snRNPs from Mouse Teratocarcinoma Cells Reacting with Polyclonal anti-Sm and anti-m₃^{2,2,7}G Antibodies and Biochemical Characterization of the snRNPs

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(Received 16 February 1987/Accepted 15 July 1987)

ABSTRACT. Highly purified Sm small nuclear ribonucleoproteins (snRNPs) in the nuclear extract of embryoid bodies and F9 cell extract that contained small nuclear RNAs (snRNAs) U1a, U1b, U2, U4, U5, U6 and X (about 90 nucleotides) were recovered from anti-Sm antibody column. Sm snRNPs were also tried to isolate immunochemically from chromatin/nucleolar digest and the post-mitochondrial cytoplasmic fraction. It was demonstrated that selective leakages of any major U-snRNPs to those fractions during aqueous cell fractionation seem unlikely. The high proportion of U1a-snRNP to U1b-snRNP in embryoid bodies and F9 cells was reproduced, using anti-Sm column. X snRNA could not be detected in the Sm snRNP fraction from human KB cell and 129 male mouse liver nuclei. Anti-2, 2, 7-trimethylguanosine (m₃^{2,2,7}G) antibody column was also demonstrated to be useful for the fractionation of U-snRNPs from embryoid bodies. Only U1a, U1b and U2-snRNP were retained by and eluted from the column. However, when the deproteinized RNA isolated from the nuclear extract of embryoid bodies was loaded onto the column, other U snRNAs (U4 and U5 snRNA) as well as the three U snRNAs were also retained by and eluted from the column. The partially purified U1-snRNP(s) from embryoid bodies was eluted at 0.18 M NH₄Cl and U2+U4+U5 as well as U1-snRNP(s) eluted at approximately 0.3 M NH₄Cl from a DEAE column. The U6 and U7/10-snRNPs were eluted at approximately 0.35 M NH₄Cl from the column.—**KEY WORDS:** mouse teratocarcinoma, U-snRNP.

Jpn. J. Vet. Sci. 49(6): 981-987, 1987

All eukaryotic cells studied so far contain U snRNAs that exist in snRNP particles [2]. In the analysis of gene regulation and cell metabolism, one area of current interest is the role played by snRNAs and snRNPs [2]. Moreover, on the basis of several recent observations [5, 8, 15], the roles of snRNAs and snRNPs are more varied and important than have been assumed.

Mouse embryoid bodies, which resemble the embryonic portion of the mouse embryo between 3 and 5 days, and embryonal carcinoma stem cells, which are derived from the embryoid bodies, are considered to be useful as a model system for studies of early mammalian development [11]. Recently, differential control of expression of U1 snRNA during mouse development has been demonstrated [9]. It is necessary,

therefore, to characterize the snRNAs and snRNPs in this system in order to clarify the mechanism(s) of gene regulation during differentiation.

Previously, we reported the isolation of U1-snRNP(s) from mouse teratocarcinoma cells [12]. In this paper, we describe the snRNP particles from embryoid bodies and embryonal carcinoma stem cells (F9) which react with polyclonal anti-Sm and anti-m₃^{2,2,7}G antibodies, and biochemical characterization of the snRNPs.

MATERIALS AND METHODS

Human KB cells, embryoid bodies (OTT6050) and F9 cells: These have already been described [10, 12].

Cell fractionation: The method to frac-

tionate the cells has been described previously [10, 12]. A crude chromatin/nucleolar pellet from embryoid bodies was suspended in a small volume of 0.5M NaCl, 50 mM MgCl₂, 10 mM Tris-HCl, pH7.4, and was digested with DNase I (chromatographically purified, Sigma Chemical Company). This digest and an original cytoplasmic fraction were clarified by centrifugation at 12,000× g for 20 min. The resulting two supernatants were used as the sources of the chromatin/nucleolar digest and the post-mitochondrial cytoplasmic fraction, respectively.

