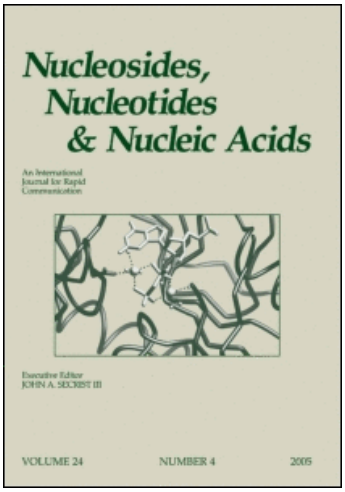

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NUCLEAR TARGETING OF ANTISENSE OLIGONUCLEOTIDES: MODIFICATION OF PRE-mRNA SPLICING OR INHIBITION OF POLYADENYLATION?

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INTRODUCTION

The mechanism by which antisense oligonucleotides inactivate target mRNA molecules is the objective of intensive studies. Until few years ago studies involving antisense oligo were based on the assumption that the decrease of mRNA, detectable using Northern blots, was a consequence of the formation of a duplex between the oligo and its target cytoplasmic mRNA molecules, attacked by RNAase H [1, 2]. Lamond and co-workers introduced the use of 2'-O-alkyl oligoribonucleotides as probes to specifically label nuclear snRNA molecules without activating RNAase H [3, 4]. Using nuclear extract from HeLa cells Dominski and Kole [5] demonstrated that 2'-O-methyl oligo targeted against β-globin pre-mRNA could correct the splicing of thalassemic pre-mRNA. Since these results have been extended using cells instead of isolated nuclei [6], it appears that at least oligoribonucleotides have the capacity to bind nuclear pre-mRNA molecules. Is this behaviour linked to the molecular structure of this class of oligo or can it be shared by the more widely used antisense oligodeoxynucleotides?

Leonetti et al. [7] microinjected antisenses in different cells observing a fast translocation into the nucleus and their concentration in discrete nuclear structures. Nuclear

diffusion of antisenses was not affected by ATP depletion and lowering of temperature, suggesting that an active process was not involved. They interpreted these data by assuming a binding of antisenses to nuclear structures. In the following years, the sensitivity provided by confocal microscopy allowed the precise localisation of antisenses in interchromatin granules (speckles). The functions of speckles are reviewed in ref. 8 and 9.

Do speckles contain poly (A) mRNA in transit to the nuclear pores? Huang et al. [10] have carried out very accurate studies to answer to this question. Their results indicate that interchromatin granule clusters (the larger speckles) contain poly (A) RNA, which does not represent nascent RNA transcript, but rather a stable RNA population having an unknown function. Are then antisense probes binding to this RNA and what is the meaning ?

Pombo and Cook [11] have recently discussed the functional significance of speckles. Two hypotheses were put forward: a) speckles could be the sites of both transcription and splicing; b) speckles could be sites having no transcriptional activity where completed transcripts accumulate and are processed. A careful confocal microscopy approach led them to conclude that most speckles are not directly involved in transcription. Zhang et al. [12] used modified oligonucleotide probes to detect nascent RNA. They concluded that splicing of pre-mRNA occurs at sites of transcription which are not coincident with intranuclear speckles. Thus neither transcription nor splicing are events occurring in speckles, a conclusion supported by recent findings by Du and Warren [13] who established a functional interaction between RNA polymerase II and pre-mRNA splicing.

Since there is a general agreement that speckles contain a large nuclear fraction of poly (A)RNA, the conclusion emerging from these data is that speckles not only accumulate the terminal products of splicing but very probably they contain poly A polymerase. Zhang et al.[12] proposed that speckles may fulfill the role of a storage site for splicing factors; however, several data support this role for coiled bodies. Recent biochemical evidence supports the idea that in speckles the terminal post-transcriptional modification of pre-mRNA is occurring. Condon and Bennett [14] used antisense directed against various domains of E-selectin pre-mRNA and detected, by Northern blots, the splicing products. One of these, directed against the 3' terminus, inhibited the splicing of the last intron causing the appearance of a novel mRNA species retained in the nucleus. It is very likely that the antisense blocked the polyadenylation step preventing the export of mature mRNA into the cytoplasm. However, this assumption requires a direct verification since O'Keefe et al. [15], using an oligo directed against snRNP, which should inhibit splicing of β-globin pre-mRNA, obtained several fragments apparently formed by endonucleolytic cleavage.

ARE PROTEINS INVOLVED IN UPTAKE OF mRNA IN GRANULES OR IN EXPORT OF POLYADENYLATED RNA ?

If mRNA is accumulated in granules (speckles) where its polyadenylation occurs, it is likely that carriers are involved. Many proteins having an high affinity toward RNA have been isolated but a role as carriers has never been postulated. We describe now recently isolated RNA binding proteins. This field has been recently reviewed [16].

