

# Antibodies specific for *N*<sup>6</sup>-methyladenosine react with intact snRNPs U2 and U4/U6

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Antibodies specific for *N*<sup>6</sup>-methyladenosine (*m*<sup>6</sup>A) were elicited in rabbits and used to study the accessibility in intact snRNPs of the *m*<sup>6</sup>A residues present in the snRNAs U2, U4 and U6. The antibody quantitatively precipitates snRNPs U2 and U4/U6 from total nucleoplasmic snRNPs U1–U6 isolated from HeLa cells, which demonstrates that the *m*<sup>6</sup>A residues of the respective snRNAs are not protected by snRNP proteins in the snRNP particles. While the anti-*m*<sup>6</sup>A IgG does not react at all with U5 RNPs lacking *m*<sup>6</sup>A, a significant amount of U1 RNPs was co-precipitated despite the fact that U1 RNA does not contain *m*<sup>6</sup>A either. Since anti-*m*<sup>6</sup>A IgG does not react with purified U1 RNPs and co-precipitation of U1 RNPs is dependent on the presence of U2 RNPs but not of U4/U6 RNPs, these data indicate an interaction between snRNPs U1 and U2 in vitro. The anti-*m*<sup>6</sup>A precipitation pattern described above was also observed with snRNPs isolation from mouse Ehrlich ascites tumor cells, indicating similar three-dimensional arrangements of snRNAs in homologous snRNP particles from different organisms.

U snRNP; RNP structure; Antibody; Splicing; *N*<sup>6</sup>-Methyladenosine

## 1. INTRODUCTION

The small nuclear RNAs U1, U2, U4, U5 and U6 are the most abundant members of the U snRNA class which is found in eucaryotic cells (reviews [1,2]). While the snRNAs U1, U2 and U5 exist as discrete ribonucleoprotein particles (snRNPs), the snRNAs U4 and U6 are organized in one and the same ribonucleoprotein complex [3,4]. The two RNAs are base paired together in the U4/U6 RNP particle [5]. Some of the U snRNP proteins are antigenic for the so-called anti-RNP and anti-Sm autoantibodies which are often developed by patients suffering from autoimmune disorders such as systemic lupus erythematosus [6,7]. Both the structure of the snRNAs and at least the antigenic domains of the snRNP proteins have been highly conserved evolutionarily.

In the cell the snRNPs appear to be involved in the processing of nuclear pre-mRNA. Experimental evidence has been provided that all the major nucleoplasmic snRNPs U1–U6 participate in principle in the splicing of nuclear pre-mRNAs [8–15]. The snRNPs U1 and U2 appear to bind to the 5' splice site and the branch point [10,16,17], respectively, while U5 RNP has been suggested to recognize the 3'-end of an intron [17,18]. The exact function of each individual snRNP particle at single steps of the splicing process which proceeds in the so-called 50 S–60 S spliceosomes [29,30], remains to be elucidated, however.

An interesting feature of the snRNAs is their high content of modified nucleosides such as pseudouridines and methylated nucleosides (review [1]). The occurrence and location of methylated nucleosides in the respective snRNAs have been highly conserved during evolution, which suggests that they may play an important role for the structure and/or function of the snRNPs. For instance, with the single exception of U6 RNA, the U

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snRNAs from all eucaryotic cells so far examined possess a 5'-capped end with the highly methylated base m<sup>3</sup>G at its 5'-terminus. The snRNAs U2, U4 and U6 display one N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) residue each and this at equivalent positions when the RNA molecules from a variety of organisms are compared (see [35] for a compilation of U snRNA sequences).

A promising approach to study the role of modified nucleosides in RNA molecules is the use of nucleoside-specific antibodies (review [19]). We here demonstrate that antibodies specific for m<sup>6</sup>A which were raised in rabbits precipitate snRNPs U2 and U4/U6, indicating that the m<sup>6</sup>A residues of the respective snRNAs are located at the surface of the intact snRNPs and are not shielded by snRNP proteins. The immune precipitation assays further reveal an interaction in vitro between snRNPs U1 and U2.

