RAT LIVER NUCLEAR SKELETON AND SMALL MOLECULAR WEIGHT RNA SPECIES

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ABSTRACT

Small molecular weight RNA species (smwRNAs) were studied in rat liver nuclei with and without chromatin as well as with and without nuclear envelope and nucleoplasm. From all the species identified, only two, N5 and 5Sb, were related to ribosomes. The others were localized exclusively in the nuclear skeleton or the spongelike network that was described in the preceding communication. This network or protein matrix contains a less abundant but exclusive set of molecules designated 5Sa, N1, and 4.5S, as well as other more abundant molecules which also exist in rat liver endoplasmic reticulum but not in polysomes or postribosomal RNP complexes. The smwRNAs behave like HnRNA; they remain located in the nuclear skeleton when nuclei are deprived of nucleoplasm and chromatin. With the information presently available, it is not possible to know whether both species are in the same or different RNP complexes and whether some of the smwRNAs contribute to the architecture of the nuclear skeleton. Distinct from any other nuclear RNA species, smwRNAs have two unique properties: facility of extraction, and resistance to nuclear ribonuclease digestion.

KEY WORDS nuclear skeleton · ribonucleoprotein complexes · small molecular weight RNA

For several years it has been known that the nucleus contains relatively small molecular weight RNAs (smwRNAs), varying in size from 80 to 200 nucleotides (5, 11, 19, 26, 27, 33, 34). They are different from the 5S which is associated with the large ribosomal subunit, the 5.5S which is hydrogen-bonded to the 28S rRNA, and transfer RNAs. Some of these have been purified and their nucleotide sequences are known (26–28). With some exceptions, they are not exclusively located in the nucleus, and in the cytoplasm they exist associated with membranes of the endoplasmic reticulum (34).[1] Their role in either transcription or translation, is unknown.

Several workers have suggested that some of these RNAs are artifacts arising from ribosomal degradation (2, 13, 32). Others have claimed that some species are exclusively associated with chromatin (20), nuclear particles that contain rapidly labeled RNA (29), nucleoli (27), nuclear skeleton (27, 34), and/or that they are free in the nucleoplasm (27, 34). In this report we present evidence

[1] T. E. Miller and A. O. Pogo. Manuscript in preparation.

that in rat liver nuclei none of these RNAs arise as a result of ribosomal degradation. Like heterogeneous RNA (HnRNA), they are located exclusively in the nuclear skeleton. Finally, they have the unique property of being highly resistant to endogenous ribonuclease digestion. A preliminary report has been presented elsewhere (21).

MATERIALS AND METHODS

Isolation of Rat Liver Nuclei

The isolation of rat liver nuclei from female rats weighing 175–200 g was performed according to the method of Pogo et al. (25). In order to dissolve lipids of the nuclear envelope, nuclei were washed twice with 40 vol of a buffer solution containing 10 mM Tris-HCl, pH 7.5, 23°C, 2.5 mM $MgCl_2$, and 0.25 M sucrose (TMS) to which the protease inhibitor, 0.5 mM phenylmethyl sulfonyl chloride or fluoride (PMSF or PMSC), was added. Triton X-100 was added to a final concentration of 1% and the mixture was incubated for 10 min at 0°C. The detergent-treated nuclear suspension was centrifuged at 1,000 *g* for 15 min, and the pellet was washed twice with 40 vol of TMS containing the proteolytic inhibitor.

RNA Extraction

Several methods of RNA extraction were tested, and the conventional method of phenol-cresol or phenol-chloroform was adopted, as no significant difference in yields of smwRNAs was observed. The phenol-cresol method was used as explained by Parish and Kirby (23). A pellet of packed nuclei was resuspended in 5 vol of TMS and frozen overnight.[2] After thawing, 0.1 vol of a solution containing 1 M NaCl, 0.1 M EDTA, 0.1 M Tris-HCl, pH 7.4, 23°C (×10 NET) was added. Enough ×1 NET was then added to give a final ratio of packed nuclei to buffer of 1:40 (vol/vol). The final suspension was made 0.2% with sodium dodecyl sulfate. An equal volume of freshly distilled phenol-cresol saturated with distilled water and containing 0.08% hydroxyquinoline was added, and the mixture was vigorously stirred for 20 min at 23°C. The aqueous and phenol phases were separated by centrifugation at 16,000 *g* for 20 min. The aqueous phase was made 0.5 M with LiCl, and an equal volume of phenol-cresol was added, mixed, and the mixture was centrifuged as described above. 2.5 vol of cold ethanol was then added, and the preparation was kept overnight at −20°C. The precipitate was collected after centrifugation at 15,000 *g* for 20 min in a Sorvall RC-2B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). After the RNA was washed with ethanol, it was dissolved in a buffer solution containing 0.1 M NaCl and 0.05 M Na acetate at pH 5.1. No contaminant DNA was detected by the Burton reaction (4) when RNA was extracted by this procedure.