SR proteins. These are a family of non-snRNP splicing factors essential for splicing in vitro, but not found in yeasts. Neugebauer et al. [17], using an immunologic approach, have identified 20 nuclear proteins which are not present in purified spliceosomes assembled in vitro. They have a unique structure in which arginine alternate with glutamate or aspartate. The authors proposed that *the alternating arginine* domain represent a common functional element of pre-mRNA splicing factors. The signals directing SR proteins into speckles have been recently identified [18].

Nuclear polyadenylated RNA-binding proteins. Wilson et al. used UV light to cross-link proteins directly bound to polyA RNA in vivo. These proteins, localized in the nucleoplasm with a pattern similar to snRNA in human cells (Nab proteins), may be required for packaging pre-mRNA into RNP structures amenable to efficient nuclear RNA processing. For example, loss of Nab2p results in alteration in both polyA tail length and nucleo-cytoplasmic transport of mRNA (see ref. 16).

Other RNA binding proteins. Hackl et al. [19] isolated a 69 kDa protein which reversibly associates with several snRNPs. Their results are consistent with the hypothesis that this protein is involved in the nuclear transport of snRNPs. Hackl and Luhrmann [20] recently reported that the 69 kDa protein is strongly concentrated in speckles as well as being distributed in the nucleoplasm.

Dreyfuss et al. [21] have reviewed the properties of hnRNP proteins. The A and B proteins in their classification are involved in shuttling between the nucleus and the cytoplasm. They contain two RNP motif RNA-binding domains and a glycine-rich auxiliary domain at the COOH terminus. This domain may facilitate protein-protein interactions.

It is clear that speckles are not sites of transcription and splicing, but they may be sites of polyadenylation. However, according to Huang et al. [10] the polyA RNA present in speckles is not a form ready to serve as cytoplasmic mRNA. Huang et al. have also proposed that this poly A mRNA is a long-lived species. Does this means that mRNAs with a long half-

life have the tendency to reside in speckles (as a reserve) while short-lived mRNA are polyadenylated outside speckles and are immediately transported to the cytoplasm? Using antisense oligo we have collected some evidence supporting this possibility. For example, comparing an antisense against cytosolic phospholipase A2 (an enzyme with a stable mRNA) with an antisense against cycloxygenase 2 (readly inducible and having a short-lived mRNA), we have detected for the first binding sites in speckles, while the second was predominantly cytoplasmic (Spisni et al., submitted).

THE POLYADENYLATION STEP

Transcription and splicing are very probably topologically associated events in the nucleus. Very little is known about the third step which follows the first two and allows the passage of mature mRNA to the cytoplasm, i.e. polyadenylation by polyA polymerase (PAP). The polyadenylation process is as important as transcription and splicing in order to correctly place mature mRNA on ribosomes. The sequences that define the poly A site in mammals contain a limited number of consensus elements. The most used is the nearly ubiquitous hexanucleotide AAUAAA found almost invariably 10-30 bases upstream of the polyadenylation site. The sequence is recognised by cleavage polyadenylation specificity factor (CPSF), while cleavage stimulation factor (CstF) recognises a GU-rich element. PAP is finally able to bind to the poly A site elongating the chain [22].

The nuclear sites, where this step along the mRNA maturation process occur, are still unidentified. Huang et al. [10] using oligo dT probes detected poly A-RNA within the speckled regions formed by interchromatin granule clusters. They suggested that modifications occurring in speckled structures enabled poly A-RNA to cross nuclear pores and to enter the cytoplasm. However, no evidence about a translocation from speckles to the cytoplasm was detectable by confocal and electron microscopy.

Does polyadenylation occurs within speckles or are these structures simply storing polyA-RNA? It is relevant that Dirks et al. [23] studying the maturation of hepatocytomegalovirus mRNA, by double hybridisation experiments, found that a substantial part of transcripts contained a poly A tail. This suggests that most of the transcripts are polyadenylated at the site of transcription, but it is not clear from this work whether polyadenylation precedes splicing. This would be in contrast with many other data of the literature.