## 2. MATERIALS AND METHODS

### 2.1. Cell growth and labeling conditions

HeLa cells (S3 strain) and mouse Ehrlich ascites tumor cells (EATC) were grown in suspension culture and labeled with [<sup>32</sup>P]orthophosphate essentially as in [3,21].

### 2.2. Preparation of the immunogen and immunization

m<sup>6</sup>A purchased from Sigma was conjugated to bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) via the periodate-oxidized nucleoside as described [22]. The immunogen m<sup>6</sup>A-BSA was characterized by spectral analysis [23]. The number of m<sup>6</sup>A residues bound per molecule of carrier protein was determined by measuring the ratio of absorbance at 260 and 280 nm, respectively [24], and was calculated to be 11 and 20 for BSA and KLH, respectively. Two randomly bred rabbits (R1280, R1281) were immunized at multiple dermal sites with 2 mg m<sup>6</sup>A-BSA in complete Freund's adjuvants on days 0, 28, 35 and 42. Rabbits were bled 1 week after final injection and after each monthly boost. Anti-m<sup>6</sup>A IgGs were purified from the rabbit sera as described [25].

### 2.3. ELISA

The specificity of anti-m<sup>6</sup>A IgGs was in-

vestigated by a microtiter ELISA. m<sup>6</sup>A-KLH was adsorbed to the wells of polystyrene multiwell microtiter plates (250 ng/well which accounted for about 60 pmol m<sup>6</sup>A) by incubation for 14 h at 4°C. After washing the wells three times with PBS, pH 7.4 (0.02 M potassium phosphate, pH 7.4, 0.15 M NaCl), the plates were saturated with 1% (w/v) ovalbumin (OVA) in PBS, pH 7.4, for 2 h at room temperature. After another three washes with PBS, pH 7.4, 50 µl of the respective inhibitor nucleoside diluted to the appropriate concentration in PBST (PBS containing 0.1% (w/v) Tween 20) was added, followed by 50 µl anti-m<sup>6</sup>A IgGs (0.1 µg/ml in PBST). The plates were incubated for 2 h at room temperature and washed three times with PBST. Bound m<sup>6</sup>A-specific antibodies were detected by incubating the plates with 80 µl of an anti-rabbit IgG-phosphatase conjugate (1:1000 dilution in PBST of the commercial stock solution, which was purchased from Sigma) for 2 h at room temperature. The plates were washed as described above, and 80 µl *p*-nitrophenyl phosphate (1 mg/ml in 0.1 M NaHCO<sub>3</sub>, pH 9.5, 0.002 M MgCl<sub>2</sub>) was added. After 2 h the absorption of the yellow-colored product *p*-nitrophenol was measured at 405 nm using a micro-ELISA-auto-reader (Dynatech).

### 2.4. Isolation of U snRNP particles and immunoprecipitation assays

snRNP particles U1, U2, U5 and U4/U6 were purified from nuclear extracts of both HeLa cells and EATC by immunoaffinity chromatography with rabbit anti-m<sup>3</sup>G IgG, essentially as in [20]. A fraction of snRNPs containing only the snRNPs U1, U2 and U5 was obtained by differential salt elution of anti-m<sup>3</sup>G antibody-bound snRNPs as outlined in detail previously [3]. Pure U1 RNPs were obtained from this latter snRNP mixture by chromatography on DEAE-Sephadex columns [26,27]. Immunoprecipitation assays and RNA analysis were performed as described in [21].

