

# Assembly and Localization of the U1-specific snRNP C Protein in the Amphibian Oocyte

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**Abstract.** To study the intranuclear localization of the U1-specific snRNP C protein and its assembly into U1 snRNPs, we injected transcripts encoding a *myc*-tagged C protein into amphibian oocytes. The distribution of protein translated from the injected RNA was essentially the same in continuous and pulse-label experiments. In both cases the C protein localized within the germinal vesicle in those structures known to contain U1 snRNPs, namely the lampbrush chromosome loops and hundreds of extrachromosomal granules called snurposomes. Oocytes were also injected with

an antisense oligodeoxynucleotide that caused truncation of U1 snRNA at the 5' end. In these oocytes, *myc*-tagged C protein localized normally in the germinal vesicle and could be immunoprecipitated together with truncated U1 snRNA. These experiments suggest that the C protein can enter the germinal vesicle on its own and there associate with previously assembled U1 snRNPs. In transfected tissue culture cells, the *myc*-tagged C protein localized within the nucleus in a speckled pattern similar to that of endogenous U1 snRNPs.

THE small nuclear ribonucleoproteins (snRNPs)<sup>1</sup> U1, U2, U4/U6, and U5 are required for pre-mRNA splicing (Steitz et al., 1988), along with other non-snRNP factors (Ruskin et al., 1988; Fu and Maniatis, 1990; Krämer and Utans, 1991). When an RNA that contains an intron is added to a suitable nuclear extract, these components assemble on the RNA to form an active splicing complex called a spliceosome (Brody and Abelson, 1985; Grabowski et al., 1985; Frendeway and Keller, 1985; Maniatis and Reed, 1987). Although the biochemical requirements and kinetics of spliceosome formation have been studied in detail in vitro, relatively little is known about the site of splicing within the nucleus. Immunofluorescent staining and in situ hybridization show that splicing components are not uniformly distributed in somatic cell nuclei (Lerner et al., 1981; Spector, 1984; Nyman et al., 1986; Carmo-Fonseca et al., 1991a,b; Carter et al., 1991; Spector et al., 1991), suggesting that either splicing itself or the assembly of splicing components takes place at specific intranuclear sites. In giant chromosomes, splicing components occur in the actively transcribing regions, namely the puffs and Balbiani rings of dipteran polytene chromosomes (Sass and Pederson, 1984; Vazquez-Nin et al., 1990) and the loops of lampbrush chromosomes from amphibian oocytes (Gall and Callan, 1989; Wu et al., 1991; Tsvetkov et al., 1992). Furthermore, Beyer and colleagues have shown by EM that transcripts from *Drosophila* genes almost certainly undergo splicing before they leave the sites of synthesis on the chromosomal DNA (Osheim et al., 1985; Beyer and Osheim, 1988). Thus, transcription and

splicing probably occur simultaneously under many conditions in the nucleus, even though the two processes are not obligately linked (Pan and Prives, 1988; Tsvetkov et al., 1992).

In the amphibian germinal vesicle (GV), splicing components are also found in hundreds to thousands of extrachromosomal granules called snurposomes (Gall and Callan, 1989; Wu et al., 1991). Three types, designated A, B, and C snurposomes, can be distinguished on the basis of morphology and molecular composition. A and B snurposomes are ~1–4  $\mu\text{m}$  in diameter, whereas Cs vary from as small as 1  $\mu\text{m}$  to giant structures 20  $\mu\text{m}$  in diameter. The A snurposomes contain only U1 snRNA and associated proteins; so far, they have been identified in GVs of the newt *Notophthalmus*, but not in *Xenopus*. B snurposomes are generally the most abundant. They are known to contain the five splicing snRNAs, several snRNP proteins, at least one essential non-snRNP splicing factor (SC35), and hnRNP proteins. The composition of the Cs is problematic. They stain more or less uniformly with antibodies directed against the Sm proteins and against the trimethylguanosine cap found on most snRNAs. Nevertheless, in situ hybridization of splicing snRNAs is limited to small internal inclusions (our own unpublished observations).

The functions of snurposomes are still obscure. Because B snurposomes and the actively transcribing loops of the lampbrush chromosomes share such a variety of splicing components, these two structures may be related in some way. Among the (nonexclusive) possibilities are that B snurposomes serve as sites for (a) preassembly of spliceosome components destined for the loops, (b) storage of snRNPs used later in embryogenesis. (c) recycling of splicing compo-

1. *Abbreviations used in this paper:* GV, germinal vesicle; snRNP, small nuclear ribonucleoprotein.

nents originally assembled on the loops, or (d) splicing itself.

One approach to understanding the relationships among the various snRNP-containing structures in the GV is to follow the incorporation of newly synthesized splicing components into these structures by microscopic techniques. If, at the same time, the assembly of snRNPs or larger complexes is followed biochemically, some idea about sites of assembly can be gained. To this end we have studied the U1-specific snRNP C protein in the GV. We synthesized *myc*-tagged transcripts from a cDNA clone of the C protein and injected these into newt and *Xenopus* oocytes. We then followed the incorporation of the newly synthesized C protein into various nuclear structures by immunofluorescence. We also demonstrated that new C protein can associate with pre-existing U1 snRNPs within the nucleus. For comparison, we examined the localization of newly synthesized C protein in somatic cells after transfection with the cDNA clone.

## Materials and Methods

### Isolation of the snRNP C cDNA

The snRNP C cDNA was isolated from a lambda ZAP expression library made from *Xenopus laevis* ovary mRNA (Tafari and Wolffe, 1990). The snRNP C coding region was one of two open reading frames in a clone selected for its ability to bind RNA. After conversion of the lambda phage to the corresponding Bluescript SK(-) phagemid, designated pl2, the entire insert was sequenced by the dideoxy chain termination method (Sanger et al., 1977). The identity of the snRNP C coding region was determined by a sequence homology search in the GenBank data base. The sequence data are available from EMBL/GenBank/DBJ under accession No. X63892.

### Construction of *myc*-tagged snRNP C Fusion Protein

To produce clone pCMA, the 6 *myc* tags of Bluescript KS(+)/MT7 (Roth et al., 1991) were fused to the carboxy terminus of the snRNP C cDNA, along with the 3' untranslated region of NO38 (Peculis and Gall, 1992).

Positive clones were identified by restriction digests and sequencing. One positive clone was used for all further studies; it was called pCMA for C protein-*Myc*-poly A. pCMA had a unique open reading frame, starting at the AUG codon of the snRNP C fragment, continuing through the 6 *myc* tags, and ending 15 amino acids further downstream at the beginning of the NO38 3' untranslated region (see Fig. 2).

### In Vitro Transcription

Plasmid pCMA was linearized at a unique *Bam*HI site downstream of the poly A tail. Capped in vitro transcripts were produced from the T7 promoter according to previously described methods (Roth and Gall, 1989). After phenol extraction and ethanol precipitation, an aliquot of the RNA was checked for size and quality by electrophoresis on a 2% agarose gel containing 1.5% formaldehyde. The remaining RNA was resuspended at 1 mg/ml and used for oocyte injections. Radiolabeled antisense transcripts of various U snRNAs were made as described by Hamm et al. (1989).