The phenol-chloroform procedure was performed according to Perry et al. (24). To 1 vol of packed nuclear pellet resuspended in 5 vol of TMS buffer solution, 0.1 vol of a solution containing 1 M NaCl, 50 mM EDTA, and 1 M Na acetate, pH 5.1, was added. The suspension was then made 0.5% with sodium dodecyl sulfate, and an equal volume of phenol-chloroform (1:1, vol/vol) saturated with a buffer solution containing 0.1 M NaCl, 5 mM EDTA, and 0.1 M Na acetate, pH 5.1, was added and the mixture was mixed vigorously for 20 min at 23°C. The aqueous and phenol phases were separated by centrifugation at 16,000 *g* for 20 min in a Sorvall RC-2B centrifuge (Ivan Sorvall, Inc.). The phenol phase and the interlayer were reextracted with the same buffer, and after centrifugation the two aqueous phases were combined. The combined aqueous phase was extracted several times with an equal volume of phenol-chloroform until there was no visible interlayer after centrifugation at 16,000 *g* for 20 min. 2.5 vol of cold ethanol were added to the final aqueous phase, the DNA fibrils were removed by spooling, and the solution was stored overnight at −20°C. The precipitate was centrifuged and washed with cold ethanol. The RNA was dissolved in a buffer solution containing 0.01 M Tris-HCl, pH 8, 23°C, 0.01 M NaCl, and 0.01 M $MgCl_2$. DNase 1 (Worthington Biochemical Corp., Freehold, N.J.) was added to a final concentration of 100 μg/ml (DNase was treated with iodoacetic acid as reported earlier [35]), and incubated at 36°C for 10 min. The RNA was extracted by phenol-chloroform as previously described.

Proteinase K Treatment of Rat Liver Nuclei and RNA Extraction

A packed nuclear pellet was resuspended in 5 vol of TMS buffer, and to this 0.1 vol of a buffer solution containing 0.5 M Na acetate, pH 5.1, and 0.05 M EDTA was added. To this suspension, proteinase K (EM Laboratories, Inc., Elmsford, N.Y.) was added at a concentration of 500 μg/ml, and the suspension was incubated at 0°C for 20 min. The suspension was made 0.5% with sodium dodecyl sulfate and incubated at 37°C for 2 h. To this, 0.1 vol of a solution containing 1 M NaCl and 0.5 M Na acetate, pH 5.1 was added, followed by an equal volume of phenol-chloroform. RNA extraction and elimination of contaminating DNA was performed as previously described.

Nuclear Disruption in a Nitrogen Cavitation Bomb

A pellet of packed nuclei (Triton-treated) was resuspended in 5 vol of TMS containing 0.5 mM PMSF or PMSC, and disrupted in a nitrogen cavitation bomb at 1.2–1.4 lb/in² as described earlier (7). The disrupted nuclei were centrifuged at 16,000 *g* for 10 min. The supernate or nucleoplasmic fraction was decanted and

[2] It was observed that freezing and thawing were essential for an efficient extraction of RNA at 23°C.

the pellet, which contained the bulk of DNA, nuclear proteins, RNA, and lipids, was resuspended in TMS in the same volume as the supernate. RNA from both fractions was extracted by the phenol-cresol method as previously described.

Chromatin Extraction of Rat Liver Nuclei and RNA Isolation

The high salt buffer treatment and DNase digestion in the presence of proteolytic inhibitor was performed as described in the preceding paper (22).