## 3. RESULTS

### 3.1. Characterization of antibodies against m<sup>6</sup>A

Antibodies against m<sup>6</sup>A were elicited in two randomly bred rabbits immunized with a conjugate of m<sup>6</sup>A and BSA. The specificity of anti-m<sup>6</sup>A antibodies from rabbit 1280 was investigated by

testing several nucleosides in a competitive microtiter ELISA for their ability to inhibit the reaction between purified anti- $m^6A$  IgG and  $m^6A$ -KLH. The high apparent affinity of the antibody for  $m^6A$  is largely determined by the presence of the  $N^6$ -methyl group on the purine ring. This is demonstrated by our findings that concentrations of the nonmethylated adenosine between  $10^4$ - and  $10^5$ -times higher were necessary to produce the amount of inhibition produced by  $m^6A$ , while 6-methylaminopurine ( $m^6Ade$ ) was almost as efficiently bound by anti- $m^6A$  IgG as  $m^6A$  (fig.1). The presence of an additional bulky methyl group at  $N^6$  ( $m^6,^6A$ ) reduces the affinity of the antibody by about one order of magnitude (fig.1). The antibody crossreacts to some extent with 1-methyladenosine ( $m^1A$ ) and also with 2'-*O*-methyladenosine (Am) (fig.1). The quantities of both nucleosides required to achieve a 50% inhibition of antibody binding were about 100–500-fold higher as compared to  $m^6A$  (fig.1). Low cross-

reactivity of rabbit anti- $m^6A$  antibodies with  $m^1A$  and Am has also been observed previously [23]. The structural basis for the reaction of anti- $m^6A$  IgG with Am in particular is not quite clear. The possibility that it is due to the presence of antibody populations which selectively recognize the ribose methyl group can be excluded, however, as 2'-*O*-methylcytidine is a very poor inhibitor (fig.1). It is important to note that the antibody did not react at all with methylated guanosines such as  $m_3G$  and  $m^2G$  (not shown), which are also present in U snRNAs.

### 3.2. Reaction of anti- $m^6A$ antibodies with isolated snRNP particles

Reactivity of anti- $m^6A$  antibodies towards the snRNPs U1, U2, U5 and U4/U6, which were purified by affinity chromatography with anti- $m_3G$  IgG, was investigated by immunoprecipitation assays. Most importantly the snRNPs U2 and U4/U6 were almost quantitatively precipitated by

