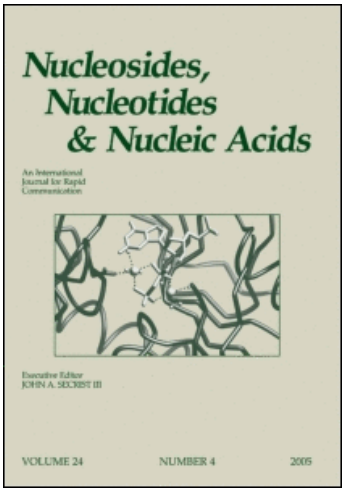

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Cleavage of Leishmania Mini-exon Sequence by Oligonucleotides Conjugated to a Dimidazole Construction

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CLEAVAGE OF LEISHMANIA MINI-EXON SEQUENCE BY OLIGONUCLEOTIDES CONJUGATED TO A DIIMIDAZOLE CONSTRUCTION

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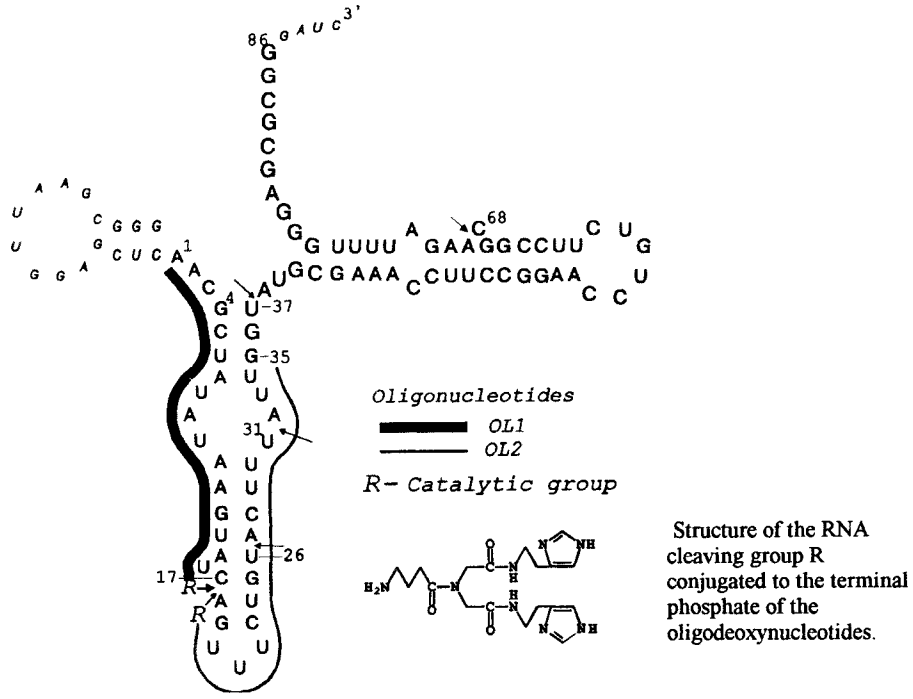
Abstract: RNA sequences derived from the *Leishmania amazonensis* mini-exon and pre-mini-exon sequences have been targeted with complementary oligonucleotides bearing a diimidazole construction mimicking active center of ribonuclease A. The conjugates were shown to cleave the target RNAs at specific positions.

Equipment of antisence oligonucleotides with reactive groups capable of hydrolyzing phosphodiester bonds in RNA is a perspective approach for improving their inhibitory potential. Catalytic mechanism of the cleavage eliminates possibilities of reactions of the compounds with biopolymers other than RNA and can provide high antisense efficiency. A straightforward approach to design small RNA cleaving catalytic groups consists in mimicking active centers of ribonucleases by organic molecules[1-7]. Covalent attachment of synthetic constructions bearing imidazole residues to oligonucleotides provides conjugates capable of cleaving complementary RNA in a sequence- specific manner[6-8]. In this communication we describe cleavage of RNA sequence derived from the mini-exon of *Leishmania amazonensis* by complementary oligonucleotides conjugated to γ-aminobutyro{N,N-bis[N'-(1H-imidazole-4-yl-ethyl)carbamoylmethyl]}amide (R).