### Oocyte Injections

Injections of oligos U1a and U2b, complementary to regions of U1 and U2 snRNAs respectively, were made as described by Pan and Prives (1988), except that injections were made into the cytoplasm instead of the nucleus (Tsvetkov et al., 1992). Oocyte injections and analysis of translation products by immunoblotting were made according to Peculis and Gall (1992). For double injections with antisense oligos and pCMA, oocytes were injected with the oligos 4–12 h before injection with pCMA. For immunofluorescence analysis of pCMA translation products, preparations of spread GV contents were made as described by Wu et al. (1991).

### Immunoprecipitation

Injected and control oocytes were held overnight in OR-2 saline (Wallace et al., 1973). For each precipitation 10–30 GVs were isolated by hand in

GV isolation buffer (83 mM KCl, 17 mM NaCl, 6.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 3.5 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub> and 1 mM DTT, pH 7.2), washed free of yolk, and transferred to an Eppendorf tube on ice. After addition of 500  $\mu$ l of NET-2 (150 mM NaCl, 50 mM Tris, pH 7.4, 0.05% NP-40), the GVs were homogenized by sonication. The extract was precleared by centrifugation for 10 min at 4°C and the supernate was used for immunoprecipitations with mAb Y12 (Lerner et al., 1981) or mAb 9E10 (Evan et al., 1985) bound to Protein A-Sepharose CL-4B beads (Pharmacia Fine Chemicals, Piscataway, NJ), following the technique described by Steitz (1989). mAb Y12 was coupled directly to the beads, but the low affinity of mAb 9E10 for protein A required that it be coupled by a secondary rabbit anti-mouse antibody. For analysis of immunoprecipitated proteins, the beads were boiled in SDS sample buffer, the extracted proteins fractionated on 7–17% gradient gels (Laemmli, 1970) and analyzed by immunoblotting (Burnette, 1981). For analysis by Northern blotting, the RNAs were extracted from the precipitated complexes following the protocol of Steitz (1989).

### RNA Isolation

Single GVs were collected in GV isolation buffer, transferred to 100  $\mu$ l TE (10 mM Tris, pH 8.0, 1 mM EDTA), 1% SDS, and sonicated. The solution was extracted twice with phenol, and twice with phenol-chloroform (1:1 vol/vol). It was then brought to 300 mM Na acetate, 5  $\mu$ g of glycogen was added as carrier, and RNAs were precipitated by addition of 2 vols of ethanol.

### Northern Blots

RNA from individual GVs or from immunoprecipitations was separated on 10% denaturing acrylamide gels, and transferred to GeneScreen membrane (DuPont Co., Boston, MA) by electroblotting (0.3 amp, overnight) in 1 $\times$  TAE (40 mM Tris-acetate, 2 mM EDTA, pH 7.4). The RNA was immobilized by UV crosslinking (1.9 mJoule/cm<sup>2</sup>) and hybridized at 65°C for 12 h with <sup>32</sup>P-labeled antisense probes in high SDS buffer (Church and Gilbert, 1984). Membranes were washed once for 15 min in 1 $\times$  SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 0.5% SDS, followed by two 15-min washes in 0.2 $\times$  SSC, 0.5% SDS at 65°C, and exposed while moist to x-ray film for 1–12 h at room temperature.

### Tissue Culture Transfection

For transfection of the *myc*-snRNP C fusion construct into tissue culture cells, the coding region of pCMA and the poly A tail of NO38 were cloned into pCMX (Umesono et al., 1991). HeLa cells were grown in MEM supplemented with 10% FCS. One day before transfection, 5  $\times$  10<sup>5</sup> cells were placed in 10-cm petri dishes containing acid-etched coverslips. Transfections were made by the calcium phosphate precipitation method (Ausubel et al., 1987). DNA precipitates were left on the cells overnight, which were then washed in PBS, covered with fresh medium, and allowed to recover for 24 h.

Cells on coverslips were fixed and permeabilized according to Fu and Maniatis (1990) except that methanol replaced acetone in the permeabilization step.

After fixation, cells were washed in 70% ethanol, and transferred to PBS. Immunofluorescent staining was performed as described by Wu et al. (1991).

### Microscopy

Microscopes and filter combinations used for phase contrast, DIC (Nomarski), fluorescence, and confocal laser scan microscopy are described in Wu et al. (1991).

### Antibodies

mAb 9E10 recognizes a 10-amino acid peptide in the human c-*myc* protein (Evan et al., 1985). mAb Y12 recognizes the Sm epitope found on several snRNP proteins (Lerner et al., 1981). Human serum 361 is known to immunoprecipitate U1 snRNPs (D. Wassarman, unpublished observation).

## Results

### Isolation and Characterization of a *Xenopus* cDNA Encoding the U1 snRNP C Protein

We have isolated a *Xenopus laevis* cDNA clone that encodes

the U1-specific snRNP C protein. The snRNP C coding region was found as one of two open reading frames in a lambda ZAP clone derived from a "baby ovary" cDNA library (Tafari and Wolffe, 1990). Sequencing of the entire insert and a subsequent search in the GenBank DNA data base showed that one of the two open reading frames coded for the *Xenopus* homologue of the human snRNP C protein. The 480-bp coding region showed 80% identity to the human cDNA (Sillekens et al., 1988) with a corresponding 92% identity on the protein level (Fig. 1).

Like its human counterpart, the *Xenopus* C protein contains a putative zinc finger cys-cys/his-his motif (Berg, 1988) near the amino terminus. The zinc finger is necessary for binding of the C protein to the U1 snRNP (Nelissen et al., 1991). Consistent with this finding, there are no amino acid differences between the *Xenopus* and human proteins in the zinc finger region (Fig. 1).

### Construction of *myc*-snRNP C Fusions

To study the intranuclear transport and localization of the C protein we wanted to inject in vitro synthesized transcripts encoding the C protein into newt and *Xenopus* oocytes and follow the translation products by immunofluorescence and immunoblotting. To distinguish newly synthesized from endogenous C protein we tagged the C protein with a 10-amino acid sequence derived from the human *c-myc* gene. The resulting fusion protein could then be detected with mAb 9E10 specific for the *myc* tag (Evan et al., 1985).

Four in-frame constructs were made that contained the C protein cDNA fused to an oligonucleotide encoding either a single *myc* tag or six tandem tags. Two constructs had the tags at the amino terminus, two at the carboxy terminus. To increase the stability of the RNAs after injection into oocytes, the 3' end of our constructs consisted of the 3' untranslated region and poly A tail of the *Xenopus* NO38 cDNA (Peculis and Gall, 1992). In vitro transcripts of all constructs were made, the RNAs were translated either in vitro, or in vivo after injection into oocytes, and the resulting fusion proteins were detected by immunoblots and by immunofluorescence of cytological preparations. In oocytes the two amino-terminal *myc* fusions accumulated in the GV, as shown by immunoblots of GV proteins, but neither could be detected on the chromosomes or other cytological structures in spread preparations of GV contents (data not shown). Presumably, therefore, the proteins were in the soluble phase of the GV. Both carboxy-terminal fusions showed strong signals on immunoblots of GV proteins and on immunofluorescently stained cytological preparations. The construct with a single *myc* tag had a calculated molecular mass of 18.9 kD, that with six tags a mass of 29 kD. On gels, both constructs showed slightly larger apparent molecular masses of 24 and 33 kD, respectively. A similar discrepancy between calculated and apparent molecular weight has also been observed for the human snRNP C protein and is believed to be caused by posttranslational modifications of the protein (Sillekens et al., 1988). For all subsequent studies we used construct pCMA, which has six tags at the carboxy terminus (Fig. 2).