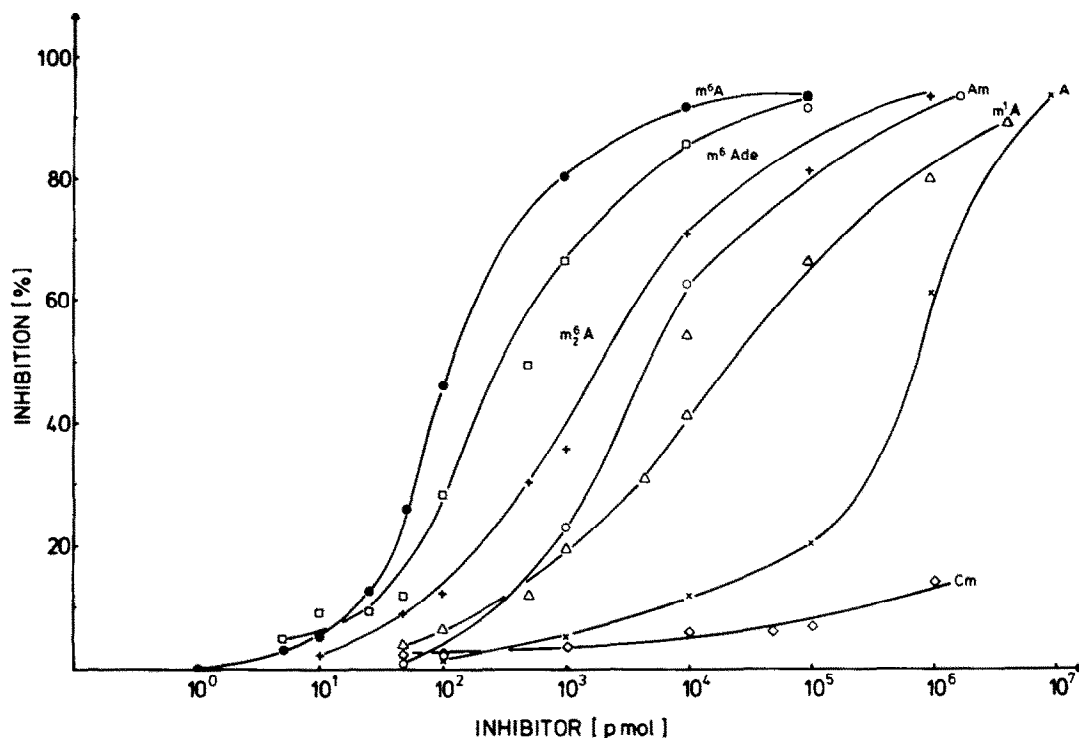


Fig.1. Competitive ELISA with anti- $m^6A$  antibodies and several competitor nucleosides. Enzyme-linked immunosorbent assays were performed as described in section 2. The decrease in binding of anti- $m^6A$  antibodies to polystyrene-adsorbed  $m^6A$ -KLH in the presence of increasing amounts of several competitor compounds as indicated in the figure is presented by inhibition curves.

anti-m<sup>6</sup>A IgG from the reaction mixtures (fig.2A, lane 2). Only minimal amounts of the two snRNP species were retained in the supernatant (fig.2B, lane 2). This demonstrates that the m<sup>6</sup>A residues of

the snRNAs U2, U4 and/or U6 are accessible for the antibodies in the intact snRNP particles and are not shielded by snRNP proteins. As the snRNAs U4 and U6 reside in one and the same

