-Cap binding site. Under the experimental conditions that we used, this accounted for the inhibition of cDNA synthesis.

3.1. *Conclusion*

The data presented here unambiguously demonstrate that reverse transcription can be selectively inhibited by antisense oligomers. Previous reports have already shown that oligonucleotide analogues can be used to interfere with cDNA synthesis. In the case of oligomers

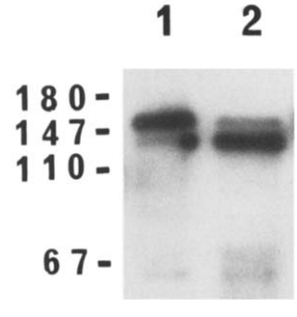

Fig. 4. Effect of cDNA synthesis inhibition on rabbit globin RNA. Primer extension from 17-SC, on a 150 nucleotide long rabbit β-globin mRNA fragment, was performed in the absence (lane 1), or in the presence (lane 2) of 17-Cap under the conditions indicated in the legend of Fig. 2. Northern blot analysis was carried out as indicated in section 2.

comprising α-anomers of nucleoside units, which cannot be elongated by reverse transcriptase, inhibition was obtained through competition with the primer [16,17]. It was also reported that phosphorothiate derivatives could prevent HIV development in cultured cells by a sequence-independent mechanism involving, at least in part, the binding of these modified oligomers to the viral reverse transcriptase [18]. The use of cross-linking reagents coupled to oligonucleotides to block reverse transcriptase has been described; platinum derivatives of oligonucleotides were able to stop the activity of reverse transcriptase working on a DNA template [19]. However, the reaction to generate cross-linked products required overnight incubation. This probably precludes the use of such oligomers in biological systems (in vitro assays with purified enzyme or cultured cells).

In our case no chemical reaction takes place between the stopper oligomer and the target RNA. But, subsequently to the binding of the stopper oligonucleotide, the template RNA is permanently modified by the RNase-H activity of the viral enzyme. It was reported that unmodified oligonucleotides did not block DNA synthesis by AMV reverse transcriptase using DNA as a template [19]. This supports our conclusion about the mechanism of cDNA synthesis inhibition as RNase-H cannot cleave such DNA/DNA hybrids. Acridine-linked oligonucleotides elicit RNase-H activity when bound to the complementary RNA sequence [20]. Moreover, it has been shown that such modified antisense oligomers can be used with intact cells, and prevent the development of the influenza virus [21] and of African trypanosomes in culture [14]. Our results indicate that they might also be of interest in the case of retroviruses.

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