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An abundant U6 snRNP found in germ cells and embryos of *Xenopus laevis*

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The particle state of U snRNPs was analyzed in oocytes, eggs, embryos and testes from *Xenopus laevis*. In each case both the relative abundance and the composition of some U snRNPs were found to differ from that of somatic cells. U2 and U6 snRNPs were the most prominent U snRNPs in germ cells and early embryos. In particular, the concentration of U6 snRNA was 10-20 times higher than that of U4 snRNA. Most of the U6 snRNA was not associated with U4 snRNA and migrated on sucrose gradients as a U6 snRNP. The structure of this novel U snRNP was analyzed. A single protein of 50 kd was co-purified with U6 snRNPs by a combination of gradient fractionation, immunodepletion with anti-Sm antibodies and immunoprecipitation with anti-6-methyl adenosine antibodies. Although the U6 snRNP did not contain Sm proteins it migrated into the nucleus when U6 snRNA was injected into the cytoplasm of oocytes. Two U6 snRNA elements have been identified. The first is essential for nuclear migration in oocytes, but not for the formation of U4/6 snRNPs *in vitro* and might be the binding site of a U6-specific protein. The second element was required for interaction with U4 snRNPs but not for nuclear targeting.

Key words: embryos/germ cells/U6 snRNP/*Xenopus laevis*

Introduction

The structure and function of the major U snRNPs (U rich small nuclear ribonucleoprotein particles) U1, U2, U5 and U4/6 have been highly conserved throughout evolution (Birnstiel, 1988). From yeast to mammals they are essential for the processing of mRNA precursors (Steitz *et al.*, 1988). Together with an unknown number of cofactors U snRNPs have been shown to associate sequentially with pre-mRNA to form functional splicing complexes (spliceosomes). The intron is excised from the pre-mRNA and the exons ligated to generate mature mRNA after spliceosome formation. One of the first steps in complex formation is the association of U1 and U2 snRNPs with conserved sequence elements present at the intron-exon boundaries and inside of the intron. U1 snRNPs interact with the 5′ splice site and U2 snRNPs with the branch point region, both interactions are at least partly dependent on direct base-pairing of the U snRNAs and the pre-mRNA (Zhuang and Weiner, 1986; Parker *et al.*, 1987). Subsequently U4/6 (or U4, U6) and U5 snRNPs bind to this pre-splicing complex, forming a functional spliceosome. The basis for their interaction is not known. During or after the cleavage-ligation reaction, U4 snRNPs are associated less tightly (or not at all) with spliceosomes, because they are no longer detectable in complexes separated on native gels (Pikielny *et al.*, 1986; Cheng and Abelson, 1987; Lamond *et al.*, 1988).

U1, U2 and U5 snRNPs are present as single particles containing a U snRNA, a set of common proteins (Sm proteins) and also U1-, U2- or U5-specific proteins (Lührmann, 1988). U4 and U6 snRNA are found in a single U4/6 snRNP (Bringmann *et al.*, 1984; Hashimoto and Steitz, 1984). The Sm proteins are bound to U4 snRNA, no U4- or U6-specific proteins have been identiied. Whether U snRNP proteins are essential for splicing and if so, for which step(s) of the reaction, is not known. Disruption of a gene coding for a U5-associated protein in yeast is lethal, indicating that some U snRNP proteins might be essential (Lossky *et al.*, 1987). However, complementation studies with U2 snRNA mutants in oocytes suggest that the binding of certain U2-specific proteins (at least for the two pre-mRNAs studied) might be dispensable for splicing (Hamm *et al.*, 1989; Pan and Prives, 1989).

The association of U4 and U6 is partly, or perhaps even entirely, due to an extensive intermolecular RNA-RNA interaction. Two complementary RNA elements (interaction domains 1 and 2) are conserved in all U4 and U6 snRNAs analyzed and are thought to play a key role in this interaction (Brow and Guthrie, 1988; Guthrie and Patterson, 1988). Experimental support for the formation of interaction domain 1 was obtained by crosslinking studies (Rinke *et al.*, 1985).

The absence of U4 snRNA from spliceosomes detected on non-denaturing polyacrylamide gels raised questions concerning the state of U6 snRNA when not part of U4/6 snRNPs. The great stability of the U4-U6 interaction observed *in vitro* (T_m ~52°C) implied that an active mechanism might be required to disrupt this complex in spliceosomes (Brow and Guthrie, 1988).

Regulation of gene expression can be achieved at the level of splicing. Alternative splicing, resulting in the production of different mRNAs from a single pre-mRNA, and regulated splicing, limiting the ability to process pre-mRNAs to certain tissues or developmental stages, are found (Leff *et al.*, 1986; Mattaj and Hamm, 1989). The mechanism of alternative or stage- and tissue-specific splicing is not understood. No sequence elements (like 5′-splice sites or branch points) specific for regulated pre-mRNAs could be identified. Sequence variants of U snRNAs expressed exclusively in germ cells and embryos would be good candidates for *trans*-acting factors involved in regulated splicing events (Forbes *et al.*, 1984; Lund *et al.*, 1985; Lund and Dahlberg, 1987). However, so far it has not been shown that the U snRNA variants form structurally or functionally distinct U snRNPs.

In this work U snRNPs from germ cells and embryos of *Xenopus laevis* were analyzed. Tissue-specific differences in the composition and relative abundance of U snRNPs were detected. U2 and U6 snRNPs were the predominant particles in germ cells. Most of the U6 snRNA was present in U6