### Intranuclear Localization of snRNP C Fusion Protein

Capped in vitro transcripts of pCMA were injected into *Notophthalmus* oocytes. The translation products were followed at various times after injection by immunofluores-

1	CAG GGA GAC GAA CGC GCC ATT ACC AAC ATG CCG AAG TTT TAC TGT	45
		6
46	GAC TAC TGC GAT ACG TAT CTC ACC CAT GAT TCT CCT TCA GTC CGT	90
7	D Y C D T Y L T H D S P S V R	21
91	AAG ACT CAT TGT AGT GGA AGA AAG CAC AAG GAG AAT GTG AAA GAT	135
22	K T H C S G R K H K E N V K D	36
136	TAT TAT CAG AAG TGG ATG GAA GAG CAG GCA CAG AGC CTA ATT GAC	180
37	Y Y Q K W M E E Q A Q S L I D	51
181	AAA ACC ACC GCC GCA TTT CAA CAA GGA AAA ATA CCA CCA ACA CCT	225
52	K T T A A F Q Q G K I P P T P	66
226	TTT GCA GCT CCG CCA GCT GGA AGT GCT ATG ATC CCA CCA CCT CCA	270
67	F A A P P A G S A M I P P P P	81
271	AGC TTG GGT GSA CCT CCT CGA CCT GGA ATG ATG CCT GCT CCC OCC	315
82	S L G G P P R P G M M P A P P	96
316	ATG GCT GGC CCT CCT ATG ATG CCA ATG ATG GGA CCA CCT CCT CCA	360
97	M A G P P M M P M M G P P P P	111
361	GGA ATG ATG CCA GTA GGC CAT GGT CCT GGT ATG AGA CCA CCC ATG	405
112	G M M P V G H G P G M R P P M	126
406	GGA GCT CAC ATG CCT ATG ATG CCT GGT CCA CCC ATG ATG OGT CCA	450
127	G A H M P M M P G P P M M R P	141
451	CCT ACA CGT CCT ATG ATG CTA CAG TCC CGA CCT GGC ATG GCA OGT	495
142	P T R P M M L Q S R P G M A R	156
496	CCT GAT CGA TAA AAC TTT TT	515
157	P D R *	159

Figure 1. Nucleotide sequence of the *Xenopus* U1 snRNP C gene and its conceptual amino acid translation. The amino-terminal portion of the molecule is identical in the human and *Xenopus* proteins. Positions at which they differ, in the middle and carboxy-terminal regions, are underlined. A putative zinc finger cys-cys/his-his motif is shown in bold face. The *Clal* restriction site, used to fuse the snRNP C encoding region to the *myc* tags, is also shown in bold face. These sequence data are available from EMBL/GenBank/DBJ under accession number X63892.

cence of spread nuclear preparations and by immunoblots of nuclear and cytoplasmic proteins. Immunoblots showed a continuous nuclear accumulation of newly synthesized C protein for up to 42 h (Fig. 3). Within the GV, the sites of highest concentration detectable by immunofluorescence changed with time (Fig. 4). At early time points, 4–10 h after injection, a weak but clearly recognizable labeling of C snurposomes was observed, whereas other structures were not detectably labeled (Fig. 4, a and b). Labeling of C snurposomes increased in intensity until ~48 h. Beginning at ~14–18 h, loops and B snurposomes showed the first signs of label (Fig. 4, c and d), which gained in intensity during the next 24–48 h (Fig. 4, e and f), with maximum labeling of all mentioned structures roughly 60 h after injection (Fig. 4, g and h). C snurposomes were usually the brightest struc-

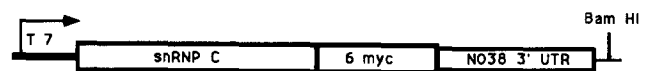
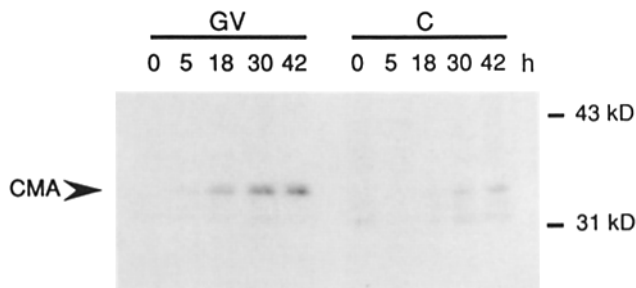


Figure 2. Diagram of the insert in plasmid pCMA. Sequences encoding 6 concatenated *myc* epitopes are fused to the 3' end of the snRNP C cDNA, followed by the 3' untranslated region of NO38 (Peculis and Gall, 1992). The whole construct is inserted in Bluescript KS(-) downstream of the phage T7 promoter. Linearization of pCMA with *Bam*HI allows in vitro synthesis of runoff transcripts from the T7 promoter.



**Figure 3.** Nuclear accumulation of the snRNP C protein. Oocytes of the newt *Notophthalmus* were injected with transcripts from pCMA. At 0, 5, 18, 30, and 42 h after injection, proteins from six GVs and two enucleated oocytes (C) were electrophoresed, transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA), and probed with mAb 9E10, which detects the *myc* sequences in the *myc*-snRNP C fusion protein. Because a GV occupies <10% of the total oocyte volume, the proteins in the cytoplasmic lanes are derived from a larger volume than those in the GV lanes. The concentration of newly synthesized C protein is, therefore, higher in the nucleus than in the cytoplasm for all time points.

tures at all time periods, although at later times the B snurposomes were occasionally stronger (Fig. 4, *i* and *j*).

The labeling of A snurposomes, which are particularly rich in U1 snRNPs (Wu et al., 1991), was inconsistent. All A snurposomes within a given preparation had comparable levels of stain, but different preparations made at the same time showed widely varying levels (data not shown).

In general, C protein translated from injected transcripts localized in the three structures known to contain U1 snRNAs and associated proteins, lampbrush chromosome loops and the A and B snurposomes (Gall and Callan, 1989; Wu et al., 1991). The strong accumulation in C snurposomes was not expected and remains somewhat of a mystery, as discussed later. Other structures that lack U1 snRNA, such as the multiple nucleoli, chromomeres, axial granules, and certain specific loops (giant loops on chromosome 2 and the so-called "sequentially labeling loops" on chromosome 11) failed to label with mAb 9E10 after CMA injection (Fig. 4, *k* and *l*). As shown before (Roth and Gall, 1989; Peculis and Gall, 1992), mAb 9E10 stains a few small loops in control GVs, but this slight background does not interfere with the patterns seen in the injected oocytes.

