Small Nuclear RNAs and Small Nuclear Ribonucleoprotein Complexes Purified from the Feline Placenta

Motoo MATSUDA, and Takatsugu YAMADA¹⁾

Laboratory for Molecular Biology, and ¹⁾Laboratory for Internal Medicine II, Azabu University School of Veterinary Medicine, Fuchinobe 1-17-71, Sagamihara 229, Japan

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ABSTRACT. Small nuclear RNAs (snRNAs) and small nuclear ribonucleoprotein particles (snRNPs) were purified from the cat placenta as an initial step in the analysis of the regulatory mechanism(s) of gene expression in the placenta. For visual observations, snRNA species including 7S, U2, U1, U4, X-I, X-II, 5S, U5, U6 and X-III were stained with acridine orange, following the extraction of RNAs with phenol from cat placental cell nuclei and their fractionation by electrophoresis on a 10% polyacrylamide gel containing 7 M urea. The small nuclear RNA U1 from the cat placenta showed approximately the same mobility as that of mouse (129/sv) liver used as a reference on the gel. The logarithms of the nucleotide lengths of cat placenta snRNAs were inversely related to their mobilities. The approximate number of snRNA molecules in the cat placenta cell nucleus was also calculated. Highly purified U1-snRNP which comprises U1 snRNA and eight polypeptide components ranging in molecular weight from 68,000 to 9,500 was immunochemically purified using systemic lupus erythematosus-anti(U1)RNP antibody column chromatography. Any polypeptide component involved in the U1-snRNP did not immunoreact with normal human IgG. No immunoreactive materials with anti(U1)RNP antibodies could be detected in cat amniotic fluid. The U1-snRNP and U2-snRNP rich fractions were separated biochemically on a DEAE Sepharose column.—**KEY WORDS:** cat placenta, snRNA, snRNP.

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The placenta produces placental hormones essential for the maintaining pregnancy [22] and pregnancy-placenta specific proteins of unknown functions [3]. It is thus an interesting organ as it actively serve various physiological functions.

Some reports have indicated that differential expression of various genes in the placenta is dramatically controlled [8, 9, 20]. Therefore, interesting and diverse molecular mechanism(s) appears to be involved in the gene expression in the placenta.

There are discrete, stable small nuclear RNAs (snRNAs) (for example 7S, U1, U2, U4, U5, U6, 4.5S snRNA and so on) ranging in size from approximate 90 to 300 nucleotides in the cell nuclei of a wide variety of eukaryotic organisms. Small nuclear RNAs and small nuclear RNA-protein complexes

(snRNPs) supposedly play some essential roles in RNA processing; splicing, poly A addition, mRNA transport and so on [2, 4, 11, 18, 23]. Since it has been suggested that some RNA molecules themselves can catalyze biochemical reactions, snRNAs may also serve as an RNA catalyst. Moreover, on the basis of several recent observations, the roles of snRNAs and snRNPs may be far more important in gene regulation than so far regarded. However, the exact functions of snRNAs and snRNPs in the cell largely remain unknown.

A study of snRNAs and snRNPs of the placenta may provide some information on their roles in regulation of gene expression. The purpose of the present research was to isolate and characterize snRNAs and snRNPs from the cat placenta.

MATERIALS AND METHODS

Nuclear isolation and fractionation of cat placenta: Nuclei were isolated from frozen cat placenta (about 50 days of pregnancy) as described by Muramatsu [17]. They were then sonicated and centrifuged to prepare a crude nuclear extract. To isolate the snRNPs, the crude nuclear extract was further purified by ultracentrifugation for 2 h, 130,000×g in a Hitachi RP65T rotor. Nuclear extract was also prepared from mouse (129/sv) liver cell nuclei.