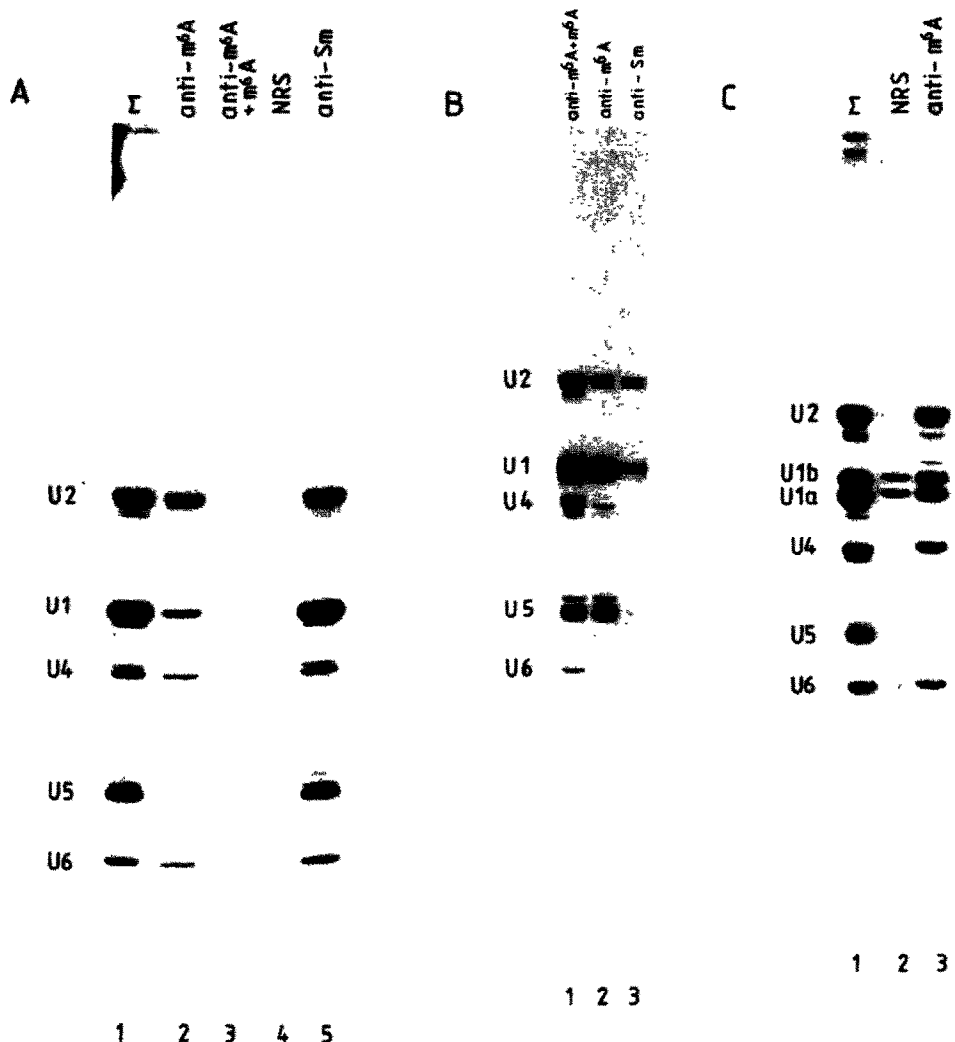


Fig.2. Reactivity of anti-m<sup>6</sup>A antibodies with purified snRNPs U1-U6. Total nucleoplasmic snRNPs U1-U6 purified from in vivo <sup>32</sup>P-labeled HeLa cells (A,B) and EATC (C) by immunoaffinity chromatography with anti-m<sup>3</sup>G antibodies were reacted with specific antibodies as indicated at the top of the lanes. Immune complexes were precipitated using protein A-Sepharose, and the extracted RNAs were analysed by electrophoresis on 10% polyacrylamide gels containing 7 M urea and TBE (0.1 M Tris-borate, pH 8.3, 0.002 M EDTA). (A) Autoradiogram of fractionated RNA species extracted from total nucleoplasmic snRNPs U1-U6 isolated from HeLa cells (lane 1) and from immunoprecipitates following reaction with anti-m<sup>6</sup>A IgGs (lane 2), anti-m<sup>6</sup>A IgGs in the presence of 10 mM M<sub>6</sub>A (lane 3), IgGs of a rabbit preimmune serum (lane 4) and human anti-Sm IgGs (lane 5). (B) Analysed RNAs purified from the respective supernatants of immunoprecipitates with anti-m<sup>6</sup>A IgGs in the presence of 10 mM m<sup>6</sup>A (lane 1), anti-m<sup>6</sup>A IgGs (lane 2) and anti-Sm IgGs (lane 3). (C) RNA species extracted from purified nucleoplasmic snRNPs U1-U6 of EATC (lane 1) and from immunoprecipitates following reaction with IgGs from a preimmune serum (lane 2) and anti-m<sup>6</sup>A IgGs (lane 3).

ribonucleoprotein particle we cannot distinguish whether only one or both of the  $m^6A$  residues react with the antibody. While no reaction at all was observed with snRNP U5, a small amount of the U1 snRNPs present in the reaction mixture (about 10%) was reproducibly precipitated by the anti- $m^6A$  IgG (fig.2A, lane 2). The latter finding is surprising insofar that U1 RNA does not contain an  $m^6A$  residue and could therefore indicate an association of U1 RNP with one of the other snRNP particles (see below). The precipitation of the snRNPs by anti- $m^6A$  IgG is specific since the rabbit pre-immune serum did not react (fig.2A, lane 4). Furthermore, reaction of anti- $m^6A$  with the snRNPs was completely abolished when the antibody binding sites were blocked with excess of nucleoside  $m^6A$  (fig.2A, lane 3). Our finding that anti-Sm autoantibodies precipitate all snRNPs U1–U6 almost quantitatively (fig.2A and B, lanes 5 and 3, respectively) demonstrates the integrity of the isolated snRNP particles.

It should be noted that the anti- $m^6A$  precipitation pattern described above is not only observed with snRNPs from HeLa cells. Qualitatively the same results were obtained with snRNPs from mouse Ehrlich ascites tumor cells (fig.2C).

### 3.3. Interaction between snRNPs U1 and U2 *in vitro*

Two possibilities may be considered to explain our finding that U1 RNP is co-precipitated by anti- $m^6A$  IgG (fig.2) despite the fact that U1 RNA itself does not contain an  $m^6A$  residue. The simplest explanation would be cross-reactivity of anti- $m^6A$  IgG with a nucleoside in U1 RNA other than  $m^6A$ . Alternatively, the co-precipitation of U1 RNP could indicate an association of U1 RNP with either snRNP U2 or U4/U6.

