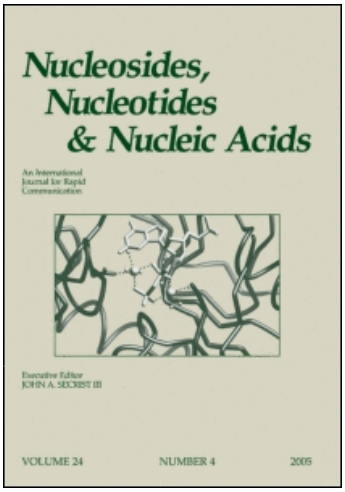

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A 38 kDa Nuclear Protein Is Involved in the Retention of an Antisense Oligonucleotide Directed Against Cytosolic Phospholipase A2

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A 38 kDa NUCLEAR PROTEIN IS INVOLVED IN THE RETENTION OF AN ANTISENSE OLIGONUCLEOTIDE DIRECTED AGAINST CYTOSOLIC PHOSPHOLIPASE A2

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ABSTRACT : Recent studies suggest that antisense phosphorothioate oligonucleotides (APO) are useful tools not only to impair gene expression, but also to modify the splicing of pre-mRNA, as the classical view that they act by suppressing the translation of mature mRNA has been challenged by several examples showing their nuclear site of action. In this work we show that an APO directed against cytosolic phospholipase A2 (cPLA2) mRNA localises in the nucleus and interacts with a specific nuclear protein.

The evidence that antisense oligonucleotides affect mRNA levels by forming a duplex with their target cytoplasmic mRNA molecules, attacked by RNase H, has been recently integrated by novel evidence indicating that APO may modify the splicing of pre-mRNA molecules[1,2,3,4]. A functional binding of antisense oligonucleotides to proteins, either sequence-specific[5] or nonsequence-specific[6], has also been demonstrated.

In the present work we investigate the mechanism of action of an APO directed against cPLA2 mRNA and designed according to the sequence proposed by Roshak and co-workers[7]. Several antisense oligonucleotides have been recently used to specifically inhibit cPLA2[8,9,10] and control the important processes in which it is involved. However, much more has to be learned about the mechanism of action of these APO.

The anti cPLA2 APO used in our studies (see Table 1 for the sequence) was biologically active: it significantly inhibited prostanoid generation and cPLA2 levels in

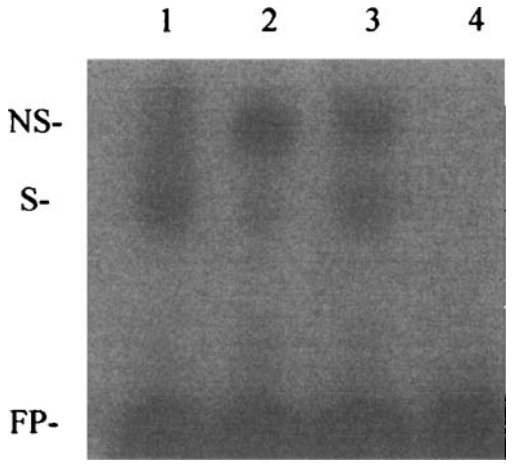

FIG. 1. Electrophoretic mobility shift assay (EMSA) carried out on HeLa cells nuclear extracts. Lane 1: Anti cPLA2 labelled oligo incubated with nuclear extract; Lane 2: competition with 50-fold molar excess of cold anti cPLA2 oligo; Lane 3: competition with 50-fold molar excess of cold control oligo; Lane 4: nuclear extract omitted. NS non specific, S specific, FP free probe. The experiments were performed by incubating 2-4 x 10^5 cpm of [γ-^{32}P] labelled anti cPLA2 oligo with 10 µg of nuclear extracts and 3 µg of poly(dI-dC)· poly(dI-dC). Competition experiments with seven mutated antisense oligos (see Table 1) were performed using a 50-fold molar excess with respect to wild type labeled antisense. The mixture, incubated at room temperature for 15 minutes before and 1 hours after addition of labeled probe, was then separate on a 5% non-denaturing polyacrylamide gel at 100 V for 90 minutes at 4°C. The gel was dried and exposed to X-ray film at -80°C with intensifying screen. The intensity of bands was evaluated by image analysis. These experiments were repeated and reproducibility was good.

LPS-stimulated human monocytes. By confocal microscopy, we observed that the 6-carboxy-fluorescein (6-FAM) labeled APO was entirely in the nucleus after 6 hours of incubation and after 9 hours it started to concentrate in discrete nuclear structures resembling to speckles, while a biologically inactive oligo remained diffused in the nucleoplasm. Speckles are subnuclear structures involved in one or more steps of pre-mRNA splicing[11]. Therefore, the biological activity of an APO localising preferentially in speckles may be due to an inhibition and/or a modification of a step of splicing occurring in these nuclear structures, leading to maturation and export of mRNA molecules. We also observed that an APO directed against cycloxygenase 2 (COX2) mRNA localised only in the cytoplasm and we detected a similar scenario either in monocytes or in

TABLE 1. IMAGE ANALYSIS OF EMSA COMPETITION EXPERIMENTS PERFORMED WITH WILD TYPE AND MUTATED OLIGONUCLEOTIDES [a]

Oligo	*Sequence*	*% of competition*[b]
Wild type	5'- TAC AGT AAA TAT CTA GGA ATG -3'	100
Mutated A	5'- **GCA** AGT AAA TAT CTA GGA ATG -3'	84,4
Mutated B	5'- TAC **GAG** AAA TAT CTA GGA ATG -3'	29,3
Mutated C	5'- TAC AGT **TGC** TAT CTA GGA ATG -3'	44
Mutated D	5'- TAC AGT AAA **ACG** CTA GGA ATG -3'	67,5
Mutated E	5'- TAC AGT AAA TAT **AGC** GGA ATG -3'	61,6
Mutated F	5'- TAC AGT AAA TAT CTA **TCT** ATG -3'	87,3
Mutated G	5'- TAC AGT AAA TAT CTA GGA **TGC** -3'	91,1

[a] The mutated bases are represented in bold. [b] The competition was evaluated analysing in transmitted light the spots obtained. The values represent the mean of three different experiments. The competiton was evaluated by using a 50 fold excess of unlabelled oligos. Two different unrelated oligos were also used as controls.

endothelial cells (HUVEC). This observation suggested that the localisation of an oligo is not cell-specific, but rather sequence-specific and prompted us to hypothesise the presence in speckles of a binding protein functioning as a carrier. Gel shift experiments revealed the presence both in HUVE and HeLa cells of an abundant 38 kDa nuclear protein binding the biologically active cPLA2 APO, but not the inactive control oligo (Fig. 1). Moreover, competition experiments with mutated oligonucleotides (Table 1) suggested that the symmetric sequence TAAAT could be involved in the binding.

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