"Preparation of N^2,N^2,7-tri-methylguanosine affinity columns." Espuny, R., Bahia, D., Cicarelli, M.B., Codony, C., Khaouja, A., Aviñó, A.M., Eritja, R., Bach-Elias, M. Nucleosides & Nucleotides 18(1), 125-136 (1999). doi: 10.1080/07328319908045599

PREPARATION OF N^2, $N^{2,7}$-TRIMETHYLGUANOSINE AFFINITY COLUMNS

Ruth Espuny, Diana Bahia, Regina Maria Barretto Cicarelli[#], Carles Codony, Amina Khaouja. Anna Maria Aviñó[ÿ], Ramon Eritja[À] and Montse Bach-Elias.[*,1]

Centro de Investigación y Desarrollo. CSIC. Jordi Girona Salgado 18-26, 08034 Barcelona. Spain. Present addresses: [#]UNESP. Faculdade de Ciencias Farmaceuticas. Araraquara, SP, Brazil. [ÿ]Dpt de Genetica Molecular, IRO (Institut de recerca oncologica), Autovia de Castelldefels km 2.7, Hospitalet de Llobregat, 08907 Barcelona. [À]EMBL. Meyerhofstrasse 1. Postfach 10.2209. Heidelberg, Germany.

[1]Phone: 34-3-400 61 34; Fax: 34-3-2045904; Email: mbebmc@cid.csic.es

Abstract: 2,2,7-trimethylguanosine (TMG) binding proteins from human cells were purified through TMG-affinity columns. TMG synthesis was improved and the TMG obtained was shown to be similar to the TMG in the *5'* cap of the UsnRNAs. The eluates obtained with TMG-affinity chromatographies ,were very different from those isolated with m^7G-affinity columns, thus suggesting that specific TMG-binding proteins were obtained. The fraction may be enriched with factors associated with import and/or hypermethylation of UsnRNPs.

The trimethylguanosine (TMG) cap is the hallmark of the UsnRNAs (U small nuclear RNAs family) (1). Major UsnRNPs (U small nuclear ribonucleoproteins) U1, U2, U4/U6.U5 are essential spliceosomal factors. UsnRNPs are complexes consisting of UsnRNAs and a very characteristic set of polypeptides (2). Spliceosomal UsnRNPs contain common and specific polypeptides. The common polypeptides present in all the spliceosomal factors of human cells are B', B, D1, D2. D3, E, F and G. The common polypeptides not only form a common structure present in all the spliceosomal UsnRNPs, the so-called UsnRNPs core (2), but they also are important signals for the biogenesis of the UsnRNPs (3). With the sole exception of U6snRNA, which is capped with MeGTP, all the major UsnRNAs contain the TMG cap in human cells. Most UsnRNAs are synthesized by the RNA polymerase II and they are later modified in the nucleus with the addition of m^7G cap (3). An active mechanism that recognizes the m^7G cap exports these UsnRNAs to the cytoplasm, where biogenesis of the UsnRNPs takes place. In the cytoplasm, core (common) polypeptides are concentrated in three RNA-free complexes (1): slow sedimenting cores, containing B polypeptide sedimenting between 2-6S; middle sedimenting cores containing [D1, D2, (E, F, and G)$_2$] sedimenting at 6S, and high sedimenting cores containing B, D3 and 69 kDa polypeptides. When spliceosomal UsnRNAs are exported to the cytoplasm, these RNA-free complexes associate with the UsnRNAs at the Sm-binding site [A(U)$_n$G] to form the core particle that contains both the UsnRNAs and the core

polypeptides B', B, D1, D2, D3, E, F and G (3). The *5'* end of the UsnRNAs is then hypermethylated and the m^7G cap changes to the m^3G cap. The core polypeptides and the m^3G cap form a bipartite nucleophilic signal that is recognized by a putative receptor (TMG-receptor) which mediates the import of the spliceosomal core particles from the cytoplasm to the nucleus (3). Recently, it has been described that both GTP-Ran and β importin might be some of the subunits of the TMG-receptor that imports TMG-capped UsnRNAs from the cytoplasm to the nucleus (4, 5). As β-importin and GTP-Ran alone are not enough to import UsnRNPs, it has been suggested that other TMG-receptor subunits or TMG-adapters are necessary to reconstitute the complete receptor (5). This work is an intent to biochemically purify the TMG binding proteins with the purpose of further studying the presence of TMG-adapters.