IgG isolation and affinity column chromatography: Autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE) and mixed connective tissue disease are known in human being and dogs as well as other animals [16, 21]. Certain anti-RNP and anti-Sm of antinuclear autoantibodies from patients with autoimmune diseases have been found to be useful for isolating snRNP complexes [13, 14]. In this experiment, sera were obtained from high-titer RNP antibodies-positive patients with SLE as defined by the American Rheumatism Association. Immunoglobulin G was isolated by ammonium sulfate precipitation followed by DEAE-cellulose chromatography from anti (UI) RNP-SLE sera as described by Lerner *et al.* [13]. In the present work, IgG fractions were coupled to cyanogen bromide activated-Sepharose 4B [14]. The affinity column consisted of 1 ml of Sepharose 4B coupled to 1 mg of IgG. The column chromatography accompanied by the stepwise elution at increasing salt concentrations was carried out by the procedure shown in Table 2.

Biochemical procedures for the U-series snRNP particles purification: DEAE-Sepharose column chromatography was conducted to bring about the partial purification of U-snRNP, as described by Kinlaw *et al.* [10].

Quantitative assays: Protein determinations were performed by the methods of Gog-

Table 1. Estimation of the number of snRNA molecules in the nucleus of a cat placenta cell

snRNA	Number of molecules
7S	2.7×10^5
U2	3.2×10^5
U1	1.0×10^6
U4	1.8×10^5
X-I	5.3×10^5
(5S)	1.6×10^5
U5	1.6×10^5
U6	1.1×10^5
X-III	1.2×10^5

stad *et al.* [6] with bovine serum albumin as the standard.

RNA and protein analysis: The pooled fractions from the column were dialyzed against 0.2% sodium dodecyl sulfate (SDS) at room temperature and distilled water at 4°C and then concentrated by lyophilization. For analysis of the RNAs, the samples were extracted with saturated phenol and precipitated with 2.5 volumes of 95% ethanol. The RNAs were analyzed by gel electrophoresis on a 10% polyacrylamide slab gel containing 7 M urea [5]. For protein analysis, SDS-8% polyacrylamide (Anderson) gel electrophoresis was carried out [1]. RNA and protein bands were visualized by acrydine orange and silver staining using commercially obtained kits, Ag-Stain Daiichi, from Daiichi Pure Chemicals Co., LTD. (Japan).

RESULTS AND DISCUSSION

Small nuclear RNAs from cell nuclei of the cat placenta were first fractionated by electrophoresis on a 10% polyacrylamide gel, visualized by acrydine orange staining as shown in Fig. 1A. Highly abundant nucleoplasmic snRNA species U1a (171 nucleotide lengths (NTs)), U1b and U2 (196) and the other three RNAs (U4, U5 and U6) with lengths of about 145, 120 and 95 residues, respectively, have been reported in mouse Ehrlich ascites cells by Lerner *et al.* [13].

Fig. 1. 10% polyacrylamide gel electrophoresis containing 7 M urea of cat placenta cell nuclei RNA (A). The RNA bands were visualized by acrydine orange staining and scanned at 550 nm (C). The relationship between RNA nucleotide length and mobility was also determined (B).

Nucleotide lengths of snRNAs in eukaryotes such as rat, human, chicken and so on have also been suggested as 294-295 for 7S RNA, 188-189 for U2 RNA, 165 for U1, 142-146 for U4, 116-118 for U5 and 107-108 for U6, respectively [4]. One of the most abundant snRNA species in most animal cells is U1 RNA. U1 snRNA in the U1-snRNP particles from cat placenta showed approximately equal mobility to that of mouse (129/*sv*) liver nuclei, which was used as a reference, on the gel (Fig. 2B). In light of these aspects of snRNA 7S, U1 and U2 snRNA were identified in the cat placenta sample (Fig. 1A and 1B). Moreover, the designation of certain other snRNAs in cat placenta was attempted on the basis of the mobility (Fig. 1A and 1B). The logarithms of the NTs of cat placenta snRNAs were inversely related to the mobility in this gel system (Fig. 1B). Fig. 1C

Table 2. Amount of protein in each eluate from the anti(U1)RNP column

	Protein Conc.
1) Materials loaded onto the column (nuclear extract)	3.50 mg
2) Flow-through from the column and buffered 0.2 M NaCl eluate	2.50 "
3) Buffered 0.5 M NaCl and 1.0 M NaCl eluate	0.34 "
4) 2.5 M MgCl ₂ , pH 7.2 eluate	0.32 "