The intranuclear distribution of endogenous U1 snRNPs was demonstrated in control, uninjected oocytes by staining with human serum 361, which specifically immunoprecipitates U1 snRNPs (D. Wassarman, unpublished observation). Serum 361 stained chromosome loops and the three types of snurposomes (Fig. 5; see also Wu et al., 1991). It consistently gave strong staining of A snurposomes, moderate staining of B snurposomes, and weak staining of C snurposomes.

To show that the intranuclear distribution of the *myc*-snRNP C fusion protein was not due to the *myc* tag alone, two additional *myc* constructs, one encoding the *Xenopus* nucleolar protein NO38, and one containing a fragment of pyruvate kinase cDNA fused to the SV-40 nuclear localization signal were injected as controls. As expected from earlier experiments (Peculis and Gall, 1992), the translation products of these two constructs accumulated in the GV,

NO38 in the nucleoli and pyruvate kinase in the soluble nucleoplasm. Both patterns were clearly distinguishable from that of the *myc*-snRNP C protein.

### Assembly of *myc*-snRNP C Fusion Protein into U1 snRNPs

To further characterize the transport and accumulation of C protein in the GV, we tested whether newly synthesized *myc*-snRNP C fusion protein was assembled into intact snRNPs. Total snRNPs were immunoprecipitated from GV extracts of injected and uninjected *Xenopus* or *Notophthalmus* oocytes and assayed for the fusion protein by immunoblotting with mAb 9E10. The immunoprecipitations were made with the anti-Sm mAb Y12 (Lerner et al., 1981). These experiments showed that the majority of the *myc*-snRNP C protein was coprecipitated with the total snRNPs, suggesting that a large fraction of it was properly assembled into intact snRNPs (Fig. 6). The minor fraction of unprecipitated *myc*-snRNP C protein which remained in the supernatant in these experiments (Fig. 6, lane 5) could represent protein that had not been assembled into snRNPs, or could result from inefficient immunoprecipitation of assembled snRNPs by mAb Y12.

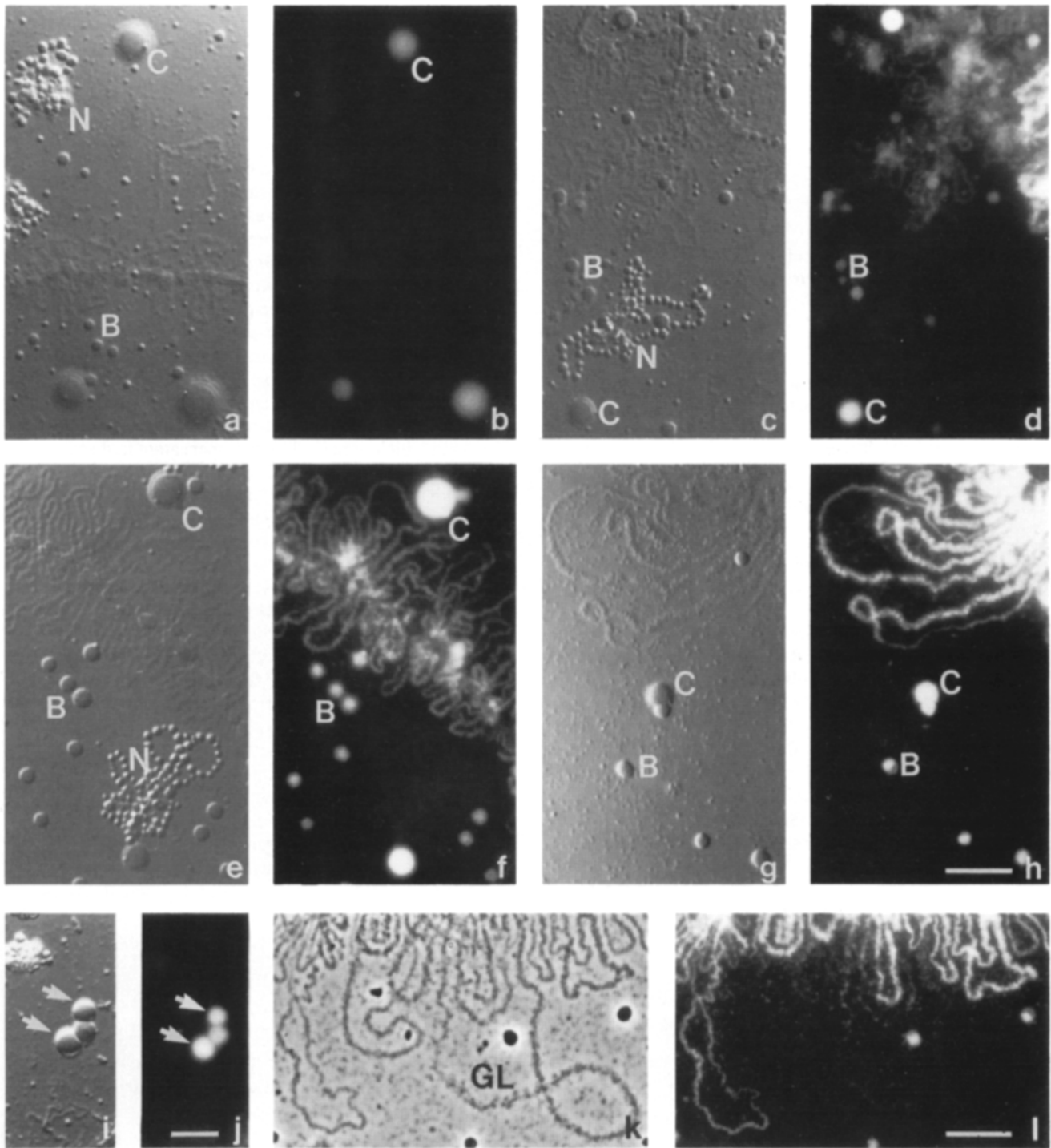
The immunoprecipitation experiments and the control injections suggest that the *myc* tag does not significantly interfere with the assembly of the fusion protein into intact snRNPs and the normal localization of these snRNPs within the GV.