Achromatinic nuclei were obtained after the treated nuclei were layered on top of a 30–68% sucrose gradient containing 5 ml of a 72.5% sucrose cushion and centrifuged in a Beckman SW27 rotor at 18,000 rpm for 18 h. Fractions of the achromatinic nuclei were pooled and diluted with 1 vol of distilled water. 2 vol of ethanol were added to the final suspension of achromatinic nuclei and the suspension was stored at −20°C overnight. The precipitate was collected by centrifugation in the Sorvall RC-2B (Ivan Sorvall, Inc.) at 4,000 *g* for 30 min. The pellet was resuspended in 5 vol of a solution containing 0.1 M NaCl, 0.05 M Na acetate, pH 5.1, and 5 mM EDTA. RNA was extracted by the phenol-chloroform method as previously described.

The first 20 fractions of this gradient were also pooled, but not diluted with distilled water; 2 vol of ethanol were added and the solution was precipitated overnight at −20°C. The precipitate was collected by centrifugation in the Sorvall RC-2B (Ivan Sorvall, Inc.) at 4,000 *g* for 30 min. The pellet was resuspended in 5 vol of a solution containing 0.1 M NaCl, 0.05 M Na acetate, pH 5.1, and 5 mM EDTA. RNA was extracted by the phenol-chloroform method as previously described. The contaminating DNA was removed by Whatman CF-11 cellulose chromatography as described earlier (10).

Sucrose Gradient Analysis of Isolated RNA

The isolated samples were resuspended in a solution containing 0.1 M NaCl and 0.05 M Na acetate, pH 5.1, layered onto a 5–40% sucrose gradient containing 0.1 M NaCl and 0.05 M Na acetate, pH 5.1, and centrifuged in a Beckman SW41 rotor at 26,000 rpm for 18 h. The gradients were fractionated with an ISCO gradient fractionator (ISCO (Instrumentation Specialties Co.), Lincoln, Nebr.) equipped with a UV absorption recorder, at 254 nm, with a 1-cm path length. Fractions of the gradient containing RNA sedimenting slower than 18S RNA were collected and precipitated by adding 2.5 vol of ethanol. The precipitated RNAs were dissolved in the appropriate buffer and tested for contaminating DNA by the procedure of Burton (4).

Polyacrylamide Gel Electrophoresis

The precipitated RNA was dissolved in a buffer solution containing 0.04 M Tris-acetate, pH 7.2, 23°C, 0.02 M Na acetate, and 2 mM EDTA. For electrophoresis under nondenaturing conditions, the method of Hodnett and Busch was employed (14). For electrophoresis under denaturing conditions, the buffer solution was made 8 M with urea, heated at 70°C for 10 min, and the electrophoresis was performed in gels containing 8 M urea.

About 40–100 μg of RNA were usually added per gel, and electrophoresis was performed at 5 mA. In a parallel gel, 5S and 4S cytoplasmic RNAs were also subjected to electrophoresis. The gels were scanned at 260 nm in an Acta III spectrophotometer (Beckman Instruments Inc., Science Essentials Co., Mountainside, N. J.) and stained with methylene blue as follows: after electrophoresis the gels were soaked in 1 M acetic acid for at least 1 h and stained in a solution containing 0.2% methylene blue, 0.2 M Na acetate, and 0.2 M acetic acid for at least 2 h. The gels were destained overnight in running water.

Determination of the Amount of Each smwRNA Species

The paper tracing procedure, as explained in the preceding paper (22), was used to measure amounts of UV-absorbing materials in the sucrose gradient and in the polyacrylamide gel. Picograms per nucleus of each molecule were calculated by knowing the amount of RNA extracted (9) and the number of nuclei. Knowing the UV absorbing material, shown as the shaded area in the respective figures, and the total UV-absorbing material in the sucrose gradient, the amount of RNA sedimenting more slowly than 18S was calculated. From this value, it was possible to estimate the amount of each RNA molecule in the polyacrylamide gels by measuring each UV absorption peak and the total area of UV absorption material above background. The general formula for these calculations is:

$$\text{pg RNA per nucleus} \times \frac{Ss}{Ts} \times \frac{Se}{Te} = \text{pg of each smwRNA species,}$$

where *Ss* is the area of total smwRNAs sedimenting in the sucrose gradient and *Se* the individual absorption peak in the polyacrylamide gels; *Ts* is the total area in the sucrose gradient and *Te* is the total UV absorption material in the polyacrylamide gels. Determinations were made in triplicate (three different gels) of the original UV scannings.