The buffered NaCl contained 50 mM Tris, 5 mM EDTA, 0.05% nonidet P40, 0.1 mM phenylmethylsulfonylfluoride and NaCl at the concentration indicated, pH 7.2.

shows a scan of the RNA bands at 550 nm. About 1×10^6 molecules of U1 snRNA and about 1×10^4 to 5×10^5 molecules each of other snRNAs are considered to be present in a single human cell [4]. The approximate number of snRNA molecules in a cat placenta cell nucleus was calculated from the value of U1 RNA in a human cell and the relative amounts of snRNAs on the gel (Fig. 1C). The results are also shown in Table 1. Some snRNAs visualized on the gel including X-I, X-II and X-III remain unidentified due to insufficient data. We cannot completely exclude the possibility that they are degradative products from other RNA species.

In the next series of experiments, fractionation of the nuclear extract by the affinity column chromatography with anti(U1)RNP antibody was carried out, since snRNA, if not all, are thought to be functional when they associate with protein moieties. To obtain highly purified U1-snRNP particles, an elution procedure with 2.5 M MgCl₂, pH 7.2, following the stepwise elution of the column with increasing NaCl concentrations was used [14]. Table 2 shows the amount of protein in each eluate from the column. Protein recovery from the column was about 90% of that of the loading materials. The RNA components of the purified U1-snRNA particles eluted from the column by 2.5 M MgCl₂ were analyzed by electrophoresis. Figures 2A and

Fig. 2. RNA (A and B) and protein (C) components of U1-snRNP purified from cat placenta by an immunochemical procedure. RNA was analysed by electrophoresis on a 10% polyacrylamide gel in the presence of 7 M urea. RNA components of U1-snRNP purified immunochemically from mouse (129/*sv*) liver nuclei were also analysed as a reference (B2). Protein was analysed by electrophoresis on SDS-8% polyacrylamide (Anderson) gel. The RNA samples were 2.5 M MgCl_2 , pH 7.2 eluate from the antibody column (A and B). The protein samples were: materials loaded onto the column (3), flow-through from the column and buffered 0.2 M NaCl eluate (4), buffered 0.5 M NaCl and 1.0 M NaCl eluate (5) and 2.5 M MgCl_2 eluate (6) (C). Bovine serum albumin (68,000), carbonic anhydrase (30,000), lysozyme (14,300) (1) and ovalbumin (43,000) and chymotrypsinogen (25,000) (2) were used as molecular weight standards. The RNA and protein bands were visualized by silver staining.

2B show U1 RNA to be essentially an RNA species present in the final 2.5 M MgCl_2 eluate. Thus, this elution procedure for obtaining highly purified U1-snRNP particles from the column is efficient and reproducible, confirming the previous results [14].

The polypeptide components in each fraction eluted from the column were analyzed by SDS-8% polyacrylamide (Anderson) gel electrophoresis. Polypeptides in the 2.5 M MgCl_2 eluate had molecular weights of 68,000, 54,000, 36,000, 35,000, 17,500, 16,500, 10,000 and 9,500 (Fig. 2C(6)). To date, no report has appeared on either immunochemical and biochemical isolation or polypeptide analysis of U1-snRNP from the cat placenta. The polypeptide patterns of

U1-snRNP purified immunochemically from the cat placenta may partly agree with the results from observations of other cells by other workers [7, 10].

The nuclear extract from the cat placenta was chromatographed on a column containing normal human IgG coupled to Sepharose 4B as a control experiment. A fraction eluted with 2.5 M MgCl_2 did not contain any polypeptide components of U1-snRNP, as mentioned above (data not shown).

Amniotic fluid from the cat was also chromatographed on an anti (U1) RNP column to determine the possible presence of U1-snRNP or any materials immunoreactive with the antibodies in the amniotic fluid. No materials immunoreactive with the antibodies were

Fig. 3. An elution profile of the nuclear extract from cat placenta on DEAE Sepharose column. A nuclear extract equivalent to 30 mg of proteins was loaded onto the column. The amount of protein in each pooled fraction appears in parentheses.