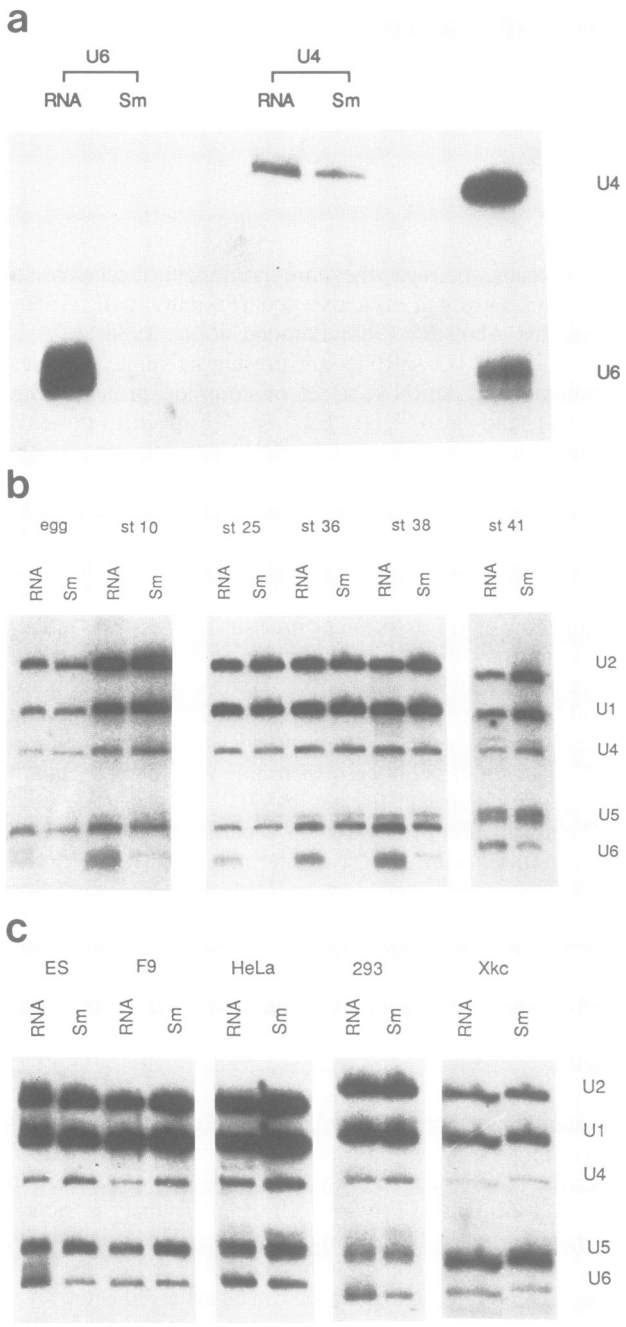

Fig. 1. U snRNAs associated with Sm antigens. (**a**) Defolliculated stage VI oocytes were used to extract total RNA or to immunoprecipitate RNPs with anti-Sm antibodies. RNAs were separated on denaturing acrylamide gels and analyzed on a Northern blot hybridized with radioactively labeled U6- or U4-antisense RNA probes. RNA extracted from oocytes injected with U4 and U6 genes together with [α-^{32}P]GTP was run as a size marker on the same gel. RNA extracted

snRNPs rather than in U4/6 snRNPs as in somatic cells. Since U6 snRNPs have not been described previously the properties of this particle were analyzed in more detail. The U6 snRNP was found in the nucleus of oocytes. A potential karyophilic signal was identified in U6 snRNA which was essential for nuclear migration but not required to form U4/6 snRNPs *in vitro*. A protein of 50 kd which co-purified with U6 snRNPs, but not with U4/6 snRNPs was detected.

Results

Free U6 in oocytes and embryos

When a *Xenopus tropicalis* U6 gene was injected into oocytes it was efficiently transcribed (Carbon *et al.*, 1987). However, U6 RNA was not precipitated by anti-Sm antibodies, even when U4 and U6 RNAs were made simultaneously by co-injection of U4 and U6 genes, although the U4 snRNA was Sm-precipitable (unpublished data). This implied that the U6 RNA made from the injected gene did not enter U4/6 snRNPs.

The particle state of endogenous U6 was analyzed to rule out the possibility of an injection artefact. For an analysis of oocyte U snRNAs it is necessary to remove the follicle cells that surround the oocytes (2000–3000/stage VI oocyte) and carry somatic nuclei. The follicle cells and the thecal layer were removed by a two-step procedure that had been shown to generate oocytes virtually free of follicle cells (Sakmann *et al.*, 1985; Alan Coleman, personal communication). RNPs were immunoprecipitated with anti-Sm antibodies or total RNA extracted and analyzed by filter hybridization (Figure 1a). Two striking observations were made. First, there was a large excess of U6 over U4 RNA (although the blot shown in Figure 1a was hybridized separately with anti-U6 or anti-U4 probes, it accurately reflected the relative concentration of U4 and U6 RNA; Figures 1b, 2b and unpublished data). Second, the majority of the U6 RNA was not precipitated by anti-Sm antibodies indicating that also most of the endogenous U6 was not part of U4/6 snRNPs.

The presence of large amounts of free U6 was not observed in somatic cells. In order to identify the transition point from the oocyte phenotype (large excess of U6, not Sm-precipitable) to the somatic phenotype (small excess of U6 over U4, at least 30–50% Sm-precipitable) eggs were fertilized *in vitro* and embryos collected at different stages

Sm antibodies or total RNA extracted and analyzed on Northern blots (Figure 1b). This showed that the state of U6 did not change during early development. Although transcription of the U snRNAs was initiated at the mid-blastula transition (stage 9) most of the U6 was not found associated with U4 before the feeding tadpole stage (stage