The first possibility was excluded by immunoprecipitation assays with isolated U1 snRNPs which were purified from the mixture of snRNPs U1–U6 by DEAE chromatography. Anti- $m^6A$  IgG did not precipitate U1 RNPs to levels above the background observed for the preimmune serum (fig.3A, lanes 4,5). As a control, nearly quantitative reaction with U1 RNPs was observed with anti- $m^3G$  and anti-Sm antibodies, which underscores the structural integrity of the U1 snRNP preparation (fig.3A, lanes 2,3). As the above results favor an interaction of U1 RNP with

one of the other snRNPs, this possibility was investigated in more detail. When a mixture of purified snRNPs U1, U2 and U5 only was reacted with anti- $m^6A$  IgG, U1 RNPs were still precipitated together with U2 RNPs (fig.3B, lane 2), and to the same extent as observed for the immunoprecipitations from total snRNPs U1–U6. Again no reaction at all was observed with U5

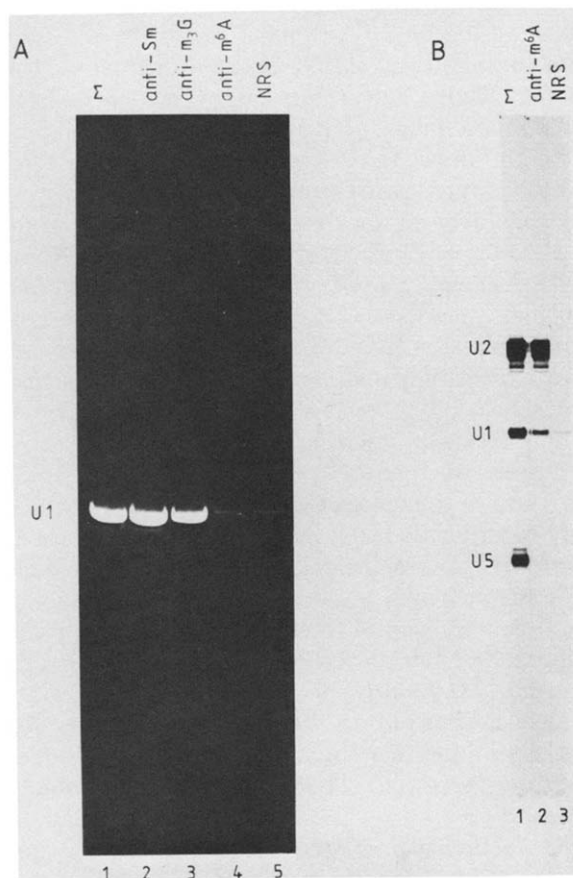


Fig.3. Reactivity of anti- $m^6A$  IgGs towards fractions of isolated snRNPs. Purified U1 RNPs (A) and a fraction comprising snRNPs U1, U2 and U5 (B) were used as a source of antigen for immunoprecipitation assays. The photograph in A shows the gel fractionated and ethidium bromide-stained RNAs extracted from pure U1 RNPs (lane 1) and immunoprecipitates obtained after reaction with anti-Sm IgGs (lane 2), anti- $m^3G$  IgGs (lane 3), anti- $m^6A$  IgGs (lane 4) and IgGs from a rabbit preimmune serum (lane 5). The autoradiograph shown in B represents the RNA species purified from the fraction encompassing U1, U2 and U5 RNPs (lane 1) and immunoprecipitates using anti- $m^6A$  IgGs (lane 2) and IgGs from the rabbit preimmune serum (lane 3).

RNPs (fig.3B, lane 2). These data indicate an interaction in vitro between isolated snRNPs U1 and U2.

