生理的イオン強度下でのショ糖勾配遠心法とRNAゲル電気泳動法を用いたマウス胚様体(OTT6050)U-snRNPの沈降特性に関する研究

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A Study of the Sedimentation Properties of the U-snRNPs of Mouse Embryoid Bodies (OTT6050) by Sucrose Gradient Centrifugation under Physiological Ionic Conditions and Electrophoretic Analysis of the RNA in Various Fractions

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ABSTRACT. Fractionation of nuclear extracts of mouse embryoid bodies (EBs; OTT6050) on sucrose gradients (5–20%) under conditions of physiological ionic strength (150 mM NaCl, 1mM $MgCl_2$) and an analysis of the RNA in various fractions by gel electrophoresis revealed that the major U small nuclear ribonucleoproteins (U-snRNPs; U1a, U1b, U2, U4, U5 and U6-snRNPs) sediment over a wide region of the gradient, although they sediment preponderantly in the light region of the gradient. This result suggests that, under these experimental conditions, some of the populations of snRNPs exist as free particles separated from large nuclear RNP particles, while some of the populations associate with them in EBs. Furthermore, all species of these major U-snRNPs appear to associate with the larger nuclear RNP particles of EBs, since all these species sediment in the heavier fractions (approximate$\geq$60S) of the 15–40%/50% gradients. The relative abundance of the various species of major U-snRNPs can also be observed to vary among the fractions of the gradients. A similar analysis of the post-mitochondrial cytoplasmic fraction showed that some leakage of the major U-snRNPs, but not the selective leakage of any particular species of U-snRNP, from the nuclear fraction, occurred during aqueous fractionation of the cells. Some species of RNA, larger than the snRNAs U1a/b and U2 respectively were also detected in the cytoplasmic fraction.—KEY WORDS: mouse embryoid body, physiological ionic strength, RNA gel electrophoresis, sucrose gradient centrifugation, U-snRNP-hnRNP assembly.

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U small nuclear RNAs (U snRNAs) are abundant and stable species of RNA which are present in a wide variety of higher eukaryotes. They are found as components of U small nuclear ribonucleoprotein particles (U-snRNPs) with an S value of approximately 10–12S and are thought to play an essential role in the processing of heterogeneous nuclear RNA (hnRNA) in the cell [10]. HnRNA also exists in association with a specific set of proteins in the form of large nuclear ribonucleoprotein particles [heterogeneous nuclear ribonucleoproteins (hnRNPs) of approximate size 50–200S] [9]. It is assumed that RNA processing occurs in these large RNP particles [7]. Associations between snRNPs and hnRNPs in the cell nucleus have been reported (*in vivo* hnRNP-snRNP assemblies), and isolation of hnRNA-snRNA-protein complexes has also been reported [1, 2, 8, 10, 15]. Investigations concerning the nature of organization of *in vivo* hnRNP-snRNP assemblies may, thus, be important in order to clarify the state of U-snRNPs in cells.

Embryoid bodies (EBs) are ascitic tumors composed of an outer ring of endoderm and inner core of embryonal carcinoma cells and resemble the embryonic portion of 3- to 5-day-old mouse embryos [12]. Differentiation of embryoid bodies result in solid teratocarcinomas which consist mainly of neuroepithelial cells. Thus, the mouse teratocarcinoma is considered to be useful as a model system for studies of early mammalian development. Månsson and Harris

attempted the molecular characterization of mouse EBs by an analysis of the complexity of poly(A)-containing hnRNA, and indicated that as many as 50% of the sequences in total poly(A)-RNA from the mouse neuroepithelial teratocarcinomas may be present in much lower concentration in the nuclear poly(A+)-RNA transcripts in EBs than are the sequences in EB total poly(A+)-RNA population, and these changes in gene expression may reflect changes in the pattern of post-transcriptional processing that is involved in gene regulation [11]. Therefore, it is worthwhile to investigate biochemically the *in vivo* hnRNP-snRNP assemblies in EBs. However, such characterization of EBs have not yet been reported. We have previously undertaken the immunochemical and biochemical characterization of snRNPs from teratocarcinoma cells [13, 14]. As part of our biochemical characterization of snRNPs, in the present paper, we describe an analysis of the overall sedimentation properties of U-snRNP particles from mouse EBs (OTT6050) by sucrose gradient centrifugation under physiological ionic conditions and of electrophoresis of the associated RNA, in a study designed to characterize the nature of *in vivo* hnRNP-snRNP-assemblies.

MATERIALS AND METHODS

EBs: The transplantable, mouse teratocarcinoma EB (OTT6050) has been maintained in syngeneic 129/*sv* male mice. EBs were recovered from the ascitic fluid of the mice.