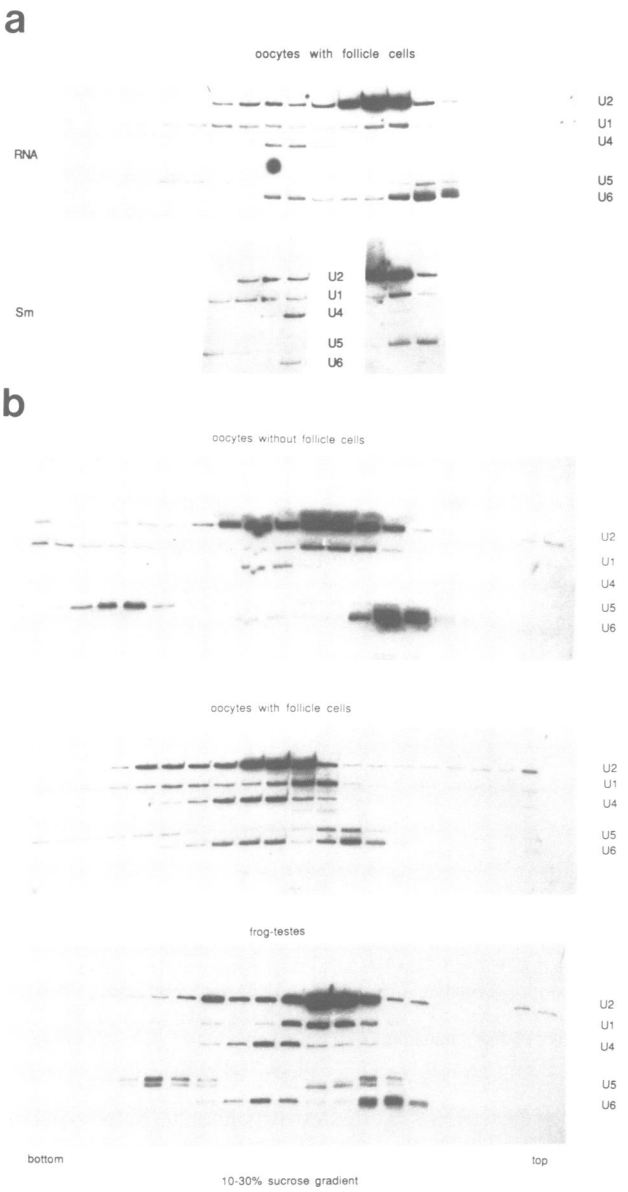

Fig. 2. Separation of U snRNPs on sucrose gradients. (**a**) Fifteen stage VI oocytes were homogenized and loaded on a 10−30% sucrose gradient. Twenty-four fractions of 500 μl were collected, RNPs were immunoprecipitated with anti-Sm antibodies from 250 μl and total RNA was extracted from 250 μl. RNAs were separated on denaturing acrylamide gels and analyzed on Northern blots as in Figure 1. The fractions from the top of the gradient are on the right of the figure. (**b**) Migration of U snRNPs of different origin. Follicle cells were manually removed from stage VI oocytes. Fifteen of these oocytes were homogenized and analyzed as in (a) (oocytes without follicle cells). Fifteen stage VI oocytes were homogenized without prior treatment and analyzed as above (oocytes with follicle cells). Testes isolated surgically from one frog were homogenized and analyzed as above (frog testes).

tated by anti-Sm antibodies, exhibiting the previously reported somatic U6 state. The 293 cells and, in particular, the ES cells contained a larger excess of U6 snRNA, although this did not reach the high level observed in embryonic tissues from *Xenopus*.

Free U6 migrates as a RNP on sucrose gradients

In order to determine whether the U6 not associated with U4 was present as free RNA or as a U6 RNP, RNPs were separated on sucrose gradients and subsequently immunoprecipitated with anti-Sm antibodies (Figure 2a, bottom panel) or total RNA extracted (Figure 2a, upper panel). The different U snRNPs were clearly resolved on the gradients and U6 migrated at two positions. One of them apparently corresponded to that of U4/6 snRNPs because this U6 migrated faster than U1 or U2, co-migrated with U4 and was precipitated by anti-Sm antibodies. The remaining U6 migrated slower than U1 or U2 at the position of the slower migrating U5 population. Immunoprecipitation showed that this U5, but not U6, was associated with Sm antigens (Figure 2a). When phenol-extracted oocyte RNA was run on a parallel gradient U4, U5 and U6 snRNA was found three fractions closer to the top of the gradient than the U6 RNP (data not shown).

These observations implied that U6 was present in two states, as a U4/6 snRNP and as a U6 RNP. The U6 RNP was apparently associated with proteins not recognized by anti-Sm antibodies.

U2 and U6 snRNPs are the most abundant U snRNPs in oocytes and testes

The experiments described above demonstrated the existence of two different U6 particles, U6 RNPs and U4/6 snRNPs. In the gradient shown in Figure 2a these U6 populations seemed to be of similar abundance, but from the blot shown in Figure 1a it would have been expected that the U6 RNP would have been the major species. However, the gradient was loaded with oocytes surrounded by follicle cells, while these had been removed from the oocytes used for the experiment shown in Figure 1a. This observation implied that there might be tissue-specific differences in the relative abundance of U snRNPs.

To address this question, RNPs from oocytes with or without follicle cells were separated on gradients (Figure 2b). When the follicle cells had been removed from the oocytes almost all of the U6 migrated as a U6 RNP and only small amounts of U4/6 snRNPs were detectable. In addition, U6 was far more abundant than U4 and the ratio of U4 and U6 was identical to that seen in Figure 1a. When U snRNPs from eggs (which do not have follicle cells) were separated on gradients, the result was exactly like that of oocytes without follicle cells (data not shown).

A further observation made was that U2 was far more abundant than U1 in oocytes after removal of the follicle cells. So U2 and U6 were the most abundant U snRNPs in oocytes, while U1 (followed by U2 and U5) was the most abundant U snRNP in cultured cells (Figure 1c). U5 snRNPs migrated at two positions in the gradients. One population co-migrated with U6 snRNPs, the other was the fastest migrating U snRNP. Since both U5 snRNP-types were precipitated by anti-Sm antibodies it was likely that the faster migrating species were associated with large U5-specific proteins (Bach *et al.*, 1989). The comparison of the two gradients leads to the conclusion that oocytes and somatic (follicle) cells have different complements of U snRNPs.

To determine whether these observations were oocyte-specific or a general property of germ cells, U snRNPs from frog testes were analyzed. The gradient with RNPs from testes was similar to the one from oocytes without follicle cells, although U4/6 snRNPs were found in larger quantities. Since the material was derived from homogenized testes containing both germ cells and somatic cells, it is likely that

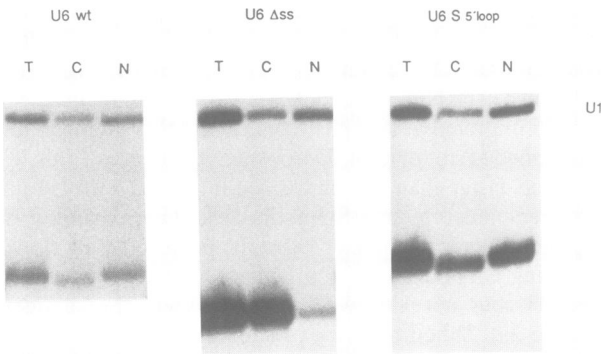

Fig. 3. Nuclear migration of U6 snRNAs. Purified U6 wt, U6 Δss or U6 S 5'loop RNA and U1 RNA (as an internal control) were co-injected into the cytoplasm of stage VI oocytes. Oocytes were separated manually into cytoplasmic and nuclear fractions 12 h later, and RNA was extracted and analyzed on denaturing acrylamide gels. One oocyte equivalent of RNA was loaded per lane (T, total RNA; C, cytoplasmic RNA; N, nuclear RNA). The mutated regions of U6 are depicted in Figure 5.