### Localization of the snRNP C Protein after Cycloheximide Treatment

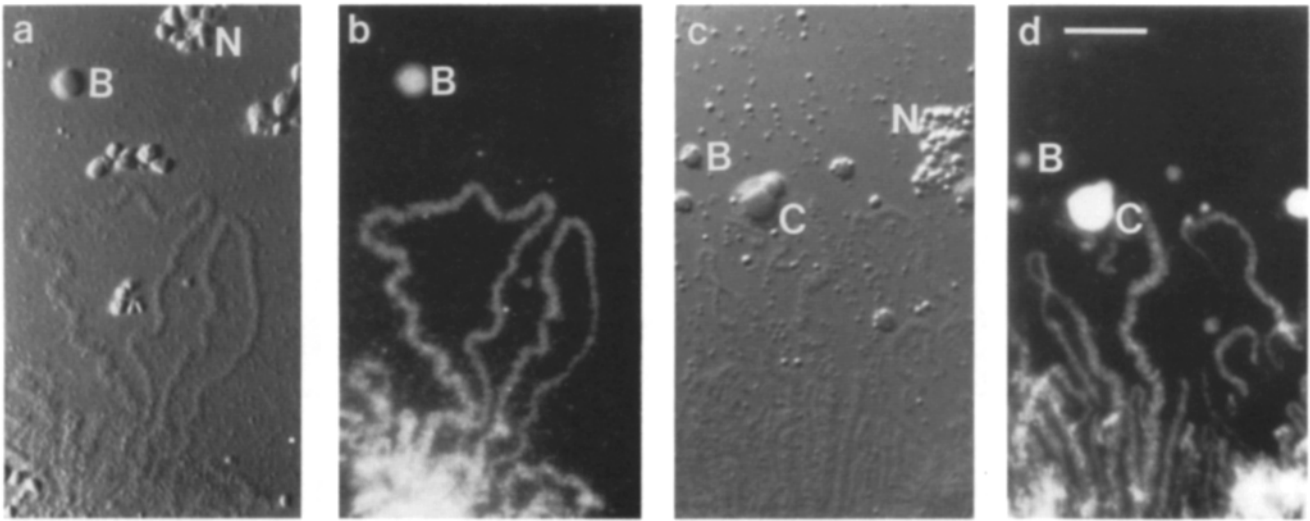
If U1 snRNPs are moving from one intranuclear compartment to another, their movement might be detected more easily after a pulse of newly synthesized protein than during continuous synthesis. Therefore, protein synthesis was inhibited by transferring the oocytes to a medium containing cycloheximide at various times after CMA injection. Subsequently, the distribution of newly synthesized *myc*-snRNP C fusion protein was monitored by immunoblotting and immunofluorescence. In this experiment, the same distribution of the fusion protein was observed in control and cycloheximide-treated oocytes. The intensity of stain, however, was markedly reduced in the cycloheximide-treated oocytes, as was the amount of fusion protein detectable on immunoblots (data not shown). The cycloheximide experiment suggests that there is little or no transport of the *myc*-snRNP C fusion protein from one intranuclear organelle to another during the time course of this experiment.

### Nuclear Transport of the snRNP C Protein

The import of most snRNPs into the nucleus, including U1, is dependent on preassembly of proteins and snRNA in the cytoplasm, with several proteins unable to enter the nucleus on their own (Andersen and Zieve, 1991). Pulse label and fractionation experiments of tissue culture cells suggest, however, that the C protein can enter the nucleus independently in somatic cells (Feeney et al., 1989). To study the nuclear transport of the C protein in oocytes, we carried out experiments in which the endogenous U1 snRNA was truncated at its 5' end by injection of the antisense oligodeoxynucleotide (oligo) U1a (Pan and Prives, 1988, 1989). 2–12 h later, CMA was injected, and the localization of



**Figure 4.** Distribution of the snRNP C protein. Spread GV contents from *Notophthalmus* oocytes prepared at 9 (a and b), 18 (c and d), 36 (e and f) and 60 (g and h) h after injection of CMA. (a, c, e, and g) Differential interference contrast (DIC) and (b, d, f, and h) immunofluorescence images in the fluorescein channel of the same region stained with mAb 9E10 to show the distribution of the *myc*-snRNP C fusion protein. (a and b) At 9 h after injection only weak labeling of C snurposomes (C) is seen. (c and d) 18 h after injection, B snurposomes (B) and lampbrush chromosome loops show first signs of labeling, which subsequently increases (e and f) until maximal labeling is reached 60 h after injection (g and h). The multiple nucleoli remain unlabeled throughout. DIC (i) and immunofluorescent (j) images of a C snurposome with two attached B snurposomes (arrows) 40 h after injection of CMA. The two B snurposomes show maximal labeling, whereas the C snurposome has already decreased somewhat in intensity. Phase contrast (k) and immunofluorescent (l) images of the giant loops (GL) found on chromosome 2. These loops do not accumulate the C protein (they also fail to label with anti-snRNP antibodies and in situ hybridization probes against U snRNAs). N, nucleolus. Bar, 10  $\mu$ m.



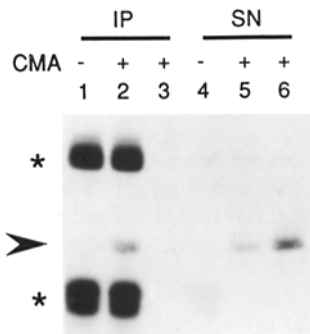
**Figure 5.** DIC (a) and immunofluorescent (b) images of lampbrush chromosome loops of *Notophthalmus* stained with serum 361, which is specific for U1 snRNPs. DIC (c) and immunofluorescent (d) images of lampbrush loops from a CMA injected oocyte stained with mAb 9E10. The distribution of endogenous U1 snRNPs (a and b) and exogenous snRNP C protein (c and d) is essentially identical. B and C, B and C snurposomes, respectively; N, nucleolus. Bar, 10  $\mu$ m.

newly synthesized *myc*-snRNP C fusion protein was monitored by immunoblotting and immunofluorescence. The cleavage of U1 snRNA was simultaneously followed by Northern blots. It has been shown previously that U1 snRNA truncated at the 5' end cannot enter the nucleus, because the trimethylguanosine cap is required for nuclear migration (Hamm et al., 1990).

As expected from earlier studies (Pan and Prives, 1988, 1989; Tsvetkov et al., 1992), the injection of the U1a oligo caused rapid cleavage of U1 snRNA, with no detectable resynthesis for up to 65 h (Fig. 7). During this time, immunoblots showed that the nuclear accumulation of the CMA translation product proceeded as in control oocytes that had

not received the U1a oligo (data not shown). The intranuclear distribution of the *myc*-snRNP C protein was similar in control and oligo-injected oocytes (Fig. 8). The only difference observed by immunofluorescence was less intense labeling of chromosome loops in oocytes injected with the oligo.

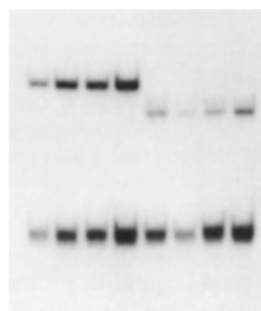
Because the *myc*-snRNP C fusion protein presumably entered the GV as free protein, yet showed the same intranuclear localization as in controls, we wanted to know whether it was associated with the truncated, inactive U1 snRNA. We immunoprecipitated U1 snRNPs from newt or *Xenopus* oocytes injected with CMA, using mAb 9E10 directed against the *myc* tag. Some oocytes had been preinjected with the U1a oligo to truncate the U1 snRNA. The immunoprecipitated material was then tested for full-length vs. truncated U1 snRNA by Northern blots (Fig. 9). As a control, all splicing snRNPs were immunoprecipitated from comparable oocytes using mAb Y12. In these experiments both truncated ( $\Delta$ U1) and full-length U1 snRNAs were immunoprecipitated by mAb 9E10 (Fig. 9, lanes 2 and 3 respectively), demonstrating that the *myc*-snRNP C fusion protein was associated in the GV with truncated U1 snRNA. The control immunoprecipitations with mAb Y12 showed that U1