Fractionation of cells and centrifugation of various fractions on sucrose gradients: The fractionation of the cells was carried out as described previously [13]. In the present experiment, the cell fractionation was carried out under conditions of physiological ionic strength (150 mM NaCl, 1–1.5 mM $MgCl_2$), because different ionic conditions may affect the conformational states of the U-snRNP structures and the nature of *in vivo* hnRNP-snRNP assemblies in the cell. For example, the dissociation of U-snRNPs from the large nuclear RNP particles is most likely promoted at higher salt concentrations. Sedimentations were carried out by centrifugation in 5–20% and 15–40% linear sucrose gradients with a 50% cushion (15–40%/50%) in the presence of 150 mM NaCl, 1mM $MgCl_2$ and 10mM Tris-HCl, pH 7.4, at 22,000 rpm (63,000×g), for 18 hr at 4°C (Hitachi SRP 28 SA rotor). Heparin (200μg/m*l*) and polyvinylsulfate (50μg/m*l*) were used as inhibitors of RNase activity.

Preparation of RNA and gel electrophoresis: Each of the fractions from the sucrose gradients was deproteinized with phenol at room temperature, precipitated with 2.5 volumes of ethanol, and analyzed by electrophoresis on 10% polyacrylamide gels [14]. The bands of RNA were visualized by staining with silver, using a commercial kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

RESULTS AND DISCUSSION

HnRNAs in eukaryotic cells are complexed with a set of proteins to form large nuclear RNP complexes. Associations between the large nuclear RNPs and snRNPs and isolation of such particles have been reported [1, 2, 8, 10, 15]. Therefore, we attempted to fractionate the nuclear extracts by centrifugation on 5–20% sucrose gradients under conditions of physiological ionic strength to determine the sedimentation properties of snRNPs from nuclear extracts of EBs. In these kinds of experiments, it is very important to ensure that fractionation of cells, lysis of nuclei and sucrose gradient centrifugation are performed under those conditions that faithfully reflect intracellular ionic conditions.

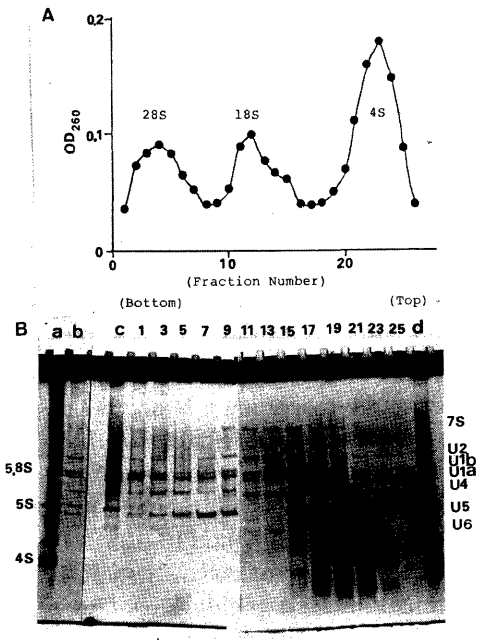

Fig. 1. Cytoplasmic RNA (28S, 18S and 4S) fractionated by centrifugation on 5–20% sucrose gradients for use as a standard (A). RNAs from sn-RNPs, obtained by the same centrifugation procedure of nuclear extract prepared from EBs were analyzed by electrophoresis (B). The lanes in the gel correspond directly to the numbered fractions from the gradients. Cytoplasmic RNA (5.8S, 5S and 4S) (a and d); nuclear RNA (b); and RNA precipitated during centrifugation (c); were also fractionated in the gel. Lanes a-9 and 11-d of B show two separate gels.

Cytoplasmic RNA extracted from EBs was used as a marker to indicate the position of the peaks of ribosomal and transfer RNA (28S, 18S and 4S) (Fig. 1A). Fig. 1B (lanes 1–25) shows the overall profiles of RNA in each of the fractions by centrifugation on 5–20% sucrose gradients of nuclear extracts, analyzed by gel electrophoresis. As clearly demonstrated in Fig. 1B, U2-, U4-, U5- and U6-snRNPs, as well as U1a- and U1b-snRNPs, sediment predominantly in the light region of the gradient (4–18S; free snRNP particles may be present with an S value of approximately 10–12S) and U-snRNPs also sediment extensively over a wide region of the gradients.

Identification of individual species of U snRNA in the fractions from the sucrose gradients was accomplished by examination of their mobilities relative to cytoplasmic RNA markers (lanes a and d in Fig. 1B), and the nucleoplasmic species of U snRNA (lanes 1–25 in Fig. 1B) could be easily identified on the gel, as shown in the figure.