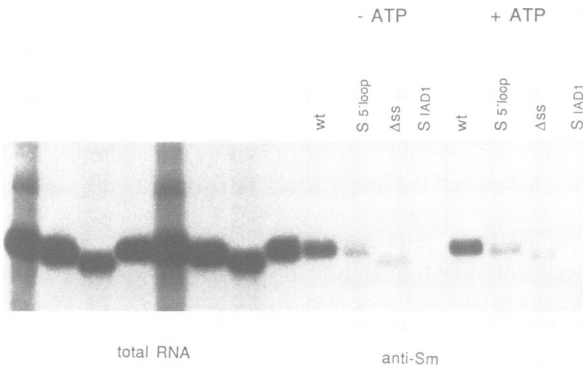

Fig. 4. Exchange of labeled T7 U6 RNAs into HeLa U4/6 snRNPs. Wild-type or mutant U6 RNAs were synthesized with T7 RNA polymerase and incubated in HeLa nuclear extract alone (−ATP) or in the presence of ATP/creatine phosphate/$MgCl_2$ (+ATP). One-half of this reaction was used to immunoprecipitate RNPs with anti-Sm antibodies (Sm), the other half to extract total RNA. RNA was analyzed on denaturing acrylamide gels.

oocytes and male germ cells contain a similar complement of U snRNPs.

Nuclear migration and interaction with U4 snRNPs

The properties of the U6 snRNP were analyzed in more detail. When the intracellular location of U6 RNPs was determined in oocytes by separating nuclei and cytoplasm manually and analyzing the RNA on Northern blots, U6 was found exclusively in the nucleus (data not shown). This raised the question of how U6 was targeted to the nucleus.

Sm-associated U snRNPs are synthesized as precursors in the nucleus. They then enter the cytoplasm and associate at least with the 'common' U snRNP proteins. Subsequently their cap structures become trimethylated and the modified U snRNPs migrate into the nucleus (DeRobertis *et al.*, 1982; Mattaj, 1988; Zieve *et al.*, 1988). Mutant U snRNAs unable to bind to the Sm proteins remain in the cytoplasm and their cap structures are not correctly modified (Hamm *et al.*, 1987; Mattaj, 1988; J.Hamm and I.W.Mattaj, unpublished data).

If all the U6 had been in U4/6 snRNPs it could have been transported due to the karyophilic signal of U4 snRNPs.

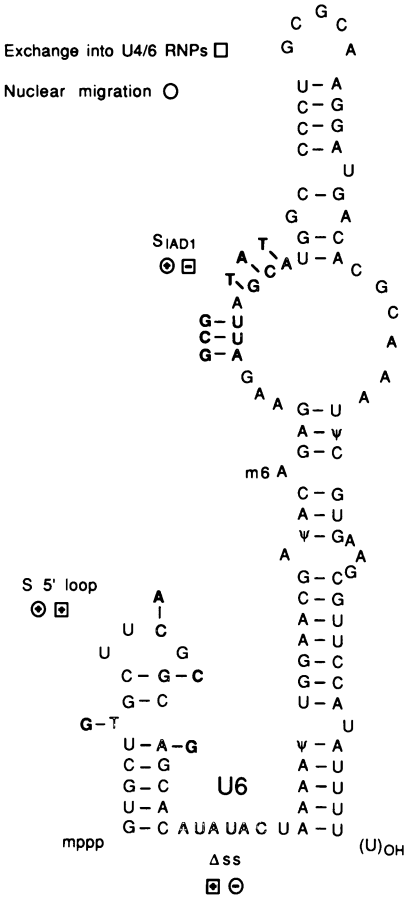

Fig. 5. Location of introduced mutations in U6 snRNA. Nucleotides substituted in U6 S 5'loop/U6 S IAD1 or deleted in U6 Δss are indicated in a secondary structure model of U6 (Rinke *et al.*, 1985). U6 ΔIAD1 is identical with construct 8 from Carbon *et al.* (1987). The effect of these mutations on nuclear migration (○) or exchange into U4/6 snRNPs (□) is represented schematically (±).

However, U6 RNPs were shown neither to be associated with U4 nor with Sm proteins. Nevertheless, U6 RNA (purified from oocytes injected with a U6 gene and [α-^{32}P]-GTP), migrated into the nucleus when injected into the cytoplasm of oocytes, exactly like U1 (Figure 3, U6 wt).

This assay offered a possibility to identify a U6 RNA element required for nuclear migration by generating a set of U6 RNA mutants and searching for an RNA unable to enter the nucleus. Four different U6 RNA regions were mutated (Figure 5); (i) four nucleotides were substituted in the phylogenetically conserved 5′ stem−loop structure (U6 S 5′ loop); (ii) six nucleotides predicted to be single-stranded and flanked by stem−loop structures were deleted (U6 Δss); (iii) six nucleotides expected to interact with U4 (based on phylogenetic comparison and crosslinking studies) were substituted (U6 S IAD1); or (iv) deleted U6 ΔIAD1). The mutations were introduced into a *X.tropicalis* U6 gene by site-directed mutagenesis, the U6 DNAs injected into oocytes together with [α-^{32}P]GTP and the RNAs gel purified. Labeled U1 snRNA was purified from oocytes injected with a U1 gene and [α-^{32}P]GTP by immunoprecipitation with U1/U2-specific antibodies. Each of the mutant U6 RNAs was injected either alone or in combination with U1 snRNA (as an internal control for migration, separation and extraction) into the cytoplasm of stage VI oocytes. All mutant U6 RNAs were stable in oocytes (data not shown). Twelve