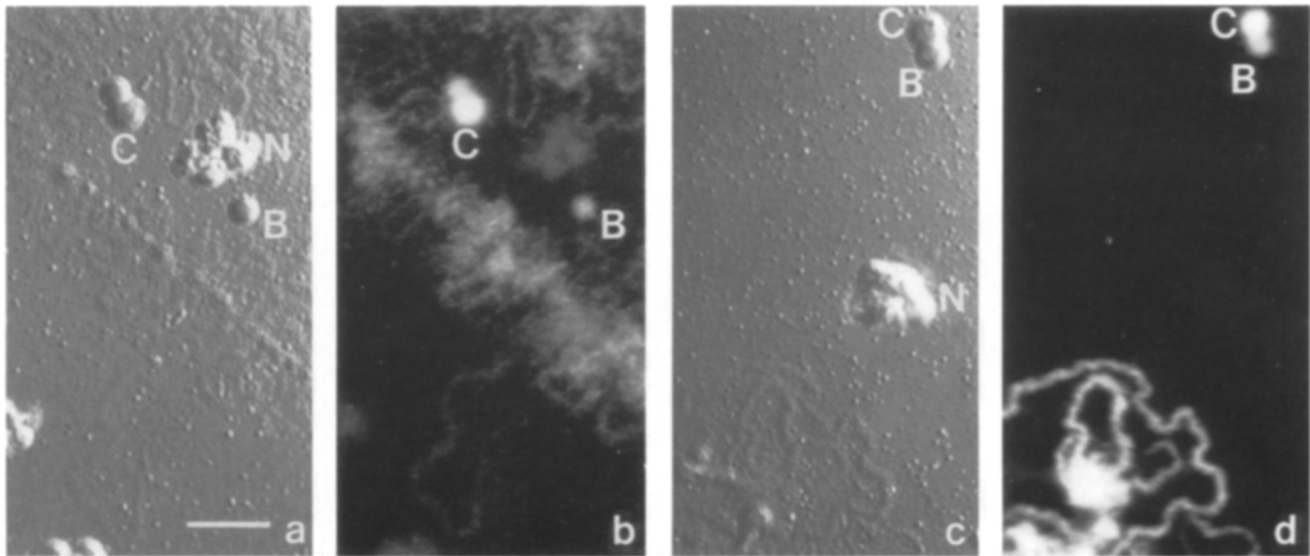
**Figure 6.** Incorporation of the *myc*-snRNP C protein into intact snRNPs. Proteins from 50 *Xenopus* GV's from CMA injected (CMA +) and uninjected (CMA -) oocytes were immunoprecipitated with mAb Y12 attached to protein A beads (lanes 1, 2, 4, and 5), or with protein A beads alone (lanes 3 and 6). The precipitates (IP) and corresponding supernates (SN) were subsequently probed with mAb 9E10,

which detects the *myc* tag. The precipitate from CMA-injected oocytes is positive (lane 2), and there is a weaker signal in the corresponding supernate (lane 5). The precipitate and supernate from uninjected oocytes show no signal (lanes 1 and 4). Protein A beads alone failed to bring down *myc*-tagged proteins from CMA-injected oocytes (lane 3), leaving the entire signal in the corresponding supernate (lane 6). The arrowhead marks the position of the *myc*-snRNP C fusion protein. Asterisks indicate IgG heavy and light chains of mAb Y12, visualized by the enzymatic detection system used for this immunoblot. Comparable results were obtained when the experiment was performed on *Notophthalmus* oocytes.

	Control		$\alpha$ U1a	
	15h	65h	15h	65h
U1	+	+	+	+
$\Delta$ U1			+	+
U6	+	+	+	+



**Figure 7.** Northern blot of snRNAs from single GV's of *Notophthalmus* isolated from control oocytes, or oocytes injected 15 or 65 h previously with the antisense U1a oligo ( $\alpha$ U1a). The blot was probed simultaneously for U1 and U6 snRNAs. The oligo injection converts U1 snRNA to a truncated form ( $\Delta$ U1), which is stable within the GV for at least 65 h. U6 is unaffected by injection of the U1a oligo.



**Figure 8.** Comparison of *myc*-snRNP C protein in GV from oocytes that had been injected with the Ula oligo and 24 h later with CMA RNA (a and b), or with CMA RNA only (c and d). Corresponding DIC and immunofluorescent images are shown of preparations stained with mAb 9E10. In both cases C snurposomes (C) and the B snurposomes (B) on their surface are well labeled. After Ula oligo injection (b) the loops are positive, but less well labeled than in the absence of the oligo (d). Nucleoli (N) are unlabeled. Bar, 10  $\mu$ m.

snRNPs with truncated U1 snRNA contained the usual complement of Sm proteins B and D (Fig. 9, lanes 4 and 5).

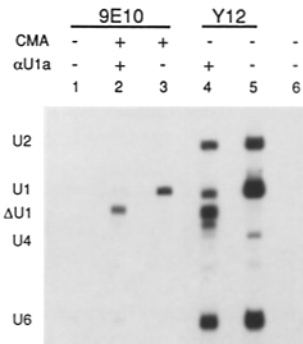
To determine whether the *myc*-snRNP C fusion protein had interacted nonspecifically with other splicing snRNPs in the GV, the Northern blots were rehybridized with probes for the other splicing snRNAs (Fig. 9). Whereas mAb Y12 immunoprecipitated U1-U6 snRNAs, including the truncated U1, mAb 9E10 precipitated only the full-length and truncated forms of U1, suggesting that the *myc*-snRNP C fusion protein interacted only with the U1 snRNP in the GV.

#### Localization of the snRNP C Protein in Somatic Cells

It has been known for some time that snRNP components are distributed in a speckled pattern in interphase nuclei (Lerner et al., 1981; Spector, 1984, 1990). Depending on the cell type, the fixation protocol, and the antibody probe, the immunofluorescence pattern can be somewhat variable, ranging from numerous discrete areas of stain with a low background to a relatively diffuse staining of the nucleus with only a few stronger patches (Fu and Maniatis, 1990; Carmo-Fonseca et al., 1991a,b; Carter et al., 1991; Spector et al., 1991). The relationship of the nuclear speckles observed in somatic cells to the A, B, and C snurposomes of the amphibian GV is not known.