Fig. 4. RNA profiles of snRNPs fractionated on DEAE Sepharose column chromatography. The RNA samples were: pooled fractions 1 (1), 2 (2), 3 (3), 4 (4) and 5 (5).

found (data not shown), although a report indicated that amniotic fluid contained some biological activity such as that of RNA-directed DNA polymerase in the placenta [19].

The nucleoplasmic U-series snRNPs were found to be U1, U2, U4, U5, and U6 snRNPs

by an indirect immunofluorescence experiment [12]. Partial purification was also made on the U-snRNPs by separating the snRNPs from each other and from the bulk of cellular proteins on the ion exchange chromatography (Fig. 3). Following chromatographic fractionation, pooled fractions 1 to 5 were each analyzed for RNA components. Figure 4 shows the RNA profiles of each pooled fraction eluted from the column. There are no snRNA components in fraction 1 and 5, being evident from Fig. 4 (1) and (5). The RNA patterns on the gel showed that the pooled fraction 2, eluted at approximately 0.1 M NH_4Cl , contained an abundant amount of U1-snRNP, and that fraction 3, eluted at approximately 0.2 M, contained a substantial amount of U2-snRNP. The U1-snRNP-rich fraction also contained a small amount of RNA components such as U2 snRNA and X-I, which was not definitely identified on the basis of their mobilities on the gel. The fraction with U2-snRNP contained U1, U4, X-II and 7S RNA, besides U2 RNA (Fig. 4 (3)). Kinlaw *et al.* isolated biochemically human Hela snRNPs containing U1 and U2 RNA [10]. In their purification, U1 and U2+U4+U6 snRNPs were eluted from a DEAE Sepharose column at 0.15 M and 0.3 M NH_4Cl , respectively. These may reflect pos-

sible differences in the characteristics of snRNPs in human and cat placenta cells. In the present research, we were not able to perform the separation of U-snRNPs by chromatography alone, as a single step. In light of this, further investigation to find some means for separating minor snRNPs from others should be carried out.

The placenta is a very active internal organ from the standpoint of gene expression; it produces placental hormones essential for the maintenance of pregnancy [22], as well as many pregnancy-placenta specific proteins whose functions are not yet to be determined [3]. However, the detailed molecular mechanism(s), by which these proteins (hormones) govern gene expression, remains to be clarified. The snRNPs described in the present paper need to be further purified and their assay systems need to be established to determine biological activity or functions of the snRNPs *in vitro* in order to elucidate the mode by which gene expression is regulated in cat placenta. The present research may be a first attempt in this direction.

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要 約

ネコ胎盤の snRNA 及び snRNP: 松田基夫・山田隆紹¹⁾ (麻布大学獣医学部分子生物学教室, ¹⁾内科学教室 II) ——ネコ胎盤における遺伝子発現の調節機構を解析する目的で, 胎盤の核内低分子量 RNA 及び核内低分子量リボ核蛋白質複合体の分離精製を試みた。ネコ胎盤細胞核から抽出した RNA を 7M 尿素を含む 10% ポリアクリルアミドゲル電気泳動法で分画すると, アクリジンオレンジ染色により 7S, U2, U1, U4, X-I, X-II, 5S, U5, U6 及び X-III RNA が検出された。U1 snRNA は対照として用いたマウス肝臓の U1 snRNA とほぼ同じ移動度を示した。胎盤 snRNA のヌクレオチド長の対数は用いた RNA ゲル電気泳動系においてその移動に正しく逆比例していた。ヒト細胞核内における報告されている U1 RNA の分子数を指標としてネコ胎盤中の細胞核当りの snRNA の分子数を概算した。SLE-抗 (U1) RNP 抗体を用いた免疫化学的手法で U1-snRNP の単離を行ったところ, U1 RNA と分子量が約 68,000, 54,000, 36,000, 35,000, 17,500, 16,500, 10,000 及び 9,500 の 8 個のポリペプチド成分が銀染色法によって検出され, これらポリペプチド成分はいずれも正常ヒト IgG とは反応しなかった。ネコ羊水中には抗 (U1) RNP 抗体と反応する成分は検出されなかった。ついで生化学的手法を用いて U1-snRNP に富む分画と U2-snRNP に富む分画が得られた。