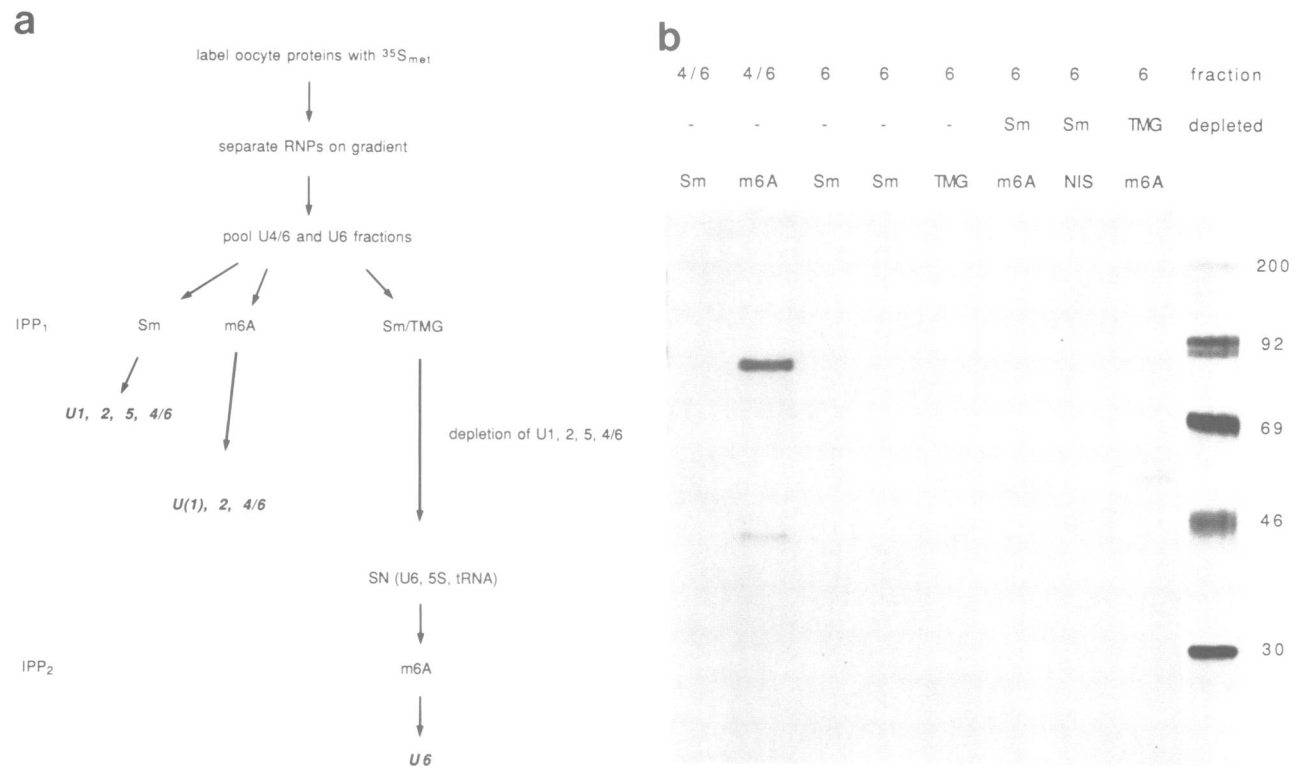

Fig. 6. Proteins co-migrating with U6 snRNA on sucrose gradients. (**a**) Purification scheme for U6 snRNPs (IPP, immunoprecipitation; SN, supernatant; 5S, 5S RNA; TMG, anti-trimethyl-G cap antibodies). (**b**) Proteins co-migrating with U4/6 or U6 snRNA. [^{35}S]Methionine-labeled proteins immunoprecipitated from U6 or U4/6 fractions of a sucrose gradient (as Figure 2a, but loaded with stage I/II oocytes) were analyzed on a denaturing protein gel and visualized by fluorography. The right-most lane shows a shorter exposure of ^{14}C-labeled mol. wt standards run on the same gel [NIS, non-immune serum; other abbreviations as in (a)].

hours later nuclei and cytoplasm were separated and RNA extracted and analyzed. Only one out of the four mutant U6 RNAs, U6 Δss, did not migrate efficiently into the nucleus (Figure 3; data not shown). This indicated that the potentially single-stranded U6 snRNA region flanked by stem−loop structures was required for nuclear migration of U6 snRNA. This region might function directly as a karyophilic signal or indirectly, by binding U6 snRNP proteins.

It was therefore of interest to determine whether the U6 snRNA element required for nuclear migration was also essential for the formation of U4/6 snRNPs. Mutant U6 RNAs were analyzed for their ability to form U4/6 snRNPs *in vitro*. For this purpose some of the U6 mutations analyzed above (S 5′ loop, Δss, S IAD1) were introduced into a T7 U6 gene (see Materials and methods) to enable the synthesis of ^{32}P-labeled T7 U6 RNAs with T7 RNA polymerase. The labeled T7 U6 RNAs were incubated in HeLa nuclear extract under conditions allowing the exchange of added U6 RNAs into pre-existing U4/6 snRNPs (Pikielny *et al.*, 1989). The amount of T7 U6 RNAs present in U4/6 snRNPs was analyzed by immunoprecipitation with anti-Sm antibodies (Figure 4). The exchange reaction was independent of added ATP, creatine phosphate and $MgCl_2$, and efficient enough to render 20−40% of wild-type T7 U6 RNA Sm-precipitable. Mutants U6 S 5′ loop and U6 Δss formed U4/6 snRNPs at a level that was reduced compared with the wild-type but significantly above the background level. In contrast, the substitution of interaction domain 1 (U6 S IAD1) completely prevented precipitation by anti-Sm antibodies, showing that this U6 region was essential for the formation of stable U4/6 snRNPs *in vitro* but not for nuclear migration in oocytes, while the reverse was true for U6 Δss (Figure 5).

A 50 kd protein co-purifies with U6 snRNA

The sucrose gradient and nuclear migration experiments described above suggested that oocyte U6 snRNA was associated with protein(s). No U6-specific antibodies were available to identify directly proteins potentially associated with U6. Therefore oocyte proteins were labeled with [^{35}S]-methionine and U6 snRNPs enriched by a combination of gradient fractionation, immunodepletion and immunoprecipitation (Figure 6a). This was possible because antibodies recognizing a base-modification (m6A) present in U6 and U2 snRNA, but not in U1, U4 or U5 snRNA were available (Bringmann and Lührmann, 1987).

Sucrose gradients were used to separate U6 snRNPs and U4/6 snRNPs. To minimize cross-contamination only the fractions including the two slowest (U6) or fastest (U4/6) migrating U6 snRNAs forms were pooled. Fractions containing U4/6 snRNPs were analyzed directly by immunoprecipitation with anti-Sm or anti-m6A antibodies. U6 snRNP fractions were depleted of U1, U2, U5 and U4/6 snRNPs with anti-Sm or anti-trimethyl-G cap antibodies (TMG) and U6 snRNPs precipitated from the supernatant with anti-m6A antibodies. Analysis of the RNA from the immunoprecipitates showed the expected results (data not shown). Proteins were eluted from precipitated U snRNPs and separated on denaturing acrylamide gels (Figure 6b). A single protein of ~50 kd co-purified with U6 snRNPs and was absent from U4/6 snRNP fractions. The strongly labeled protein of ~90 kd precipitated by anti-m6A antibodies from U4/6 snRNP fractions was unlikely to be a component of U4/6 snRNPs, because this protein was not precipitated by anti-Sm antibodies and was also detected after nuclease treatment (see below). We did not detect other U