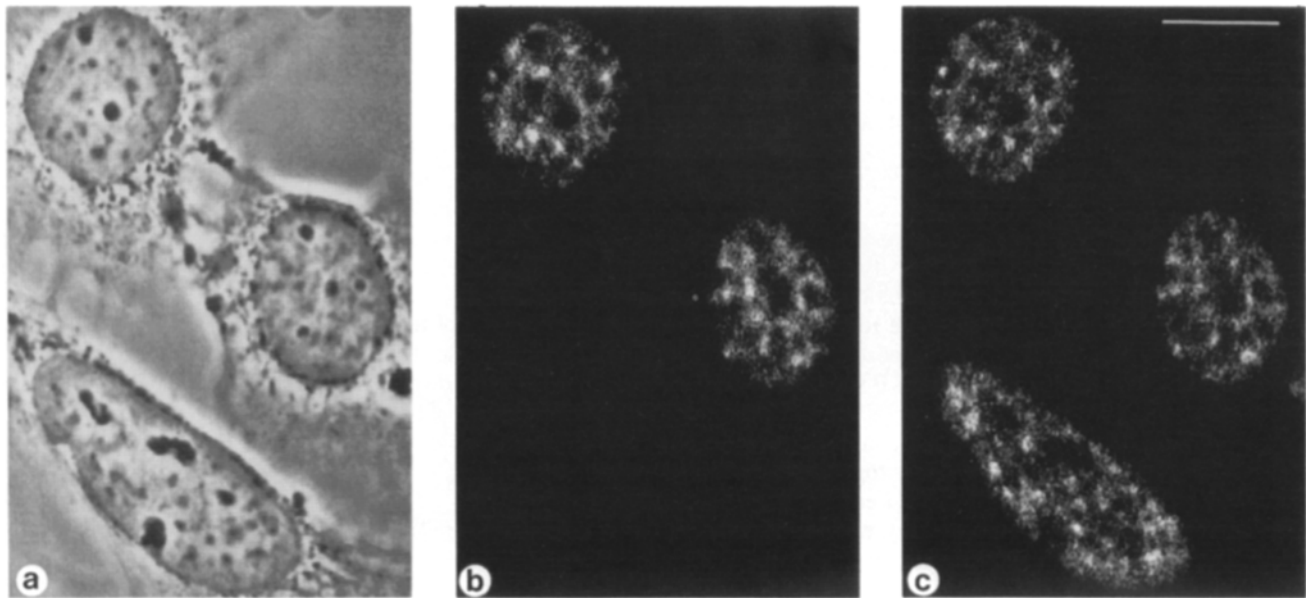
To study the intranuclear localization of the *myc*-snRNP C fusion protein in somatic cells, we transfected HeLa cells with a plasmid expressing the entire CMA coding region. The cells were grown on coverslips, fixed with formaldehyde, and stained simultaneously with mAb 9E10, which stains only the fusion protein, and with serum 361, which reveals all U1 snRNPs. As expected, transfected as well as nontransfected nuclei were stained in a punctate pattern by serum 361 (Fig. 10 c). In the transfected cells, mAb 9E10 stained in a similar pattern (Fig. 10 b). Serum 361 stains both the fusion protein and endogenous U1-specific proteins. Thus, in the transfected cells, regions stained by mAb 9E10

must also be stained by 361, although the converse would not necessarily be true. In fact, within a given transfected nucleus, the two stains were colocalized (Fig. 10, b and c), demonstrating that new snRNP C protein is found wherever there are U1 snRNPs. Nucleoli were unstained in all cases.



**Figure 9.** *Xenopus* oocytes were injected with the Ula oligo ( $\alpha$ U1a +), RNA encoding the *myc*-snRNP C protein (CMA +), or the oligo and subsequently the RNA. snRNPs from hand-isolated GV were immunoprecipitated with mAb 9E10 (lanes 1–3), mAb Y12 (lanes 4 and 5), or no antibody (lane 6), the snRNAs extracted with phenol/chloroform, and electrophoresed on a 10% acrylamide/8 M urea gel. The RNAs were transferred to a GeneScreen filter, and probed for U1, U2, U4, and U6 snRNAs. mAb 9E10 reacts with the *myc* tag in the *myc*-snRNP C fusion protein. Because mAb 9E10 precipitates both truncated (lane 2) and full-length U1 snRNA (lane 3), we conclude that the fusion protein can interact in the GV with the truncated RNA, presumably by exchange into previously assembled U1 snRNPs. mAb Y12 recognizes the Sm epitope and precipitates all major snRNAs (lane 5, but data not shown for U5 snRNA). Because it also precipitates the truncated version of U1 snRNA in injected oocytes (lane 4), Sm proteins are presumably associated with the truncated RNA. The presence of full-length U1 in lane 4 is probably due to poor injection of the oligo into one of the oocytes in this sample. Because mAb 9E10 immunoprecipitates poorly, extracts from 30 GV were used with this antibody compared with five GV with mAb Y12. In addition, the autoradiographic exposure for lanes 1–3 was twice that for lanes 4–6. Comparable results were obtained when the experiment was performed on *Notophthalmus* oocytes.

must also be stained by 361, although the converse would not necessarily be true. In fact, within a given transfected nucleus, the two stains were colocalized (Fig. 10, b and c), demonstrating that new snRNP C protein is found wherever there are U1 snRNPs. Nucleoli were unstained in all cases.



**Figure 10.** Distribution of endogenous U1 snRNPs and transiently expressed snRNP C protein in the same nuclei, 24 h after transfection. HeLa cells were fixed in paraformaldehyde and Triton X-100 following the protocol in Fu and Maniatis (1990). Phase contrast (*a*) and immunofluorescent (*b* and *c*) images of the same nuclei. (*b*) Nuclei from two transfected cells showing the distribution of transiently expressed *myc*-snRNP C fusion protein, detected with mAb 9E10 followed by a fluorescein-labeled secondary antibody (not all cells express the protein). (*c*) The same field showing the distribution of endogenous U1 snRNPs in the lower nucleus and endogenous + exogenous snRNP C protein in the upper two nuclei. Detected with human serum 361, which stains all U1 snRNPs, followed by a rhodamine-labeled secondary antibody. Note the colocalization of label in panels *b* and *c*, which were taken with the Zeiss confocal laser scanning microscope. Bar, 10  $\mu$ m.

## Discussion

### The U1-specific snRNP C Protein

Cell fractionation and immunoprecipitation experiments have shown that the C protein is a specific constituent of U1 snRNPs (Lelay-Taha et al., 1986; Bringmann and Lührmann, 1986). The *Xenopus* cDNA isolated by us encodes a protein with 92% sequence identity to the human C protein (Sillekens et al., 1988). Both the *Xenopus* and human proteins contain a *cys-cys/his-his* motif at the amino terminus that could form a zinc finger (Berg, 1988). In vitro assembly experiments have shown that the putative zinc finger region is important for the binding of the human protein to U1 snRNPs (Nelissen et al., 1991). Consistent with the functional significance of this region, the *Xenopus* and human sequences are identical for the first 67 amino acids, including all of the putative zinc finger region. We found that *myc*-C fusion proteins with either one or 6 *myc* tags at the amino terminus accumulated in the GV, but did not localize in structures known to contain U1 snRNPs. The *myc* tag may have affected the formation or stability of the zinc finger itself, or it may have interfered sterically with binding of this region to an attachment site on the U1 snRNP. By contrast, *myc*-C fusion proteins with the tag at the carboxy terminus accumulated in the GV and localized on specific intranuclear structures.

### Localization and Assembly of the C Protein

Within the GV the *myc*-tagged C protein was detectable by immunofluorescence in the lampbrush chromosome loops and in the snurposomes, structures known from earlier

studies to contain U1 snRNPs (Wu et al., 1991). Conversely, immunofluorescent staining was not seen in structures that lack U1 snRNPs, such as the nucleoli, chromomeres, axial granules, and certain specific loops. When other *myc*-tagged constructs were injected into the oocyte, entirely different patterns of localization were observed (Roth and Gall, 1989; Peculis and Gall, 1992).

Immunoprecipitations confirmed that most of the newly synthesized C protein was associated with intact U1 snRNPs. We conclude, therefore, that the *myc*-tagged fusion product behaved normally in the GV in the sense that it was incorporated into U1 snRNPs and its intranuclear distribution was similar to that of endogenous U1 snRNPs.