Immunoaffinity column chromatography with anti-Sm and anti-m₃^{2,2,7}G antibodies: The IgG was isolated from the sera obtained from high-titer Sm antibodies-positive patients with systemic lupus erythematosus as defined by the American Rheumatism Association. The conditions for immunoaffinity chromatography were described earlier [10, 12]. U-snRNPs were eluted with 2.5 M MgCl₂, pH7.2, after a stepwise elution of the anti-Sm antibody column with increasing concentrations of NaCl. Purified rabbit anti-m₃^{2,2,7}G IgG was provided by Dr. Reinhard Lührmann [1]. The anti-m₃^{2,2,7}G antibody column was also constructed by linking the IgG fraction to CNBr-activated Sepharose 4B (Pharmacia). The materials bound to the anti-m₃^{2,2,7}G column were eluted stepwise with solutions containing 0.2 M, 0.5 M, 1.0 M NaCl and the materials still retained were eluted with 2.5 M MgCl₂, pH7.2. All solutions were adjusted to 1 mM 2-mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride shortly before use.

Biochemical fractionation of U-snRNPs from embryoid bodies: DEAE-Sepharose column chromatography was carried out as described previously [12, 13].

Preparation of RNA samples and electrophoresis of RNA: The pooled fractions from the columns were dialyzed against 0.2% sodium dodecyl sulfate at room

temperature and against distilled water at 4°C, followed by lyophilization. Deproteinized RNAs were analyzed by gel electrophoresis in a 10% polyacrylamide gel containing 7 M urea [3]. The RNA bands were visualized by silver staining using a commercial kit (Wako Pure Chemical Industries, LTD.).

RESULTS

Anti-Sm autoantibodies have been reported to recognize snRNPs that contain U2, U4, U5 and U6 snRNAs, as well as U1-snRNP in various mammalian cells. First we demonstrated that human Sm snRNPs could be purified with our anti-Sm antibody column, as shown in Fig. 1A, in order to confirm the quality of the column that we prepared for this series of experiments. Fig. 1A (lane 3) shows that human anti-Sm antibody column can recognize human U1, U2, U4, U5 and U6-snRNPs quite efficiently. As shown in lane 2, the nuclear extract from KB cells contains 7S, 4S, U7, U8, U9 and U10 snRNA and/or snRNP, as well as those species detected in the Sm fraction. Fig. 1B shows that logarithms of the nucleotide lengths of the snRNAs are inversely proportional to their mobilities on the gel.

Then, we analyzed Sm snRNPs of embryoid bodies and F9 cells derived from the inner core of the embryoid body using the anti-Sm antibody affinity column. Fig. 2A shows the electrophoretic profiles of the RNAs from Sm snRNPs purified from the nuclear extract of embryoid bodies on the anti-Sm column. Fig. 2A clearly demonstrates that U2, U1b, U1a, U4, U5, U6 and X snRNA species are present in the final eluate with 2.5 M MgCl₂. Little of these RNAs was eluted from the column with lower concentrations of NaCl than 1 M (data not shown). The size of X snRNA, which is not a precedent for the RNA,

Fig. 1 A: Analysis of cytoplasmic RNA (1), nuclear RNA (2), and RNA species in Sm snRNPs purified immunochemically from human KB cell nuclei (3). B: The relationship between the nucleotide lengths of the RNA species and their mobilities.

Fig. 2 Analysis of RNA from U-snRNPs of the nuclear extract (A), the post-mitochondrial cytoplasmic fraction (B), and the chromatin/nucleolar digest (C) of embryoid bodies eluted from the anti-Sm column.

designated X, in Sm snRNP, was estimated to be approximately 90 nucleotides. When anti-Sm antibody column chromatography was used, the observation previously reported that the ratio of U1a-snRNP to U1b-snRNP was high in the U1-snRNP(s) from embryoid bodies [12] was reproduced (Fig. 2A).