Materials and Methods

Oligonucleotides: 16-mer pATACTTATATAGCGTT, complementary to MEX and MED RNA (FIG. 1) at position 1-16 (OL1) and 17-mer CAATAAAGTACAGAAACp, complementary to the RNAs at position 19-35 (OL2)



Structure of the RNA cleaving group R conjugated to the terminal phosphate of the oligodeoxynucleotides.

Fig.1 Target MEX RNA (1-35) and MED RNA (1-86) and the oligonucleotide conjugates. Phosphodiester linkages attacked by the oligonucleotide conjugates are indicated by arrows.

were synthesized by standard methods. Conjugation of the catalytic group to the 5'-end of OL1 and to the 3'-end of OL2 was performed according to a published procedure[9].

Oligonucleotides were analyzed by electrophoresis on a polyacrylamide/urea gel, following by 5' end-labeling with γ-[^{32}P] ATP (Biosan, Russia) and T4 polynucleotide kinase, according to standard procedures[10].

Target RNAs were RNA fragments corresponding to the *Leishmania amazonensis* mini-exon RNA (MEX-RNA, 35 nt) and pre-mini- exon RNA (MED-RNA, 86 nt), deleted of 4 modified residues present at the 5'-end of the natural sequence. Corresponding DNA sequences were cloned downstream of the T7 RNA polymerase promoter into pBluescript II KS plasmid, cleaved by the SacI and BamHI restriction

endonucleases. The plasmids were linearised by BamHI restriction endonuclease before transcription. RNAs were transcribed using AmpliScribe T7 Transcription kit (TEBU, Epicentre technologies). MEX and MED RNAs prepared in this way were flanked by additional sequences derived from the vector: GGGCGAAUUGGAGCUC at the 5'-end and GAUC sequence at the 3'-end. The transcripts were 5'-end labeled with γ -[^{32}P] ATP. After the labeling, the RNAs were purified by electrophoresis in 12% denaturing polyacrylamide gels. The RNAs were eluted from the gels by 125 mM ammonium acetate at pH 6,0 containing 0,5 mM EDTA and 0,025% SDS. After ethanol precipitation, RNAs were dissolved in water and stored at -70^{0}C.

Reaction mixtures contained 0.1 mM 5'-end labeled RNAs (50 000 Cerenkov c.p.m.) and oligonucleotide derivatives at different concentration, dissolved in 10 μl of 0.05 M imidazole buffer pH 7,0 containing 100 mM NaCl and 1 mM EDTA. After incubation at 25^{0}C, 10 μl of 9 M urea containing 0,1 % bromophenole blue and xylene cyanole was added and samples were subjected to electrophoresis through a 12 % denaturing polyacrylamide gel.

Results and discussion

In trypanosomatid protozoan parasites responsible for severe diseases in animals and man, every mature mRNA starts on the 5'-side by an identical sequence, termed spliced leader or mini-exon (MEX RNA)[10]. This RNA piece is contributed by a short pre-RNA (Mini Exon Derived, MED RNA), whose mini-exon sequnce constitutes the 5'-end. The mini-exon sequence represents an attractive target for antisense oligonucleotides as a single oligonucleotide can suppress synthesis of every protein in these infectious agents[11]. We investigated cleavage of the Leishmania mini-exon sequence by antisense oligonucleotides conjugated to a diimidazole construction capable of catalysing hydrolysis of phosphodiester linkages in RNA. FIG. 1 shows structures of the target RNAs and oligonucleotide conjugates with the diimidazole construction at the terminal phosphate. The conjugates were targeted so as to deliver the catalytic groups to the sequences containing dinucleotides CA and UA which are highly susceptible to hydrolysis by imidazole conjugates[2,5]. FIG. 2 displays cleavage patterns of the 5'-end labeled RNA targets by the oligonucleotide conjugates R-OL1 and R-OL2. It is seen, that the conjugates cleave the RNA at the target site, C17. In the case of R-OL1, low