The TMG nucleoside here obtained is similar to the *5'* end cap of the UsnRNAs. Our objective was to purify TMG binding proteins by a novel biochemical approach in order to better characterize factors that participate in the biogenesis of UsnRNPs. We prepared a TMG-affinity column and developed an easy purification method to prepare TMG binding proteins from cytoplasm. First, we had to synthesize high amounts of the non-commercial TMG nucleoside, and to demonstrate that the obtained TMG nucleoside was essentially similar to the TMG cap of the UsnRNAs. TMG was synthesized from $m^{2,2}G$ by methylation of position 7 of the ring with dimethyl sulfate. TMG and $m^{2,2}G$ were easily separated and purified with a SP-Sephadex ion-exchange column. The procedure rendered a yield of 50% of the initial compound $m^{2,2}G$, and NMR data confirmed that the substance obtained was TMG (data not shown). To demonstrate that the TMG here synthesized was similar to the TMG found at the 5' cap of the UsnRNPs, TMG or $m^{2,2}G$ nucleosides conjugated to the carrier KLH were used to immunize rabbits and mice. Mice were immunized with $m^{2,2}G$-KLH conjugates for two reasons: 1) this nucleoside was used as immunization control, using a nucleoside different to TMG and 2) to study whether anti-$m^{2,2}G$ antibodies could recognize TMG. Four mice, M1, M2, M3 and M4, were immunized with $m^{2,2}G$-KLH conjugate. Sera from all four mice showed low sera titers against TMG-BSA (not shown). Immunoprecipitation assays using nuclear extracts were performed with the sera from the four mice. To perform immunoprecipitation assays we used both mice and human nuclear extracts, as the TMG cap of the UsnRNPs is conserved during evolution. from yeast to human cells (1). Fig. 1A shows immunoprecipitation with sera from mice M1 and M2 (lanes 1 and 3, respectively) and a parallel inhibition assay by adding 10 mM TMG to the immunoprecipitation sample (lanes 2 and 4, respectively). Lanes 1-4 in Fig 1A show that the mice sera did not recognize the TMG cap of the UsnRNPs from mice nuclear extracts. M3 and M4 mice rendered identical results, and pre-immune sera from Ml-M4 showed background levels similar to those with the immunized Ml-M4 sera (not shown). M1 and M2 mice were re-immunized once with TMG-KLH conjugate, whereas that M3 and M4 were re-immunized once with $m^{2,2}G$-KLH conjugate, and blood was obtained from the four animals 7 days after the reimmunization. Sera from the two latter mice (M3 and M4) after re-immunization did not immunoprecipitate UsnRNPs from mouse nuclear extract (not shown), but surprisingly, one injection of TMG-KLH to mice M1 and M2 induced the synthesis of anti-TMG antibodies that recognized the cap of the UsnRNPs (see Fig. 1A. lanes *5* and 7, respectively). The reaction

of these antibodies was specific, since it could be inhibited with free 10 mM TMG (Fig. 1A lanes 6 and 8, respectively). These results indicated that the methylation of the position of the guanosine ling is essential to obtain a good immune response to the TMG cap of the UsnRNPs. As the antibodies obtained against the $m^{2,2}G$ did not cross-react easily with TMG, we suggest that $m^{2,2}G$ and TMG nucleosides have important structural differences that are finely discriminated by the immune system of the animals. As the final goal of this study is to synthesize a column capable to purify TMG-binding proteins, the immunization results with $m^{2,2}G$-conjugate indicated that TMG could not be replaced for $m^{2,2}G$, and that the more successful results should be obtained with TMG-affinity columns than with $m^{2,2}G$-affinity columns.