#### 4. DISCUSSION

Our results demonstrate that the m<sup>6</sup>A residues of the snRNPs U2 and U4/U6 are accessible for anti-m<sup>6</sup>A antibodies in the isolated particles. This indicates that the m<sup>6</sup>A residues cannot be necessary for the binding of snRNP proteins themselves. It is more likely, therefore, that these methylated adenosines might participate in creating a binding site for other factors with which the respective snRNPs interact in the cell nucleus. In this respect it should be noted that in hnRNP-snRNP complexes the 5'-terminal 32 nucleotides of U2 RNA, which include the U2 m<sup>6</sup>A residue, are protected against micrococcal nuclease digestion, whereas they are degraded when isolated snRNPs are treated with the nuclease [28], indicating that this area is involved in complex formation with other components of hnRNPs. It will be of interest to investigate whether in 50–60 S spliceosomes, which are known to contain U2 RNPs [17,31] and which are functionally better defined than the mixture of hnRNPs, the residue of U2 RNA will be accessible for the anti-m<sup>6</sup>A antibody. The antibody should also be very helpful to elucidate further the function of the U4/U6 RNP particle in processing of pre-mRNAs in vitro.

In addition to its potential applications for studies on the functional role of the m<sup>6</sup>A residues of snRNPs U2 and U4/U6, the anti-m<sup>6</sup>A antibody can also be used as a powerful tool for the isolation and fractionation of the respective snRNPs by immunoaffinity chromatography [27].

Another interesting result which emerged from the present study is the finding that, although U1 RNA does not possess an m<sup>6</sup>A residue, U1 RNPs were co-precipitated to some extent when isolated snRNPs were reacted with anti-m<sup>6</sup>A antibodies (fig.2). Since purified U1 RNPs themselves were not reactive with anti-m<sup>6</sup>A IgG and, furthermore, U1 RNPs were co-precipitated with U2 RNPs even in the absence of snRNPs U4/U6 (fig.3), our results favor an interaction between snRNPs U1 and U2 in vitro. The above findings were surprising insofar that we have so far never observed the reciprocal effect, viz. co-precipitation of U2 with

U1 RNPs when purified endogenously labeled snRNPs had been reacted with anti-RNP autoantibodies or with monoclonal antibodies against U1 RNP-specific proteins. Along the same lines autoantibodies directed against the U2-specific protein A' precipitated only U2 RNPs [32,33]. This indicates that the binding of antibodies to the proteins somehow interferes with the interaction between U1 and U2 RNPs, while this appears not to be the case for the binding site of anti-m<sup>6</sup>A IgG on the U2 RNP particle.

Our data are in good agreement with a recent report by Mattaij et al. [34], who were able to co-precipitate a fraction of the other snRNP species from extracts of *Xenopus* oocytes with monospecific antibodies against U1- or U2-specific proteins in each case. The interaction between the two snRNPs could only be detected when either U1 or U2 RNA was labeled with very high specific activity by amplified transcription of the respective snRNA genes microinjected in *X. laevis* oocytes [34].

The snRNPs U1 and U2 may associate in vitro by protein-protein, protein-RNA and RNA-RNA interactions. In this respect it is interesting to note that snRNAs U1 and U2 show some sequence complementarity, raising the possibility that intermolecular base pairing might be one factor of the interaction. In support of this possibility we have recently been able to crosslink the snRNAs U1 and U2 upon irradiation of purified snRNPs U1–U6 with UV light in the presence of the double-strand-specific psoralen reagent (Wenz, J., Rinke, J. and R.L., unpublished).

The question as to whether the observed interaction between isolated snRNPs U1 and U2 in vitro, though specific, is functionally meaningful cannot be answered from the present investigations. Given the fact that snRNPs U1 and U2 recognize the 5' splice site and the branch point of the intron, respectively, together with the finding that both RNPs are assembled into the 50–60 S active splicing complexes (U1 RNP at least transiently) [10,16,17,29–31], it is tempting to suggest that by interaction with each other in the spliceosomes the two RNPs might help to bring the intron's 5'-end and the branch point into close proximity. The possibility of crosslinking the snRNPs U1 and U2 together in the spliceosomes will be a crucial test for such a model.

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