It is possible that this RNA profile of U-snRNPs in the embryoid bodies may reflect the artifactual leakage of some major U-snRNP species to the chromatin/nucleolar pellet and/or cytoplasmic fraction during aqueous cell fractionation. Then, the crude chromatin/nucleolar digest or the post-mitochondrial cytoplasmic fraction was loaded onto the anti-Sm column, and the results obtained are shown in Fig. 2B and C. Since the electrophoretic profiles of RNAs of the major U-snRNPs (U2, U1b, U1a, U4, U5 and U6) purified immunochemically from the nuclear extract of embryoid bodies were substantially similar to those of both the chromatin/nucleolar digest and the post-mitochondrial cytoplasmic fraction, it seems unlikely that any specific leakages of given major U-snRNP species from the nuclei during aqueous cell fractionation occurred. However, some minor U snRNAs and some RNA materials stained by silver, some of which must be nonspecific contamination, were also detected in the chromatin/nucleolar digest and the post-mitochondrial cytoplasmic fraction (Fig. 2B and C) but not in the nuclear extract (Fig. 2A).

When the cell extract from F9 cells was loaded onto the anti-Sm column, U2, U1b, U1a, U4, U5, U6 and X snRNA species

Fig. 3 Analysis of RNA from U-snRNPs from F9 cell extract (2) eluted from the anti-Sm column. Cytoplasmic RNA of embryoid bodies (1) was also analyzed.

were detected in the Sm fraction, as shown in Fig. 3. This indicates that a very similar profile of Sm snRNPs was obtained from F9 cell extract and the nuclear extract of embryoid bodies.

For further characterization on U-snRNPs of mouse teratocarcinoma cells, we attempted to purify Sm snRNPs immunochemically from the liver nuclei of 129 syngeneic male mice (129/sv), which frequently develop teratoma and are host mice for the transplantable teratocarcinoma, OTT6050. X snRNP could not be detected immunochemically in the liver nuclei (data not shown).

Major and minor U-series snRNAs studied so far are capped at the 5'-termini [2, 14]. Next, antibodies elicited by $m_3^{2,2,7}G$ were used to characterize the U-snRNPs in the nuclear extract of embryoid bodies. As shown in Fig. 4A, only U2, U1b and U1a-snRNPs in the nuclear extract of embryoid bodies could be retained by the anti- $m_3^{2,2,7}G$ column. However, when the deproteinized RNA isolated from the nuclear extract of embryoid bodies was loaded onto the anti- $m_3^{2,2,7}G$ column, other U snRNA species (U4 and U5) as well as the

Fig. 4 Analysis of RNA from U-snRNPs in the nuclear extract (A) and from the deproteinized RNA isolated from the nuclear extract (B) of embryoid bodies retained by and eluted from the anti- $m_3^{2,2,7}G$ antibody column. The samples in the lanes are: 0.2 M NaCl eluate (1), 0.5 M NaCl eluate (2), 1.0 M NaCl eluate (3), 2.5 M $MgCl_2$, pH7.2 eluate (4), materials loaded onto the column (5) (A) and 1.0 M NaCl eluate from the column (B).

three U snRNAs (U2, U1b and U1a) were also retained by and eluted from the column (Fig. 4B).

Finally, in order to characterize snRNPs from embryoid bodies biochemically, a nuclear extract from embryoid bodies was fractionated on a DEAE-Sephacrose column by a linear 0.05–1.0 M NH_4Cl gradient elution as described previously [12], and in this study we pooled each fraction 1 through 7 (Fig. 5A). Sometimes faint bands of U1 snRNAs could be detected in the pooled fractions 2 and 6 (lanes (3) and (7) in Fig. 5B). Fig. 5B also clearly demonstrates the electrophoretic profiles of the RNA from partially purified U1-snRNPs in the pooled fraction 3 eluted at around 0.18 M NH_4Cl (lane (4)) and of the RNA from U2+U4+U5-snRNP as well as U1-snRNPs in the pooled fraction 4 eluted at approximately 0.3 M NH_4Cl (lane (5)). An RNA component whose migration corresponds to