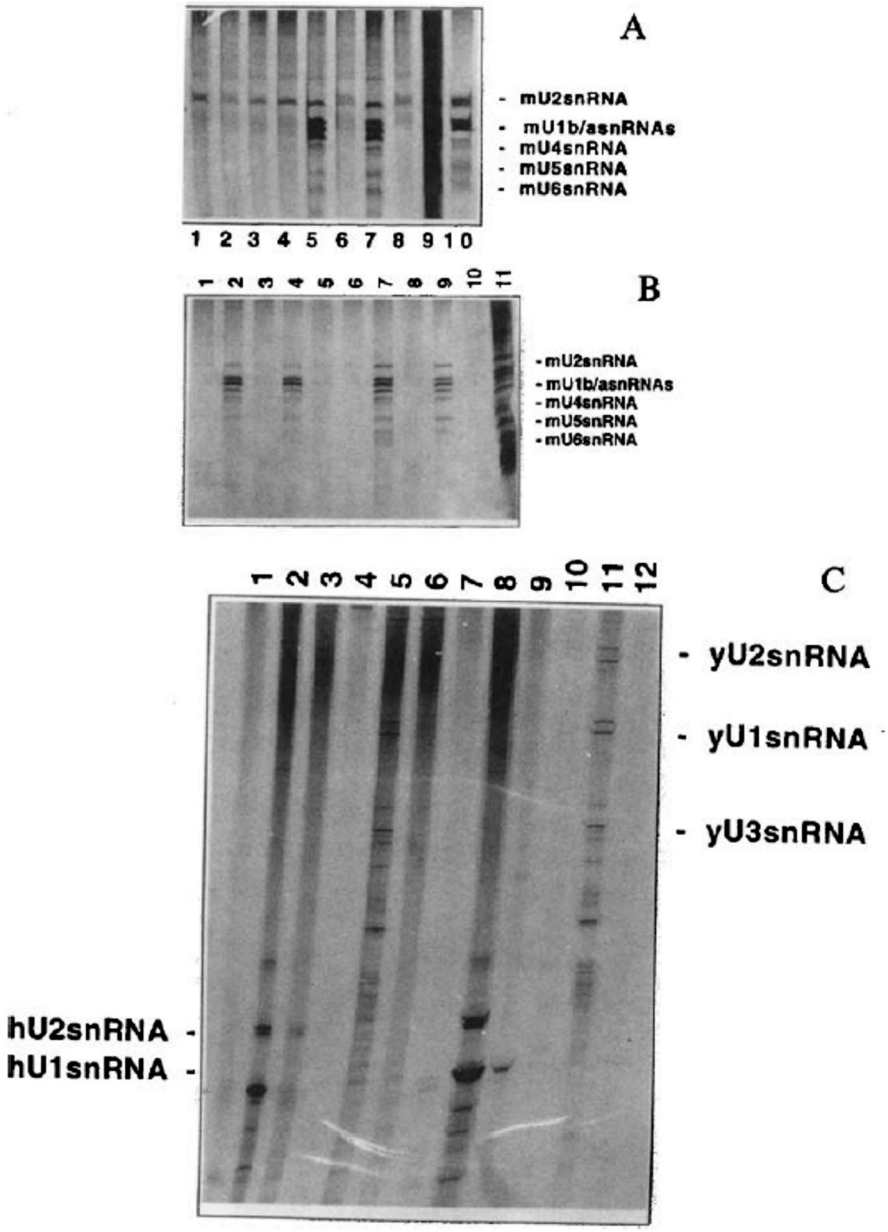

FIG. 1 : Characterization of the synthesized TMG product. (A) Immunoprecipitation assays with sera from mice M1 and M2 immunized with $m^{2,2}G$-KLH conjugate (lanes 1- 4) and sera of mice M1 and M2 re-immunized with the TMG-KLH conjugate (5-8). Lanes 2,4, 6 and 8 were