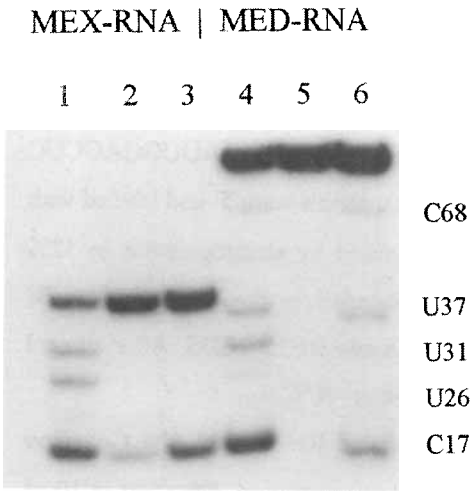


Fig.2 Cleavage of the RNA conditions: 5 hours, 25°C, 0.05 M imidazole pH 7 by oligonucleotide conjugates bearing the diimidazole construction R. The RNAs were incubated with R-OL1 (lanes 1, 4), R-OL2 (lanes 2, 5) or with R-OL1 + R-OL2 (lanes 3, 6). The incubation.0, containing 100 mM NaCl and 1 mM EDTA. Concentration of the RNAs, 0.1 μM; concentration of the conjugates, 10 μM. Arrows at the right indicate positions of the cleavage

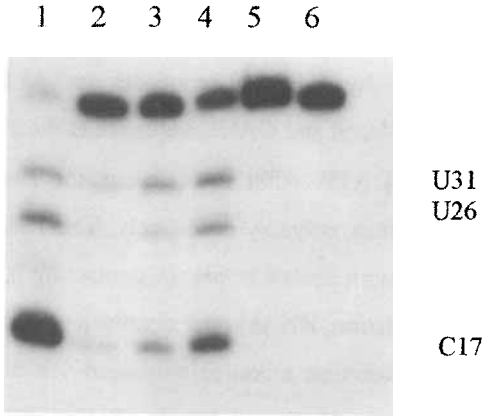


Fig. 3 Cleavage of the MEX RNA by the oligonucleotide conjugate R-OL1. Lanes 1, 4, 5, reactions with 50 μM, 10 μM and 2 μM R-OL1, incubation for 5 hours. Lanes 2, 3, 4, cleavage of MEX RNA by 10 μM R-OL1, incubation for 1.5, 3 and 5 hours, respectively. Lane 6, RNA incubated without reagents. Incubation conditions were as described in the legend to FIG. 2.

intensity cuts occur also at positions U26 and U31 in MEX RNA (Lane 1) and U37 and C68 in MED RNA (Lane 4) due to formation of nonperfect complexes with partially complementary sequences in the target . This conclusion is supported by the data of the experiment, where the RNAs was incubated with the two oligonucleotide conjugates simultaneously. In this case, no cleavages at positions U26 and U31 were observed, apparently because they were masked by hybridzation to the R-OL2 (lanes 3, 6). Spontaneous degradation of RNA in these experimental conditions in the absence of the reagents, in the presence of oligonucleotides without the imidazole group or in the presence of the free group in the absence of oligonucleotides was negligible.

The conjugate R-OL1 cleaves the target more efficiently, as compared to R-OL2. Earlier, in experiments with $tRMA^{Phe}$ we have observed, that the diimidazole construction R functions more efficiently, when it is attached to the 5'-phosphate of oligonucleotides, apparently, due to geometrical factors[6,7].

FIG.3 shows effects of incubation time and concentration of the conjugate R-OL1 on the cleavage of MEX RNA. 5 hours incubation of the RNA with the 50 mM conjugate had affected 80% cleavage of the target. The described experiments demonstrate that the oligonucleotide conjugates with diimidazole construction R can cleave RNA efficiently in mild conditions.

Acknowledgements

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