Fig. 5 DEAE-chromatography of snRNPs. A dialyzed nuclear extract was subjected to chromatography on a DEAE column as described previously (reference [12]) and each fraction eluted was pooled as shown in A. The RNA samples in the lanes shown in B are: cytoplasmic RNA of embryoid bodies (1), the pooled fraction 1 shown in A (2); 2 (3); 3 (4); 4 (5); 5 (6); 6 (7); 7 (8).

that of X snRNA was also detected in the pooled fraction 3 (lane (4)), although only faintly visible. Recently, in addition to the snRNAs designated U1 through U6, U7, U8, U9 and U10 snRNAs have been isolated from rat and HeLa cells [14]. Lane 6 (the pooled fraction 5 eluted at approximately 0.35 M NH_4Cl) shows that embryoid bodies carry U6-snRNP and may also carry U7 and U10-snRNPs. An RNA component whose migration corresponds to that of U8 snRNA was also seen in the electrophoretic profile of the pooled fraction 3 (lane (4)). Lanes 4 and 5 show that the high ratio of U1a-snRNP to U1b-snRNP

could be reproduced also using biochemical procedure.

DISCUSSION

In this study, both immunochemical and biochemical characterization of snRNPs from mouse teratocarcinoma cells (embryoid bodies OTT6050 and F9 cells) were carried out. The highly purified U-snRNPs containing the snRNAs U1a, U1b, U2, U4, U5, U6 and X were recovered from the anti-Sm antibody column by elution with 2.5 M MgCl_2 , pH 7.2. As previously described [12], the proportion of U1a-snRNP to U1b-snRNP was observed to be high in the embryoid bodies and F9 cells, when analyzed on the anti-Sm antibody column. This predominance of U1a-snRNP in the population of U1-snRNPs from embryoid bodies and F9 cells was also observed in the population isolated biochemically.

Gallinaro and Jacob [4] showed that U1 RNA molecules are found in the chromatin/nucleolar pellet (~approximately half of the cellular content), and Gurney and Eliceiri [6] also indicated that U1 RNA usually leaks out to the cytoplasmic fraction (~10% of the cellular content), during aqueous cell fractionation. Therefore, it is meaningful to test the status of U-snRNPs in these fractions. In this experiment, we attempted to fractionate the chromatin/nucleolar digest and the post-mitochondrial cytoplasmic fraction on the anti-Sm column. Since electrophoretic RNA profiles of the major U-snRNPs obtained immunochemically from those fractions look quite similar to those from the nuclear extract, any selective leakages of given major U-snRNP species to the chromatin/nucleolar pellet and the cytoplasmic fraction during aqueous cell fractionation seem unlikely. Since some minor U snRNAs were detected only in the fractions other than nuclear extract, cell fractionation procedure will have to be

improved in order to analyze minor U-snRNPs of the embryoid bodies. With regard to U8-snRNP, a large amount of U8 snRNA was detected in the Sm snRNP fraction obtained immunochemically from the chromatin/nucleolar digest. This is in agreement with the recent observation by Reddy *et al.* [14], who found U8 snRNA to be enriched in nucleolar preparations from Novikoff hepatoma.

There is no precedent for the RNA, designated X in Sm snRNPs, which was visualized in the immunochemically obtained Sm snRNP fraction. Since the X snRNA in the Sm snRNP fraction could not be detected immunochemically in human KB cells and 129 male mouse liver, X may be specific to teratocarcinoma cells. However, we cannot rule out the possibility that X snRNA is a degradation product of major U snRNA or RNA immunoreacted nonspecifically, so that further characterization, including sequencing, is required for the exact identification of this species.