A similar conclusion can be drawn about the behavior of *myc*-tagged C protein in transfected cells. In this case, the C protein localized in the nucleus in a punctate pattern identical to that of endogenous U1 snRNPs (Fig. 10).

### Kinetics of Organelle Labeling

Although it is relatively easy to follow the bulk movement of newly synthesized C protein from the cytoplasm into the GV, and to determine that it is assembled into intact snRNPs, it is difficult to predict the immunofluorescent staining patterns expected for individual nuclear organelles. These patterns will depend on several factors, including (*a*) how the newly synthesized protein enters the GV and whether it can exchange with protein in preexisting snRNPs, (*b*) whether and how rapidly snRNPs are moving from one organelle to another, (*c*) whether any of the organelles are essentially storage granules with little or no turnover, and (*d*) whether experimental overproduction of a protein disturbs the nor-



mal distribution. The situation in an oocyte, which grows slowly over weeks with little morphological change except size, differs from that in rapidly dividing tissue culture cells, where one expects the distribution of old and new protein to be identical after one or two cell doublings.

With these considerations in mind, one can examine the immunofluorescence patterns in the GV at various times after injection of transcripts. There was a general rise in the intensity of fluorescence over the course of an experiment, consistent with the increasing amount of newly synthesized protein detectable in the GV by immunoblots (Figs. 3 and 4). Beyond this, there were changes in the relative intensity of different structures. In general, C snurposomes labeled first, followed by the chromosome loops and B snurposomes. The A snurposomes also labeled, but in an inconsistent manner from experiment to experiment. Among these structures, the chromosome loops and B snurposomes together account for the great majority of fluorescence (Wu et al., 1991).

If the differences in relative intensity were due to movement of U1 snRNPs between components, the effect should be accentuated in a pulse-label experiment. We therefore carried out the cycloheximide experiment, in which protein synthesis was shut down ~3–8 h after injection of the transcript. In this case, the overall level of fluorescent staining was reduced, reflecting less newly synthesized protein, but the pattern of labeling was similar to that in the experiments without cycloheximide. That is, C snurposomes labeled first, followed by loops and B snurposomes.

The absence of a clear-cut progression of label from one structure to another, and the fact that the labeling pattern was essentially the same with continuous and pulse label, suggested that the newly synthesized C protein might enter all U1 snRNPs more or less simultaneously. In other words, there might be exchange of new for old C protein in intact U1 snRNPs in the GV, as was shown to occur *in vitro* when human C protein was incubated with intact U1 snRNPs (Nelissen et al., 1991). To test this possibility, an antisense oligo that causes truncation of U1 snRNA was injected, and 2–12 h later transcripts for the C protein were injected. When the GV contents were immunoprecipitated with mAb 9E10 against the *myc* tag, newly synthesized C protein was coprecipitated with truncated U1 snRNA. Earlier experiments with the Ula oligo (Pan and Prives, 1988, 1989; Prives and Foukal, 1991; Tsvetkov et al., 1992) showed that truncation of U1 snRNA is complete within ~30 min, the oligo itself degrades with a half-life of ~10 min, and there is no significant synthesis of new U1 snRNA during the ensuing 1–2 d. There is presumably no pool of free U1 snRNA in the oocyte, and even if there were free U1 snRNA in the cytoplasm, it could not enter the nucleus after truncation (Hamm et al., 1990). Thus we feel confident that all of the truncated U1 snRNA in the GV was present in preassembled U1 snRNPs from the beginning of the experiment. This being the case, newly synthesized C protein must have entered the GV on its own, as shown earlier by Feeney et al. (1989) for tissue culture cells. Once inside the GV, the new C protein exchanged with old C protein present in U1 snRNPs; or it entered U1 snRNPs in the GV that lacked C protein at the beginning of the experiment. In either case, the new C protein would be associated with truncated U1 snRNA. This pathway for entry of new C protein into the GV would explain why all structures that contain U1 snRNPs became la-

beled more or less simultaneously and why there was little difference between continuous and pulse-label experiments except in total amount of label. The differences between structures in rates of labeling could be caused by differences in accessibility of U1 snRNPs for exchange.

The antisense injection experiments raise another question about accumulation of the C protein in the nucleus. Under normal circumstances the C protein is restricted to the nucleus, yet it has no obvious nuclear localization signal of the type described for SV-40 large T antigen, nucleoplasmin, or other nuclear proteins (reviewed in Dingwall and Laskey, 1991). Because of its small size (29 kD) the C protein may be able to diffuse through the nuclear pores (Paine et al., 1975). Once inside the nucleus, it may remain because of its association with U1 snRNPs.

### C Snurposomes

Although most of our observations on the intranuclear distribution of newly synthesized C protein are consistent with a simple exchange of new for old C protein in each of the nuclear organelles, the labeling of C snurposomes was more complex. C snurposomes are bipartite structures, consisting of a matrix and one or more inclusions of various sizes (Callan and Gall, 1991; Gall, 1992). *In situ* hybridization shows that splicing snRNAs are limited to the inclusions (unpublished), even though the matrix stains strongly with mAb Y12 against Sm proteins and mAb K121 against the trimethylguanosine cap of snRNAs (Gall and Callan, 1989; Wu et al., 1991). Human serum 361, which reacts with the C protein, stains the entire C snurposome weakly (Wu et al., 1991). Thus we did not expect to see much accumulation of C protein in the C snurposomes in the injection experiments. However, the C snurposomes were the first structures detectable by immunofluorescence and they remained as bright as the loops and B snurposomes during the first 24–48 h. After that time, the C snurposomes sometimes lost label relative to the loops and B snurposomes. These results suggest the possibility that some of the C protein that enters the GV passes first through the C snurposomes. Because the *myc*-tagged C protein is clearly in the matrix of the C snurposomes, which has little or no U1 snRNA, it may occur as free protein in this organelle. In another study involving the intranuclear localization of newly synthesized NO38, normally a strictly nucleolar protein, some deletion constructs exhibited strong labeling of the C snurposomes (Peculis and Gall, 1992). Although these observations are at present fragmentary, they hint that C snurposomes may play some role in the distribution of proteins within the nucleus.

### A Snurposomes

The snRNP composition of A snurposomes is simpler than that of B's or C's. Of the splicing components for which we have tested, they contain only U1 snRNA and associated proteins (Wu et al., 1991). We were, therefore, surprised by the inconsistent labeling of these organelles with the *myc*-tagged C protein. Our assessment of the A snurposomes was complicated by the fact that they can vary greatly in number and size, and can be morphologically indistinguishable from B snurposomes (Wu et al., 1991). It is thus possible that we confused A and B snurposomes in some preparations where they were equally labeled. A and B snurposomes can be dis-

tinguished unequivocally in control GV's by the brighter staining of A's with human serum 361, which specifically recognizes U1 snRNPs. Unfortunately, this serum recognizes the C protein, and gave the same staining pattern as the anti-myc antibody in GV's from injected oocytes. Thus it could not be used to distinguish A and B snurposomes in the injection experiments. We can conclude that newly synthesized C protein enters A snurposomes, but beyond this, our observations do not suggest a specific role for these structures.

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