In more detail, the sedimentation profile reveals the following peaks: U1a-, U1b-, and U2-snRNPs, around fraction 17; U4-snRNP, around fractions 3 and 17; U5-snRNP, around fractions 7 and 19; U6-snRNP, around fraction 19; and particles that contain 7S RNA, around fractions 13–15 (Fig. 1B). A small amount of U-snRNP is also found to be precipitated in the bottom of the tubes during centrifugation (lane c in Fig. 1B). The relative abundance of the various species of U-snRNP can also be observed to vary among the fractions of the gradient shown in Fig. 1B. Table 1 summarizes the result on the relative abundance of the various species of U-snRNP.

Furthermore, by centrifugation on 15–40%/50% sucrose gradients, we found that the major U-snRNPs from EBs also sediment at the lower region of the gradient (the region could correspond to $\geq$60S, as described by Harmon F. *et al.* [7], though we do not determine the correct S value in the region because of no appropriate reference marker at present) (lanes 1–5 in Fig. 2). The result that the major U1a-, U1b-, U2-, U4-, U5- and U6-snRNPs sediment together with in the lower regions of the gradients strongly suggests that all these major species of U-snRNPs associate with the larger nuclear RNP particles in the nuclei of EBs. U-snRNPs, if not associate with the larger RNPs, would be expected to sediment in the upper regions of the gradients.

Although we do not know the extent of

Table 1. Relative abundance of various species of U-snRNP in the 5–20% sucrose fractions of nuclear extracts of embryoid bodies

nuclear extract										
	U1a/b	>	U2	>	U4,	U5	>	U6		
#3	U1a/b	>	U4	>	U5	>	U2	>	U6	
#7	U5	>	U1a/b	>	U4	>	U2	>	U6	
#11	U1a/b	>	U2, U4		>	U6	>	U5		
#15	U1a/b	>	U2	>	U4,	U5,	U6			
#17	U1a/b	>	U2	>	U4	>	U5	>	U6	

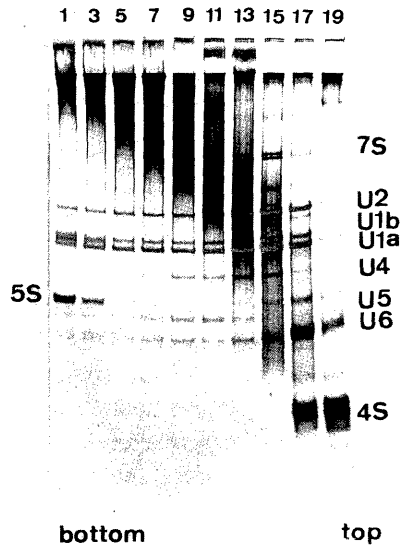

Fig. 2. Analysis of RNA from snRNPs obtained by centrifugation on 15–40%/50% sucrose gradients of nuclear extracts prepared from EBs. The lanes in the gel correspond directly to numbered fractions in the gradient.

the recovery of RNA from each fraction of the gradient in this experiment, and we certainly cannot exclude some variation in the extent of recovery, the typical, overall profiles of RNA, as shown in Figs. 1 and 2, were reproduced in three separate experiments.

Complexes formed between U-snRNPs and hnRNPs are presumed to be held together by RNA-RNA, protein-RNA, and protein-protein interactions and such complexes have been shown to be easily dissociated by salt [5]. Therefore, when such complexes are studied *in vivo* by fractionation on sucrose density gradients, it is very important that the gradients are prepared under ionic conditions that reflect the intracellular milieu. For example, if higher than physiological ionic strengths are used, the artifactual dissociation of U-snRNPs from the complexes will most likely occur, and the U-snRNPs will sediment in the light region of the gradient (10–12S). If lower ionic strengths are used, U-snRNPs will sediment more rapidly. Thus the present attempt on sedimentation analysis of such complexes was performed under physiological ionic conditions. As a result, the observed sedimentation properties of U-snRNPs of EBs during sucrose gradient centrifugation and the subsequent electrophoretic analysis of the RNA should faithfully reflect the intracellular status of complexes between U-snRNPs and hnRNPs *in vivo*. From our present experiments on the sedimentation properties of such complexes, it is suggested, first, that some members of the populations of U-snRNPs exist as free particles, some of them are associated with the large nuclear RNP particles (which may correspond to the monomeric particles of hnRNPs described by H. Gallinaro and M. Jacob [4]), and some are associated with the larger nuclear RNP particles (which may be the heterogeneous assemblies of U-snRNPs and hnRNPs that are thought to be the sites of processing of RNA, and in which splicing may take place in the nuclei of EBs). Second, it is suggested that all major species of U-snRNP are associated with the larger nuclear RNP particles. This suggestion im-

plies that all species of at least the major nucleoplasmic U-snRNPs (since we cannot detect minor species of U-snRNP in EBs by our present method) are component parts of larger nuclear RNP particles. Third, the result that relative abundance of the various species of major U-snRNPs is observed to vary among the fractions of the gradients suggests the characteristically diverse nature of U-snRNP-hnRNP complexes. From our results, therefore, it appears that further detailed clarification on the molecular level of the nature of the larger nuclear RNP particles in EBs will lead to a fuller understanding of the characteristics of the assemblies of U-snRNPs and hnRNPs *in vivo*.