competed with 10 mM TMG nucleoside, and lanes 1,3,5 and 7 were not subjected to such competition. M1 serum was used in lanes 1.2.5 and 6; M2 serum was used in lanes 3, 4, 7 and 8. The nuclear extract used was that obtained from EATC mouse cells (for lanes 1-9) and human nuclear extract for lane 10. Lane 10 was immunoprecipitated with the same sera that was used in lane 7 (from re-immunized M2). "m" indicates mouse. Lane 9 is the same amount of EATC nuclear extract used for each immunoprecipitation. The mobility of the human UsnRNAs is similar to that of the mUsnRNAs and it is therefore not shown in the figure. (B) Immunoprecipitation assays with pre-immune sera of M5 and M7 mice and sera from the M5-M8 mice immunized with TMG-KLH conjugate (lanes 1-10); lane 11 is the same nuclear extract used in each of these immunoprecipitations. The nuclear extract used was obtained from EATC mouse cells. Lane 1 and lane 6 were M5 and M7 pre-immune sera, respectively. Lanes 2 and 3, M5 serum; lanes 4 and 5, M6 serum; lanes 7 and 8, M7 serum; lanes 9 and 10, M8 serum. Lanes 3, 5, 8 and 10 were immunoprecipitations competed with 10 mM TMG and lanes 2, 4, 7 and 9 were immunoprecipitations without TMG. "m" indicates mouse. (C) Immunoprecipitation assays with polyclonal rabbit sera from rabbit R331 (lanes 1-6) and rabbit R327 (lanes 7-12). Pre-immune sera were used in lanes 1, 4, 7 and 10. Lanes 2, 5*,* 8 and 11 were obtained from the rabbit immunized with TMG-KLH conjugate and lanes 3, 6, 9 and 12 were experiments identical to those described in lanes 2, *5,* 8 and 11, although carried out in presence of 10 mM TMG. In lanes 1-3 and 7-9, a HeLa nuclear extract was used as the source of antigen; and in lanes 4-6 and 10-12, total yeast extract was used as the source of antigen. "h" means human and "y" means yeast. Fig. 1A and 1B are 10% polyacrylamide /6 M urea gels and Fig. 1C is a 6% polyacrylamide/6 M urea gel. All the gels were silver stained.

We modified the synthetic procedure of the nucleoside conjugates that appeared in previous reports (6). Mainly, we observed that the conjugation of the TMG to the carrier protein improved in higher hydrophobic conditions (DMSO and ethanol) and increasing the temperature of oxidation and conjugation to 37 ºC*.* As this conjugation step would be the chosen method to covalently bind the nucleoside to a Sepharose matrix, it was important before preparing the TMG-affinity column to study whether the modification of the conjugation method affected the integrity of the TMG nucleoside. To better study this question, four mice (M5, M6, M7 and M8) and two rabbits were immunized with the improved TMG-KLH conjugate. Fig. 1B shows that all four mice showed immune response to the TMG cap of the mouse UsnRNPs, indicating that sera from the four animals immunoprecipitated UsnRNPs (see Fig. 1B lanes 2, 4, 7 and 9, respectively), and that the immune recognition was inhibited in presence of 10 mM TMG (Fig. 1B, lanes 3, 5, 8 and 10, respectively). Pre-immune sera from M5-M8 mice showed background levels similar to those in the lanes competed with TMG (Fig. 1B shows the pre-immune sera from M5 and M7 mice, lanes 1 and 6 respectively). Similar results were obtained with the sera from the two rabbits (R331 and R327) immunized with the improved TMG-KLH conjugate. Fig. 1C shows that sera from both rabbits recognized the TMG cap of the human UsnRNPs (lane 2 for R331 and lane 8 for R327), whereas the corresponding preimmune sera did not (lane 1 for R331 and lane 7 for R327). The UsnRNPs immunoprecipitation was inhibited in presence of 10

mM TMG (lane 3 for R331 and lane 9 for R327). Similar results were obtained when UsnRNPs from yeast were immunoprecipitated with both sera (Fig. 1C, lanes 4-6 for R331, and immunoprecipitation was inhibited in presence of 10 mM TMG (lane 3 for R331 and lane 9 for R327). Similar results were obtained when UsnRNPs from yeast were immunoprecipitated with both sera (Fig. 1C, lanes 4-6 for R331, and lanes 10-12 for R327). All these results indicated that the improved method of conjugation did not affect the integrity of the TMG nucleoside bound to the protein carrier; thus, an anti-TMG cap antibody was easily obtained in the six animals immunized with the TMG-conjugate. These results indicated that the improved method of conjugation of the TMG to amine groups could be the method of choice to covalently bind the nucleoside TMG to amino-derivatived Sepharose

TMG nucleoside was covalently bound to EAH-Sepharose 4 B using the improved conjugation method here described, i.e., ribose oxidation in presence of ethanol and DMSO at 37 ºC and conjugation to the amino-deiivatived column at 37 ºC. In order to reduce binding of proteins that are recognizing guanosine or m^7G and to reduce the presence of Sepharose-binding proteins (non-specific binding) in the fractions eluted from the TMG-affinity column, a pre-column with m^7G covalently bound to Sepharose was prepared in a similar manner.