This is the first analysis of U-snRNPs from mouse teratocarcinoma cells with antibody specific for $m_3^{2,2,7}G$. It was clearly demonstrated that only 3 U-snRNPs (U1a, U1b and U2) of the major U-snRNPs of embryoid bodies which were immunoreactive with anti-Sm antibody were retained by and eluted from anti- $m_3^{2,2,7}G$ column. It seems unlikely that the absence of $m_3^{2,2,7}G$ caps of the U4 and U5 snRNAs in the snRNP particles is responsible for the lack of immunoreactivities of them with anti- $m_3^{2,2,7}G$ antibodies, since those deproteinized snRNA species as well as U1a, U1b and U2 snRNAs reacted with the antibodies. Since the antibody is reported to react predominantly with snRNP particles containing the snRNAs U1a, U1b, U2, U4 and U5 from Ehrlich ascites cells [1], it may be considered that the immunoreactions of the 5'-terminal cap regions of U4 and U5 snRNAs are hindered in the snRNP parti-

cles in the embryoid bodies.

In this study, we attempted to purify partially U-snRNPs from embryoid bodies by the procedure of Kinlaw *et al.* [7]. Previous results indicated that the purity of U1-snRNP fraction is around 90% based on the amounts of U1 snRNAs (see Figure 3 in our previous report [12]). The purity of our present U1-snRNP fraction is estimated to be as high as in the previous case, in spite of co-detecting of U8-snRNP in the fraction. The U2-snRNP fraction, also containing U4, U5 and U1-snRNPs from embryoid bodies, was eluted from the DEAE column at 0.3 M NH_4Cl as previously described by Kinlaw *et al.* [7]. We do not know if the late eluting materials from DEAE chromatography (lane 7 in Fig. 5) at approximately 0.4–0.5 M NH_4Cl are intact assemblies of snRNPs or not.

Recently, Reddy *et al.* detected the U7, U8, U9 and U10-snRNPs in HeLa cells and Novikoff hepatoma by anti-Sm antibody immunoprecipitation, as minor U-snRNPs [14]. We also detected snRNAs whose migration patterns correspond to those of U7, U8, U9 and U10 snRNA, respectively, in embryoid bodies by anti-Sm column and/or DEAE chromatography. With respect to U8-snRNP, the detection in DEAE fraction was not reproducible but sometimes it was observed clearly, as shown in Fig. 5.

In this study, we attempted to characterize snRNPs from embryoid bodies and F9 cells. Further molecular characterization of U snRNAs and U-snRNPs including size, nature and association with other heavier particles, and an elucidation of their functions in the embryonal carcinoma cells will lead us to a full understanding of the roles of snRNAs and snRNPs during differentiation.

ACKNOWLEDGEMENT. We thank Dr. R. Lührmann for anti- $m_3^{2,2,7}G$ IgG. We also thank Dr. H. Fujitani for his invaluable help. M. Matsuda is a research fellow of Cancer Research Institute, Kanazawa University.

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要 約

多クローン性抗 Sm 抗体および抗 $m_3^{2,2,7}G$ 抗体と反応するマウステラトカルシノーマ細胞の snRNP とその生化学的性状：松田基夫・山田隆紹¹⁾（麻布大学・獣医学部・分子生物学，¹⁾内科学Ⅱ）——マウス胚様体核抽出液および F9 細胞抽出液から抗 Sm 抗体カラムを用いて高純度の Sm snRNP（U1a, U1b, U2, U4, U5, U6 と X-snRNP（約90ヌクレオチド RNA））が得られた。同様の方法で、胚様体のクロマチン/核小体消化物とミトコンドリアを除去した後の細胞質画分からも、メジャーな U-snRNP に関して核抽出液画分中から得られたものと類似の RNA のプロファイルが得られた。抗 $m_3^{2,2,7}G$ 抗体カラムを用いて胚様体 U-snRNP の分画を行ったところ、U1a, U1b と U2-snRNP のみがカラムに吸着され溶出された。また DEAE カラムを用いて胚様体 U-snRNP の粗分画を試みたところ、U1 および U2-snRNP 分画の他にマイナーな U-snRNP も検出された。