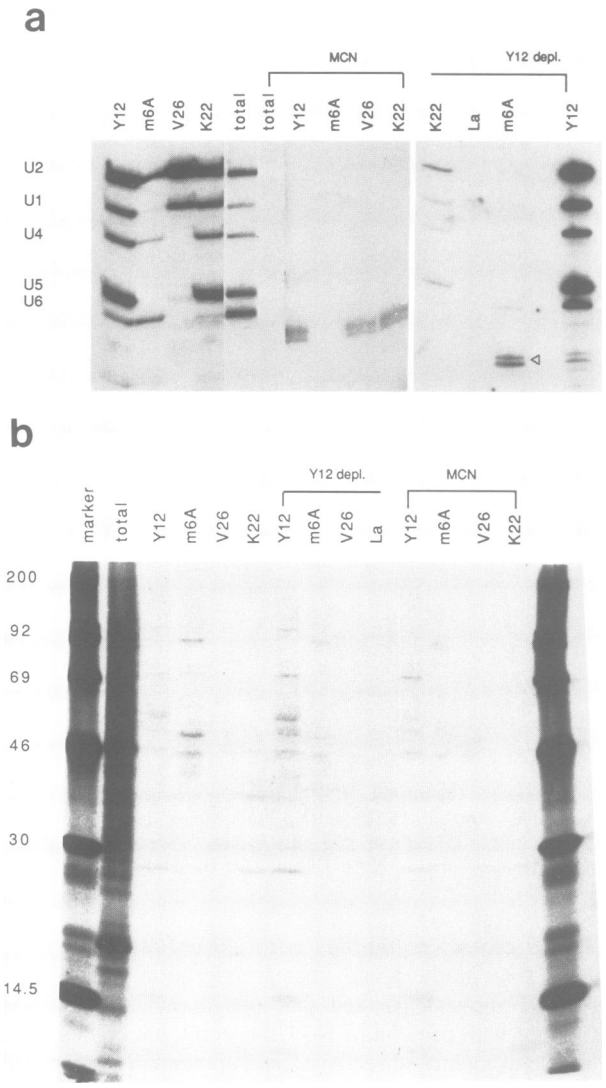

Fig. 7. Proteins co-precipitated with U6 snRNA from total oocytes. Proteins of stage I/II oocytes were labeled with [^{35}S]methionine and RNPs immunoprecipitated with anti-Sm (Y12, K22), anti-m6A (m6A) and anti-U1/U2 (V26) antibodies either directly or after micrococcal nuclease treatment (MCN). Alternatively, oocyte extracts were depleted with anti-Sm antibodies (Y12) and aliquots of the supernatant subsequently analyzed by immunoprecipitation with anti-m6A, anti-La or anti-Sm (K22) antibodies. RNA was extracted from half of the protein-A−Sepharose bound precipitate and analyzed on Northern blots as in Figure 1b. (**a**) The arrowhead indicates precipitated degradation products of U6 snRNA. Proteins were extracted from the remainng half, separated on denaturing gels and processed as in Figure 6b (**b**).

snRNP proteins (in the Sm precipitates) at the exposure level used in this experiment.

A simpler procedure was used to get more information about the U6 snRNP and its relation to the 50 kd protein. RNPs were immunoprecipitated with a variety of antibodies directly from oocytes with or without prior micrococcal nuclease treatment or after depletion with anti-Sm antibodies. In addition to anti-m6A antibodies (m6A) and monoclonal anti-Sm antibodies (Y12), sera V26, K22 and anti-La were used. V26 is a U1/U2-specific serum and was included because anti-m6A antibodies precipitate not only U6 and U4, but also U1 and U2 (Bringmann and Lührmann, 1987). Anti-La antibodies were tested because U6 snRNA has been reported to be transiently associated with the La protein (Rinke and Steitz, 1985), and serum K22 because it recognizes a 50 kd protein on Western blots of HeLa nuclear proteins (Habets *et al.*, 1989). Both the RNAs as well as the proteins precipitated were analyzed.

The RNA analysis (Northern blot, Figure 7a) revealed that only the anti-m6A antibodies precipitated U6 snRNPs, as judged by an excess of U6 over U4 snRNA in the precipitates (e.g. compare lane m6A and lane Y12; Figure 7a, left panel). Micrococcal nuclease treatment largely destroyed all the U snRNAs; only some degradation products of U1 or U2 were detected. Depletion with anti-Sm antibodies followed by anti-m6A precipitation resulted in a fraction containing U6 snRNA and a small amount of U2 snRNA. The amount of intact U6 snRNA obtained after immunodepletion was often relatively low due to degradation of U6 snRNA not associated with U4 during the anti-Sm depletion. However, stable degradation products of U6 (whose identity was verified by hybridization with a U6-specific probe alone) were precipitated by anti-m6A antibodies (Figure 7a, arrowhead). These fragments were large enough to contain both the potential protein binding site and the m6A residue required for precipitation. Neither anti-La antibodies nor serum K22 precipitated U6 snRNPs.

When the proteins present in the same precipitates were analyzed, the 50 kd protein appeared only in those containing an excess of U6 over U4 snRNA (Figure 7b). This protein was detected by direct precipitation with anti-m6A antibodies with or without prior depletion with anti-Sm antibodies. It was not observed after micrococcal nuclease treatment, showing that the precipitation was dependent on association with RNA. It was not precipitated by V26, a U1/2 snRNP-specific serum, ruling out the possibility of being a so far undetected U1- or U2-specific protein.

The direct precipitation from oocytes also showed that the contaminating protein of 45 kd present in all precipitates and in the control serum precipitate from the U6 snRNP gradient fraction (Figure 6b) was an abundant protein. It was one of the most intensely labeled oocyte proteins (see total, Figure 7b). The proteins precipitated by anti-Sm antibodies (Y12, K22), which migrate below 30 kd and around 14.5 kd are likely to be *Xenopus* homologs of B, D, E and F. These experiments showed that the 50 kd protein is present in the U6 snRNP gradient fraction and can be precipitated from this or directly from oocytes with anti-m6A antibodies. The anti-m6A precipitation is susceptible to micrococcal nuclease digestion. Taken together the results of the co-purification studies were compatible with the presence of a 50 kd protein in U6 snRNPs but not in U4/6 snRNPs.