Both columns had the same bed (around 4 ml) and were connected to each other in such a way that the flow-through of the m^7G-affinity column was immediately applied to the second TMG-affinity column. The m^7G-column has two finalities a) to deplete G- and m^7G-binding proteins and b) to deplete proteins that could be interacting with the ribose moiety of the guanosine. The cytoplasmic extract from HeLa cells was different to the previously described S100 splicing fraction (7), as shown in the Material and Methods. After the sequential loading of the cytoplasmic extract, first to m^7G-affinity column and second to the TMG-affinity column, each column was disconnected and separately washed. The proteins bound to the m^7G-affinity column or to the TMG-affinity column were specifically eluted by competition with free m^7G or TMG, respectively, and collected in four different fractions (1 ml) that were analyzed in 15% polyacrylamide/ 0.1% SDS gels. The elution with the free nucleoside rendered native proteins. Fig. 2 shows the proteins eluted from both columns: from the TMG-affinity column (lanes 2-5 corresponding to the first-to-last eluted fraction) and from the m^7G-affinity column (lanes 6-9, corresponding to the first-to-last eluted fraction). The comparison of lanes 2-5 with lanes 6-9 in Fig. 2 revealed that the pattern of m^7G-binding proteins is clearly different from that of the TMG-binding proteins. The initial fractions eluted from the m^7G-affinity column (lanes 6-9) could contain the previously described CBP20 and CBP80 (8), but they were not further characterized as these m^7G-binding proteins were not the purpose of this work. TMG-binding proteins showed a complex pattern and we marked 6 polypeptides (marked with a dark dot in Fig. 2 lanes 3 and 4) as the most prominent in the TMG eluate (Fig. 2, lanes 2-5). Their predicted molecular weights were, from top to bottom: 97 kDa; 52.6 kDa; 50.2 kDa; 33 kDa; 25.3 kDa; 19.1 kDa and 17.9 kDa. These six polypeptides were observed in the four fractions eluted from the TMG-affinity column (compare lanes 2-5) and they are named as TMG-binding proteins. The protein pattern is quite different both from that of the m^7G-binding proteins (compare lanes 2-5 with lanes 6-9 in Fig. 2), and from the protein pattern of the initial cytoplasmic fraction (not shown).

In summary, we assume that the proteins described in Fig. 2, lanes 2-5 are TMG-binding proteins (specifically eluted from a TMG-affinity column) with a specific and native elution with TMG free nucleoside; besides, and these proteins are not eluted from the TMG affinity columns with m^7G free nucleoside. Our preliminary results with Western blots indicated that β-importin is present in TMG-binding fractions (D. Bahia, personal communication). This preliminary result is very interesting, since β-importin seems to be necessary for UsnRNPs import and it could be one of the subunits of the import complex (5). However, our results did not indicate whether β-importin binds directly to the TMG cap; therefore, β-importin could be present in TMG eluates by its association with a real TMG binding protein. Finally, we intend to use this fraction to study the different activities related with the TMG cap, such as UsnRNPs import, hypermethylation of the UsnRNA cap and other possible new activities not yet described. We are currently carrying out experiments to characterize these activities.