Gurney and Eliceiri indicated that U1 RNA usually leaks into the cytoplasmic fraction (~10% of the cellular contents), during aqueous fractionation of the cells [6]. Eliceiri suggested that newly synthesized precursors to snRNA are detected first in the cytoplasm, before they are eventually found in the nucleus [3]. Thus, it is of interest to investigate the status of U-snRNPs and/or U snRNAs in the cytoplasmic fractions. Therefore, the post-mitochondrial cytoplasmic fractions were also fractionated by centrifugation on 15–40%/50% sucrose gradients. Fig. 3 shows that some leakage of U-snRNPs from the nuclear fraction into the cytoplasmic fraction certainly occurred during aqueous fractionation of the cell, but no selective leakage of any particular species of U-snRNP was detected. Fig. 3 also reveals some species of RNAs longer than U1a/b and U2 snRNAs, respectively (in lanes 15–19, they are indicated by arrow heads). Some of the longer species may be cytoplasmic precursors to the snRNAs U1a/b and U2. We do not know at present whether these RNAs are free or whether they are complexed with any of proteins that make up the snRNPs. More experiments are in progress to resolve these questions.

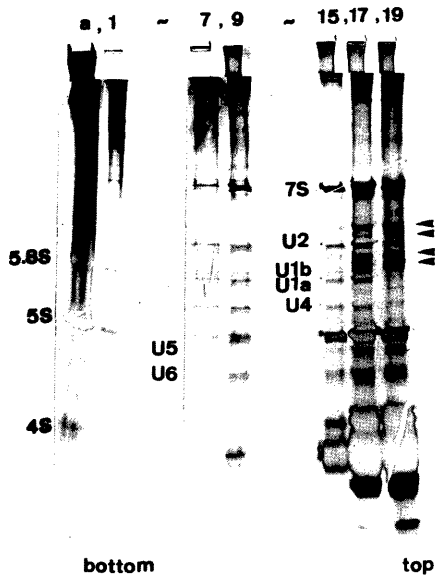

Fig. 3. Analysis of RNA from each fraction obtained by centrifugation on 15–40%/50% sucrose gradients of the post-mitochondrial cytoplasmic fraction prepared from EBs. The lanes in the gel correspond directly to numbered fractions in the gradient. Total cytoplasmic RNA was also fractionated in the gel (a).

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要　約

生理的イオン強度下でのショ糖勾配遠心法とRNAゲル電気泳動法を用いたマウス胚様体（OTT6050）U-snRNPの沈降特性に関する研究：松田基夫（麻布大学獣医学部分子生物学教室）——マウス胚様体核抽出液のショ糖勾配遠心法（5-20%）とRNAゲル電気泳動法による分画実験でU-snRNP（U1a, U1b, U2, U4, U5とU6-snRNP）は4-18s領域を中心として沈降するが，一部はグラジェントの広い領域に渡っても沈降する事が明らかとなった．これは胚様体U-snRNPは大きなリボ核蛋白質複合体（large nuclear RNP）から遊離した状態で存在する部分とassociateした状態で存在する部分がある事を示唆している．また分画されたグラジェントのフラクション間で，U-snRNPのそれぞれの分子種の相対量に関して差異が認められた．更にこれらすべてのU-snRNP分子種が15-40%/50%ショ糖勾配遠心法でチューブの底に近い領域（≥60s）で検出され，larger nuclear RNPとassociateしている事が示唆された．ミトコンドリア除去後の細胞質画分を用いた同様の実験で，水溶液中での細胞分画の際の核分画からのU-snRNPの漏出が示唆されたが，U-snRNPの特定の分子種に関する特異的な漏出は認められなかった．他の細胞系で「UsnRNA前駆体がまず細胞質に現われる」事が報告されているが，この前駆体に相当するかもしれないU1a/bとU2 snRNAよりそれぞれ長いいくつかの胚様体RNA分子種が細胞質画分をショ糖勾配遠心法で分画した際のいくつかのフラクション中で多量に検出された．また生理的イオン強度下でこの様な沈降特性を観察する事の重要性について考察した．