Discussion

U6 snRNPs and U4/6 snRNPs

The analysis of *Xenopus* germ cell U snRNPs revealed the presence of two different U6 snRNA-containing RNPs: U6 snRNPs and U4/6 snRNPs. The majority of the U6 snRNA in germ cells and embryos was found in U6 snRNPs; only small amounts of U4/6 snRNPs were detected. In the follicle cells surrounding the oocytes and in various cultured cell lines most of the U6 snRNA was part of U4/6 snRNPs. Since in germ cells and embryos the concentration of U6 snRNA was at least 10−20 times higher than that of U4 snRNA, U6 snRNA not associated with U4 snRNA could have been present as free U6 snRNA, as a U6 snRNP or bound to other U snRNPs (e.g. U5).

The U6 snRNP was, however, neither precipitated by anti-Sm antibodies nor did it co-migrate with a major U snRNA on sucrose gradients in a way indicative of intermolecular interactions. Because U6 snRNA not associated with U4 migrated faster than free U4, U5 or U6 snRNA on sucrose gradients, U6 is most likely present as a U6 snRNP (also see below). Although it is at present unclear whether U6 snRNPs are abundant in germ cells and embryos of other species, mouse embryonic stem cells were found to have a larger U6:U4 ratio than other cell lines.

Non-coordinated transcription of U4 and U6 genes could be due to different RNA polymerases involved in their expression. U6 is an RNA polymerase III transcript, while U4 genes are transcribed by RNA polymerase II. Since RNA polymerase III activity is high in oocytes this could result in the accumulation of U6 snRNA (relative to U4) in oocytes and early embryos. At the midblastula transition (MBT, stage 9) RNA polymerase II transcribed U snRNA genes are activated (Newport and Kirschner, 1982). This might have resulted in an increased production of U4 snRNA and an equalization of the abundance of U4 and U6 snRNA. The analysis of U snRNPs and U snRNAs from different embryonic stages showed, however, that the concentration of U6 snRNA remained higher than the concentration of U4 snRNA until after stage 38, demonstrating that transcription of U4 and U6 genes is not coordinated in oocytes and embryos in order to generate equal amounts of both RNAs.

The excess of U6 over U4 snRNA observed differed between individual experiments. The ratios seen in Figure 1b correspond to the minimum detected. In other experiments the ratio was higher (Figure 1a or Figure 2b, oocytes without follicle cells). This could have been due to individual variation between frogs or to a comparatively low stability of U6 snRNPs. The latter alternative is favored for the following reasons. (i) If, instead of direct RNA extraction, whole cell extracts were prepared from oocytes, eggs or embryos, only the U6 snRNA present in U4/6 snRNPs was found in the final extract, showing that U6 snRNPs were lost or degraded during the preparation (unpublished observations). (ii) If embryos used for immunoprecipitation of U snRNPs were homogenized in the absence of RNase inhibitors, the amount of U6 snRNA obtained was significantly lower. For this reason it is not possible to rule out the possibility that U6 snRNPs persist in embryos of stage 41 or later, but remain undetected due to degradation despite the presence of RNase inhibitors.

Nevertheless, the U6 snRNP was the major U6 snRNA-containing particle in germ cells and embryos determined by gradient analysis or direct extraction. The high abundance of U2 snRNPs relative to U1 snRNPs was limited to oocytes, eggs, pre-MBT embryos and testes. Embryos collected from stage 9 or later had a U1:U2 ratio similar to that of somatic cells, while pre-MBT embryos gave results identical to those of oocytes without follicle cells or unfertilized eggs: high concentrations of U2 and U6 snRNA and U6 mostly not associated with U4.

Requirements for nuclear migration of U6 snRNA and formation of U4/6 snRNPs

In order to participate in the splicing process U snRNPs have to be located in the nucleus. This is achieved for U1, U2, U4 and U5 snRNAs by binding of the Sm proteins to a conserved sequence element, the Sm-binding site. U snRNAs appear transiently in the cytoplasm, where they associate with the Sm proteins. U6 snRNA does not have an Sm-binding site, so how can the U6 snRNA not associated with U4 become localized in the nucleus? The most simple explanation would be that U6 snRNA carries a karyophilic signal functionally equivalent to an Sm-binding site.

Nuclear migration studies with mutant U1 and U2 snRNAs showed that deletions or substitutions that destroyed the Sm-binding site interfered with the ability to enter the nucleus. Other mutations, including those preventing binding of U1- or U2-specific proteins did not influence nuclear translocation. In analogy to these studies an RNA element in U6, which was required for nuclear migration, was identified. Various mutations (deletions and substitutions) were introduced into U6 genes, mutant U6 RNAs transcribed in oocytes, purified and reinjected into the cytoplasm and their intracellular location determined. Only one out of four tested mutations (U6 Δss, Figure 5) affected nuclear migration, implying that nucleotides 21–26 were directly or indirectly (by serving as a protein binding site) involved in translocation.

The region of U6 snRNA deleted in U6 Δss showed structural homology to Sm-binding sites. It was a region predicted to be single stranded in the secondary structure model of U6 and was flanked by stem–loop structures. Taken together with the observations that U6 snRNA migrated as a U6 snRNP on gradients and that the region deleted in U6 Δss was not accessible for oligonucleotide-directed RNase H cleavage of U6 in HeLa nuclear extracts (Black and Steitz, 1986), the nuclear migration of U6 snRNA might be due to binding of protein(s) to nucleotides 21–26. The binding could generate a karyophilic signal similar to that caused by binding of Sm proteins to Sm-binding sites in other U snRNAs. The binding of protein to U6 snRNA could have various effects on the ability of U6 to interact with U4. Protein binding to U6 snRNA could have a positive, negative or neutral effect on the interaction with U4 snRNPs. The mutant RNA U6 Δss should be unable to form U4/6 snRNPs if the protein bound to U6 were essential for particle formation. U6 mutants were analyzed for their ability to exchange into pre-existing U4/6 snRNPs. U6 Δss, which was unable to migrate into the nucleus, could form stable U4/6 snRNPs (although at a lower rate than the U6 wt). However, mutation of interaction domain 1 (U6 S IAD1) prevented any detectable exchange into U4/6 snRNPs. U6 S IAD1 was selected to test for specificity because this region was proposed to interact with U4 snRNPs based on phylogenetic comparison and also from crosslinking studies (Rinke *et al.*, 1985; Brow and Guthrie, 1988). These experiments showed that nuclear migration of U6 snRNA and formation of U4/6 snRNPs required different elements in U6 snRNA. If nucleotides 21–26 were the binding site of U6-specific protein(s), the presence of this binding site was apparently not essential to form stable U4/6 snRNPs. Whether binding of U6-specific protein(s) was necessary to disrupt U4/6 snRNPs could not be deduced from these studies and has not been tested.