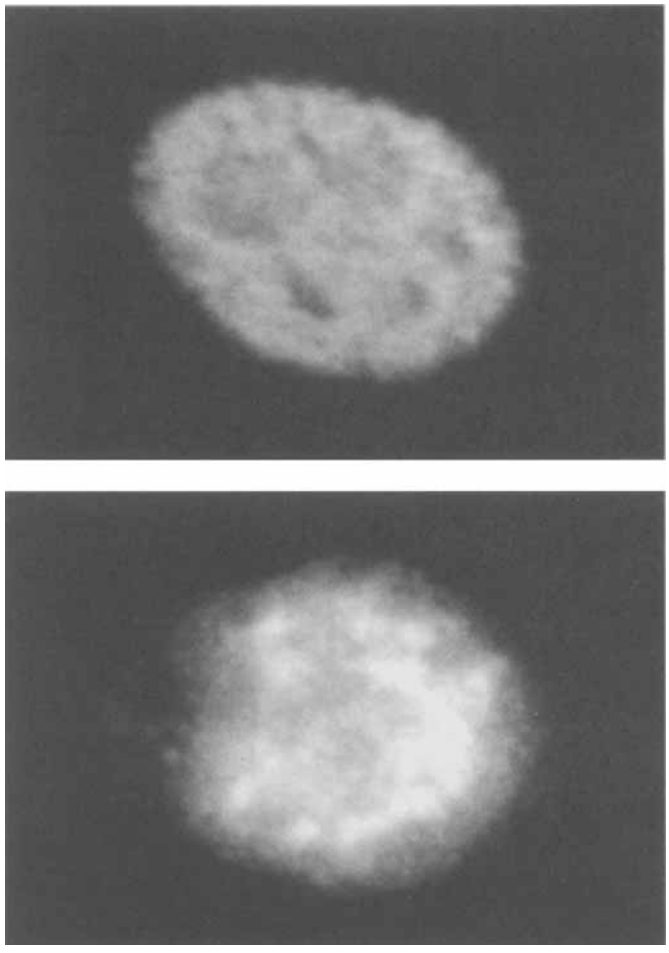
It has been proposed that polyadenylation is involved in the export of RNA to the cytoplasm [21], thus some mRNA may persist in the nucleus because they are slowly polyadenylated or not adenylated at all [24]. A second possibility is the retention in nuclei of incompletely spliced mRNA [21-24].

HnRNP AND snRNA AS TARGETS OF ANTISENSE OLIGO

It has to be pointed out that antisense have the potentiality to react either with snRNA or with hnRNP with high affinities. Several examples of these kinds of interactions are available in the literature. Already before 1987 oligos directed against snRNP have been used to demonstrate their involvement in splicing reactions. The rationale was to construct hybrids between specific sequences of snRNA and deoxynucleotides in order to activate RNase H and to destroy the duplex [24]. Buvoli et al. [25] reported experiments based on the observation of Swanson and Dreyfuss [26] regarding the binding preference of human hnRNP protein A1 for the 3' splice site of some introns to which oligo were directed.

CONCLUSIONS

Although speckles are still enigmatic objects, some of their functions begin to be established. Thus, their capacity to accumulate splicing factors either ribonucleoproteic (snRNP and hnRNP) or proteic (SR proteins) is an undisputed fact in the recent literature. For example, Misteli et al. [27] using the powerful approach based on the use of GFP (green fluorescent protein) labeling and time-lapse confocal microscopy, have demonstrated that an essential splicing factor of the SR family is stored in speckles, but that it is secreted to the sites of splicing when gene transcription is activated. This conclusion has been challenged by studies showing that antibodies to splicing factors stain speckles when they are used in standard conditions, but they stain the sites of nucleoplasmic splicing when used in diluted forms [28]. This is reminiscent of our findings when we compared an antisense oligo to a control construct (Griffoni et al., submitted). The antisense after passively diffusing in the nucleoplasm was rapidly accumulated in speckles, while the control biologically inactive oligo failed to be taken up by speckles (FIG.1). Thus, the presence of a specific carrier on speckles is likely and its properties and nature could be deduced examining those of the



A

B

FIG. 1. Nuclear localisation of antisense oligonucleotide against initiation translation site of cytosolic phospholipase A2 (cPLA2), panel B, vs. nuclear localisation of a biologically inactive oligonucleotide (panel A). An antisense oligonucleotide, inhibiting LPS-stimulated cPLA2 and thromboxane A2 formation in human monocytes, labeled using 6-carboxy-fluorescein (6-FAM), was incubated for 9-12 hrs with human monocytes and localisation was detected by confocal microscopy (all sections) on cells fixed with 4% paraformaldehyde (Griffoni et al., in preparation). Panel B shows the accumulation of fluorescence in nuclear discrete spots resembling speckles (see refs. 8, 9). The number of speckles was rather variable and in panel B from 11 to 15 speckles could be detected. Panel A shows that a biologically inactive oligonucleotide is taken up in the nucleoplasm, but it failed to localise in speckles. Similar results have been obtained using human endothelial cells (Griffoni et al., in preparation).

oligodeoxynucleotide-binding protein recently described [29]. This carrier MAC-1, a heparin-binding integrin, which is probably just the first of a family of carriers for oligonucleotides, might be present also on speckles.

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