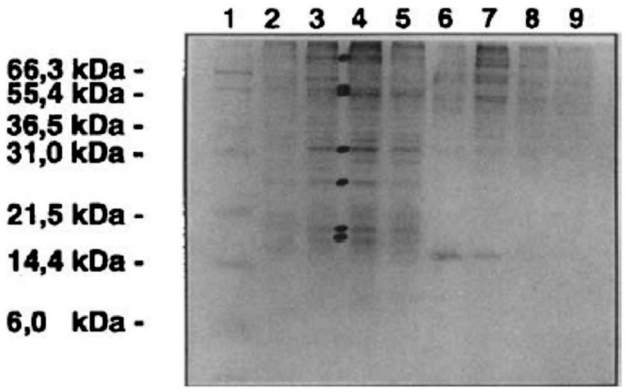

FIG. 2*:* Purification of TMG-binding proteins. Lane 1, molecular weight markers (M) described to the left of the figure. A m^7G-affinity column and a TMG-affinity column were sequentially loaded with cytoplasmic extract. Then, each column was separately washed and eluted respectively with free m^7G nucleoside and free TMG nucleoside, respectively. Lanes 2-5 were protein fractions eluted from the TMG-affinity column by competition with free TMG nucleoside; lanes 6-9 were eluted from m^7G-affinity column with free m^7G nucleoside. Lanes *2-5* represent the four fractions from the elution profile, 2 being the first and 5 the last. Lanes 6-9 represent the four fractions from the elution profile, 6 being the first and 9 the last. The most prominent proteins in TMG eluate (lane 3 and 4) were marked with a dark dot, and the molecular weight of the marked proteins is, from top to bottom: 97 kDa; 52.6 kDa; 50.2 kDa; 33 kDa; 25.3 kDa; 19.1 kDa and 17.9 kDa. The proteins were run in a 15% polyacrilamide/0.l% SDS gel and stained with Coomassie blue.

EXPERIMENTAL SECTION

Abbreviations used: TMG, N^2, $N^{2,7}$-trimethylguanosine; TEAB, Et_3NHC0_3; EATC, Ehrlich ascites tumor cells; DMS, dimethyl sulfate; DMSO, dimethyl sulfoxide.

Synthesis of N^2, $N^{2,7}$-trimethylguanosine (TMG, or m_3G or $m^{2,2,7}G$ or 2,2,7-trimethylguanosine).

The TMG precursor was N^2, N^2-dimethylguanosine ($m^{2,2}G$), which was synthesized as previously described 9. The synthesis of TMG from $m^{2,2}G$ was performed essentially as previously described with some modifications lo. In brief: 80 mg of $m^{2,2}G$ were dissolved in 1726 ml of dimethylacetamide (99%) and 54 ml of dimethyl sulfate (DMS). The methylation reaction was allowed to proceed for 12 h at room temperature. Then, 2 ml of TEAB pH 8.3 (Et_3NHC0_3) was added and loaded to a SP Sephadex G-25 (Pharmacia), at 4 ºC (the bed column was 29 ml, and the column size had a diameter of 2.5 cm and a height of 6 cm) equilibrated with 5 mM TEAB pH 8.3. The column was washed with 5 mM TEAB pH 8.3 until the flow-through, which contained the $m^{2,2}G$ that had not converted to TMG, did not show any significant absorbance at 260 nm. TMG was eluted with 50 mM TEAB pH 8.3 and was subjected to 3-4 cycles of lyophilization and subsequent dissolution in water, in order to remove the TEAB buffer. Normally the synthesis reaction rendered a yield of 50% of the initial compound $m^{2,2}G$. NMR data confirmed that the compound obtained was m_3G (data not shown).

Synthesis of the TMG-HSA (human serum albumin) and TMG-KLH (keyhole limpet hernocyanin).

The method previously reported by Erlanger and Beiser (6) was modified. Four mg of TMG was dissolved in 106 ml of ethanol and 69 ml of dimethyl sulfoxide (DMSO). Then 4 mg of $NaIO_4$ in 200 ml of H20 was added to the initial TMG mixture, and the oxidation reaction was allowed to proceed for 90 minutes at 37 ºC in the dark. Then, 14 ml of 1 M ethylene glycol was added and the mixture was allowed to further react for 10 min at 37 O C in the dark. Four mg of the carrier protein (HSA or KLH) dissolved in 1 ml of 0.4 M $NaHC0_3$ pH 9.1 was added drop by drop. The mixture was adjusted to pH 9.1 with 1 M $NaHC0_3$ pH 9.1, and allowed to react for 2 h at room temperature in the dark. Then, 20 mg (powder) of *tert*-butylamineborane was slowly added and allowed to react for 45 min at 4°C. The conjugation was centrifuged for 10 min at 12.000 g and then desalted with a PD-10 column (Pharmacia). $m^{2,2}G$-HSA and $m^{2,2}G$-KLH conjugates were prepared in a similar way to that described for the TMG conjugate.