A protein co-fractionates with U6 snRNPs

Since no U6-specific antibodies were available, potential U6 snRNP-specific proteins were identified by fractionation of U snRNPs and analysis of proteins that co-purified with U6 snRNPs. U6 and U4/6 snRNPs were separated on sucrose gradients. U6 snRNP-containing fractions were pooled and depleted from remaining U1, 2, 5 and 4/6 snRNPs with anti-

Sm or anti-trimethyl-G cap antibodies. U6 snRNPs were then precipitated with anti-m6A antibodies, and the proteins eluted and analyzed. A single protein of ~50 kd co-purified with U6 snRNPs. A protein of the same size was also detected when particles were precipitated without prior fractionation, although the background of proteins binding non-specifically to the protein-A–Sepharose beads was considerably higher (Figures 6b and 7b).

The precipitation of the 50 kd protein was shown to be dependent on intact RNA. The protein was not precipitated by anti-La or anti-Sm antibodies and co-migrated with U6 snRNPs on sucrose gradients. When RNAs precipitated by the antibodies shown in Figure 7 were analyzed by silver staining, no RNA apart from U6 was specifically precipitated by anti-m6A antibodies; small amounts of 5S RNA and tRNA were present as contaminants in all precipitates (data not shown).

Although these data do not represent proof of the association of the 50 kd protein with U6 snRNA, the observations are highly suggestive and can potentially be tested by purifying larger amounts of this protein and testing for binding to U6 snRNA. To obtain better evidence for the association of the 50 kd protein with U6, the effect of oligonucleotide-directed RNase H cleavage of U6 snRNA on co-precipitation of the 50 kd protein was tested. These attempts were unsuccessful because the cleavage conditions lead to extensive proteolysis in crude oocyte extracts and to non-specific RNA cleavage in gradient fractions.

Implications

So far the only indication for *trans*-acting factors that might be involved in regulated splicing were embryonic U snRNA sequence variants found in frog, mouse and human. It has not been shown yet that these variants form particles that differ in their structure or function from their somatic counterparts. Here it was demonstrated that the relative abundance of U snRNAs can be tissue-specific. U6 snRNA was suggested to play a key role in the splicing process because it is the U snRNA whose sequence is best conserved between *Saccharomyces cerevisiae* and mammals (Brow and Guthrie, 1988). The yeast U snRNPs homologous to U1, U2, U4 and U5 show extensive structural but only limited sequence similarity. In the splicing of the SV40 T-antigen pre-mRNA, one of the best studied examples of alternative splicing, the ratio of the alternative splicing products small t and large T is different in *Xenopus* oocytes and in HeLa cells. Concomitantly, the branch points utilized in these two cell types are different (Noble *et al.*, 1987). Since U2 snRNPs are likely to interact directly with the branch point, changes in the abundance and/or composition of U2 snRNPs could affect branch point selection. Experimental tests of the functional significance of altered U snRNP compositions in oocytes should be possible.

Materials and methods

Sucrose gradients

Oocytes, eggs or testes were homogenized in 600 μl TKM (50 mM Tris–HCl, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$) and spun for 5 min in an Eppendorf centrifuge. Four hundred microliters were loaded on a 10–30% TKM–sucrose gradient (V = 12 ml) and centrifuged for 21 h (SW40, 35 000 r.p.m., 4°C). Twenty-four fractions of 500 μl were collected. Protein was extracted with phenol–chloroform and RNA precipitated with ethanol for total RNA analysis.

Immunoprecipitations

Immunoprecipitations were performed as described previously (Hamm *et al.*, 1987), but at 4°C and TKMPP (TKM, 0.1% Nonidet-P40, 0.1% sodium azide) was used instead of IPP_{500}.

Antibodies

Y12 is a monoclonal anti-Sm antibody (Lerner *et al.*, 1981), anti-m6A is a rabbit serum (Bringmann and Lührmann, 1987), V26 is a patient serum recognizing various U1- and U2-specific proteins (Habets *et al.*, 1985) and K22 is a patient serum recognizing Sm proteins, U1-specific proteins, N and an unidentified protein of 50 kd (Habets *et al.*, 1989). The human anti-La antibody was a gift from D.Williams.

Northern blots

RNA was separated on denaturing 8% acrylamide gels, transferred to Genescreen and hybridized with a mixture of antisense probes against U1, 2, 4, 5 and 6 (Hamm *et al.*, 1989).

Embryos

Mature *Xenopus* females were injected with 100 U pregnant mare serum gonadotropin (Chrono-Gest PMSG 500: Intervet). Between 2 and 10 days later, egg laying was induced by injecting 1000 U human chorionic gonadotropin (HCG, Sigma). Eggs were fertilized *in vitro* as described earlier (Newport and Kirschner, 1982). Ten embryos were homogenized in 1 ml TKMPP (+200 U RNasin), spun for 5 min in an Eppendorf centrifuge and 500 μl used for immunoprecipitation and 500 μl for total RNA extraction.

Cells

One million cells were sonicated in 1 ml TKMPP (+200 U RNasin), spun for 5 min in an Eppendorf centrifuge and 50 μl used for immunoprecipitation or total RNA extraction.

Protein labeling

Between 30 and 60 stage I/II oocytes were incubated overnight at 19°C in 200 μl Barth's medium containing 0.5 mCi [^{35}S]methionine, washed three times in 500 μl TKM, homogenized in 200 μl TKM, spun for 5 min in an Eppendorf centrifuge and the supernatant was loaded on a gradient or directly used for immunoprecipitations. Proteins were separated on 15% denaturing acrylamide gels.

Exchange reactions

T7 U6 genes were constructed and labeled T7 RNAs made as described for T7 U1 genes and T7 U1 RNAs (Hamm *et al.*, 1987). *Dra*I restriction sites were generated by inserting three A-residues at the 3′ end of the coding region of U6 genes. Fifty nanograms of ^{32}P-labeled T7 U6 RNA was incubated together with 15 μl of HeLa nuclear extract (Dignam *et al.*, 1983) for 1 h at 42°C in a total vol of 30 μl (10 mM HEPES, pH 7.9, 10% glycerol, 0.1 mM EDTA, 0.25 mM DTT, 400 mM KCl). In the experiments labelled '+ATP' additional components were included (1.7 mM $MgCl_2$, 0.7 mM ATP, 16.7 mM creatine phosphate).

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