These calculations are possible as all extracted smwRNAs are isolated from the sucrose gradient and electrophoresis in 4% gels showed that all these mole-

cules enter the gel, and for short periods of electrophoresis, no molecules run off the gels.

Electron Microscopy

Suspensions of Triton-treated and untreated nuclei were fixed and embedded as explained in the preceding communication (22). Electron micrographs were made with a Philips 201 electron microscope at original magnifications in the range of ×3,000 -×70,000.

RESULTS

SmwRNA in Nuclei With and Without Nuclear Envelopes

The quantitation and distribution of different nuclear smwRNAs as well as other RNAs species cannot be accurately made due to the major difficulty of knowing how much is extracted. Moreover, activation of endogenous ribonucleases during nuclear disruption and fractionation makes these determinations in subnuclear fractions unreliable. However, smwRNAs have two important properties, facility of extraction and high resistance to digestion by endogenous ribonucleases. Several procedures of extraction were tested, ranging from the recent report by Holmes and Bonner (15), which employed 8 M urea, to the more conventional methods, which used phenol-cresol (23) and phenol-chloroform (24); all of them yielded the same amount and pattern of distribution. Furthermore, the smwRNAs can be extracted equally at low and high pH, as well as at room temperature and at 65°C. Moreover, it was found that when nuclei were incubated with proteinase K at 37°C for 2 h, smwRNAs were the only molecules which survived. Thus, all of the smwRNAs can still be identified by polyacrylamide gel electrophoresis after this drastic treatment (Fig. 1). In view of these unique properties, it is not unreasonable to assume that determination and distribution of smwRNAs in intact nuclei as well as subnuclear fractions reflect, to a large extent, true values.

The smwRNA species that we identified in rat liver nuclei, both treated and untreated with Triton X-100, are shown in Fig. 2. Also shown is the UV profile of stable, high molecular weight RNAs: 18S, 28S fractionated in a sucrose gradient (Fig. 2, inset). Because different laboratories have assigned different nomenclatures for the same molecule, and because none of the species that we studied were hydrogen-bonded to 32S or 28S