Nuclear and cytoplasmic extracts from human cells.

Nuclear extracts from HeLa cells, (cells provided by 4C Company, Computer Cell Culture Center S.A., Mons, Belgium) were obtained following the method of Dignam et al. (7). Cytoplasmic extracts were also obtained by the method of Dignam et al. (7): after hypotonic treatment of the HeLa cells in buffer A, 10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and disruption in a homogenizer (Dounce B) the broken cells were centrifuged for 10 min at 100 *g*. The supernatant (S1) was then carefully removed with a pipette. Then, the nuclear pellet was centrifuged again at 25,000 *g* for 20 min, this second supernatant (S2) was carefully removed from the pellet, and S2 was mixed with S1. The cytoplasmic extract used in this work corresponded to the mixture of S1 and S2 supernatants. The cytoplasmic extract was stored at -80 ºC until needed. The nuclear extracts from EATC (Ehrlich ascites tumor cells) mouse cells were prepared as previously described (11).

Total yeast extracts.

Total yeast extract was obtained from strain EJ101 as described elsewhere (12).

Immunization of mice and rabbits with TMG-KLH or m2P2G-KLH conjugates.

Two rabbits, R327 and R331, were subcutaneously immunized with 2 mg of TMG-KLH conjugate (in 1 ml PBS) mixed with 1 ml of either complete or incomplete Freund adjuvant. Rabbits were immunized with complete Freund adjuvant on day 0, and with incomplete Freund adjuvant on days 21, 35 and 42. On day 49 blood was taken from the animal's ears. Sera was obtained by centrifugation of the blood and their titer assayed by ELISA, which wells contained the TMG-HSA conjugate. Four mice were immunized with $m^{2,2}G$-KLH conjugate (M1-M4) and four more mice with TMG-KLH conjugate (M5-M8) as following: 400 ml of nucleoside-conjugate (the final quantity injected was 400 mg) in PBS mixed with complete Freund adjuvant (1:1, v/v) was intraperitoneally injected to each mice on day 0, and the animals were then immunized again on days 14 and 21. On day 28 blood was taken from the tail vein. Sera was obtained by centrifugation of the blood and their titer assayed by ELISA, which wells contained the nucleoside-HAS conjugate. The four animals injected with the $m^{2,2}G$-KLH conjugate were injected once more on day 35; two with TMG-KLH conjugate (M1 and M2) and the other two with $m^{2,2}G$-KLH conjugate (M3 and M4). On day 42, blood was taken from these four animals. All the experiments described here were performed with mouse sera from 28 days, with the exception of the mice M1-M4, that were re-immunized and whose sera from 42 days was also assayed.

Preparation of TMG-Sepharose and m^7G-Sepharose affinity columns.

Two affinity columns were prepared: one that contained the nucleoside m^7G covalently bound to Sepharose and a second one with the nucleoside TMG covalently bound to Sepharose. Both columns were obtained using the same procedure: 250 mg of each nucleoside was dissolved in 6.6 ml of ethanol and 4.3 ml of DMSO. Then, 250 mg of $NaIO_4$ in 12.5 ml of H_20 was added to the TMG initial mixture, and the oxidation reaction was allowed to proceed for 90 min at 37 ºC in the dark. Then, 875 ml of 1 M ethylene glycol was added and the mixture was allowed to react for another 10 min at 37 ºC in the dark. NaCl (0.49 g) and $NaHC0_3$ (0.28 g) were next added to the nucleoside oxidation reaction. Meanwhile, 3 ml EAH-Sepharose 4 B (Pharmacia) were washed four times with 50 ml of 0.5 M NaCl; five times with 50 ml of 0.5 M NaCl/ 0.2 M $NaHCO_3$ pH 9.1 and finally resuspended in 3 ml of 0.5 M NaCl/ 0.2 M $NaHC0_3$ pH 9.1. The oxidized nucleoside solution was mixed (drop by drop) with the resuspended resin, and the binding was allowed to proceed by rotation for 3 h at room temperature in the dark. Then, 1.25 g (powder) of *tert*-butylamineborane was slowly added and allowed to react by rotation for 1h at 4°C. The resin was washed with 50 ml of 0.5 M NaCl and 50 ml of H_20 and the column was equilibrated with TMG-buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 150 mM NaCl, 0.5 mM DTT, 0.5 mM PMSF and 5% glycerol). Both resins were mounted inside two 2.5 cm (diameter) columns. The TMG-Sepharose and m^7G-Sepharose affinity columns were stored in presence of 0.02% NaN_3 / TMG buffer.

Purification of TMG binding proteins through m^7G-Sepharose and TMG-Sepharose affinity columns.

The m^7G-Sepharose column was connected to the TMG-Sepharose column. Before loading, cytoplasmic extract (250 ml) was dialyzed against TMG-buffer. The dialyzed fraction was first loaded on the m^7G-Sepharose column, and the flow through of this column was loaded on the TMG-column overnight. Then the two columns were disconnected one from the other and each column was washed separately with 10 bed-volumes of TMG-buffer. The m^7G-Sepharose column was eluted with 10 mM m^7G in the TMG-buffer and TMG-column was eluted with 10 mM m_3G in TMG-buffer; the latter fraction contained the TMG binding proteins.

Immunoprecipitation of nuclear extracts.

Immunoprecipitation of the UsnRNPs from nuclear extracts with anti-TMG was essentially performed as previously described (13). Protein A or Protein G was used with sera from rabbit or mice, respectively.

Protein and RNA gels. Protein and RNA gels staining.

Protein and RNA gels were performed as previously described (11). Protein gels were stained with Coomassie Blue and RNA gels were stained with silver, as described (11).

ACKNOWLEDGMENTS

This work was supported by PGC grant no PB92-0004, and partially by a grant from the Marató TV3 and Asociación Española Contra el Cáncer. We also thank the Alexander von Humboldt Foundation for providing initial help with several equipment. We thank J.M. Barrios. X. Jimenez and R. Perez for technical assistance, and all members of the M. Bach-Elias group for comments about the manuscript. C. Codony was the recipient of a fellowship from PGC; R.B. Cicarelli was the recipient from a fellowship from CAPES and Spanish MEC; A. Khaouja was the recipient of a fellowship from the Moroccan government and Diana Bahia was recipient of a CNPq fellow. We also thank Martí Cullell for revising the manuscript.

REFERENCES

1. Birnstiel, M.L. (ed.) *Structure and function of major and minor small nuclear ribonucleoprotein particles.* Springer-Verlag Press, Berlin **1988.**
2. Luhrmann. R., Kastner, B., and Bach, M. *Biochim. Biophys. Acta,* **1990,** *1087,* 265.
3. Zieve, G. W. and Sauterer, R. A. *Crit. Rev. Biochem. Mol. Biol.,* **1990,** *25,* 1.
4. Palacios, I., Weis. K., Klebe, C., Mattaj, I. W., and Dingwall, C. *J. of Cell Biol.,* **1996,** *133,* 485.
5. Palacios, I., Hetzer, M., Adam, S. A., and Mattaj, I. W. *EMBO J.*, **1997,** *16*, 6783.
6. Erlanger. B. F., and Beiser, S. M. *Proc. Natl. Acad. Sci. USA,* **1964,** *52,* 68.
7. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. *Nucl. Acids Res.,* **1983,** *11,* 1475.

8. Visa, N., Izaurralde, E., Ferreira, J., Daneholt, B., and Mattaj, I.W. *J. of Cell Biol.,* **1996,** *133*, *5.*
9. Aviñó, A. M., Mayordomo. A., Espuny, R., Bach, M., and Eritja, R. *Nucleos. Nucleot.,* **1995,** *14*, 1613.
10. Saponara, A. G., and Enger, M. D. *Nature,* **1969,** *223,* 1365.
11. Bach**,** M., Krol, A., and Luhrmann, R. *Nucl. Acids Res.,* **1990,** *18*, 449.
12. Fabrizio, P., Esser, S., Kastner, B., and Luhrmann, R. *Science,* **1994,** 264, 261.
13. Steitz, J. *Methods Enzymol.,* **1989,** *180*, 468.