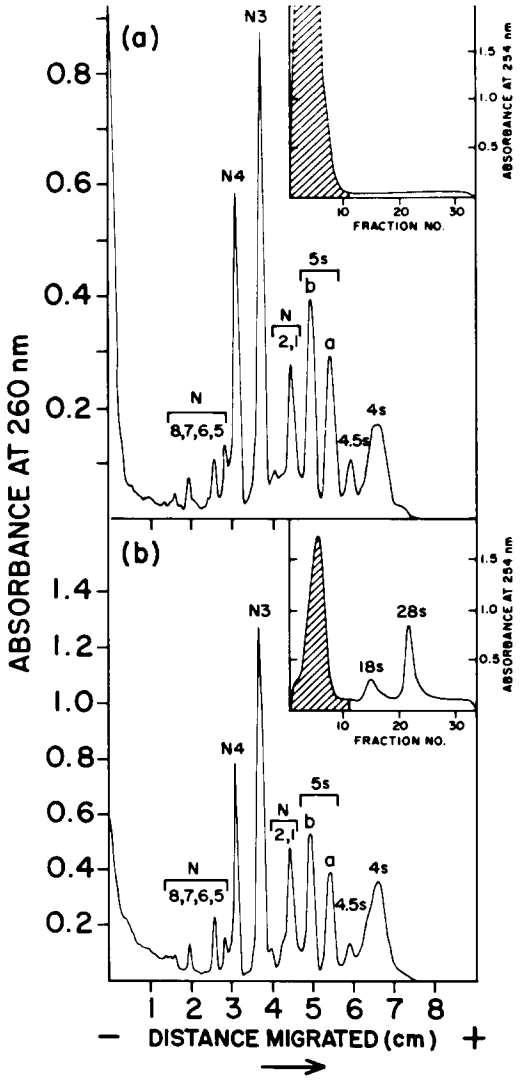


FIGURE 1 Effect of endogenous ribonuclease digestion on smwRNAs. (*a*) Nuclei were incubated with proteinase K for 2 h at 37°C and extracted by the phenol-chloroform method as explained in Materials and Methods. (*b*) Nuclei were not incubated and RNA was extracted as in (*a*). Inset shows UV profile of sucrose gradient sedimentation. Electrophoresis was performed with the material sedimenting more slowly than 18S (shown by shaded area) in 10% polyacrylamide gels for approximately 3 h.

rRNA (all smwRNAs were extracted at room temperature), it was decided, for the sake of clarity, to enumerate them according to their relative mobilities with respect to the well-charac-

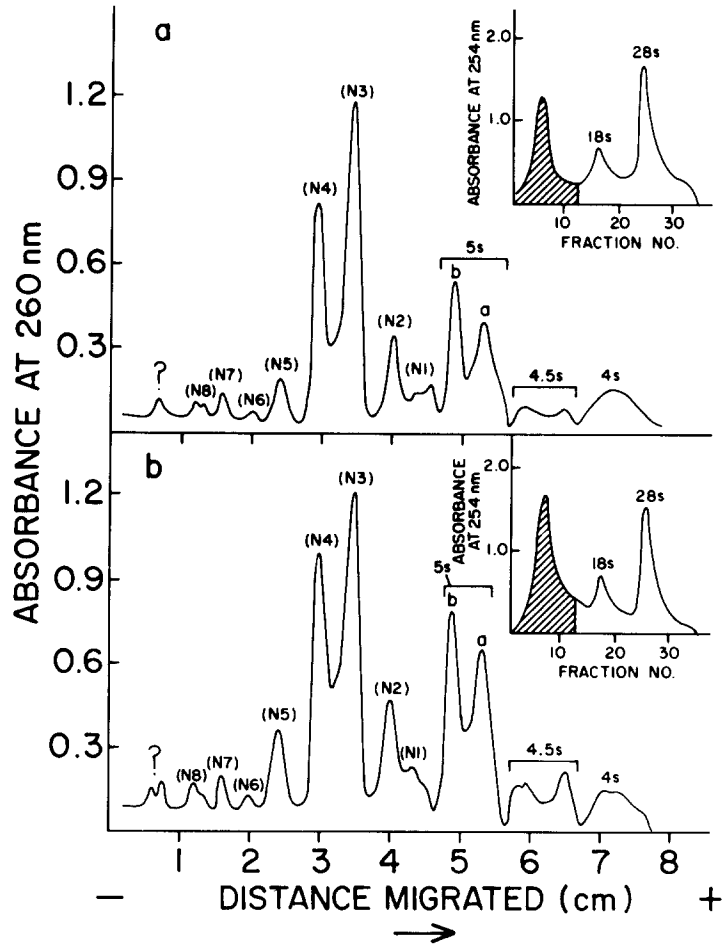


FIGURE 2 RNA extracted from Triton-treated and untreated nuclei. Nuclei were treated with Triton X-100 as explained in Materials and Methods, and RNA was extracted from both treated and untreated nuclei by the phenol-cresol method. Inset shows the sucrose gradient sedimentation. RNA sedimenting more slowly than 18S (shown by shaded area) was used for electrophoresis analysis. The samples were denatured as explained in Materials and Methods, and electrophoresis was performed in 10% gels containing 8 M urea for twice the time that it took the dye (bromophenol blue) to reach the end of the gel (~6 h). (*a*) Triton-treated nuclei, (*b*) untreated nuclei.

terized 5S and 4S cytoplasmic RNAs. Because we extracted them from the same type of cell and resolved them with the same electrophoretic technique, it was possible to make an unambiguous assignment. In general, the major species that we identified were similar to those studied in other cells by Zieve and Penman (34), as well as Ro-Choi and Busch (27).

The experiment described in Fig. 2 indicates that dissolution of lipids of the nuclear envelope did not alter the pattern and distribution of 18S, 28S, and smwRNAs species. A similar observation was made by Dingman and Peacock (5). It seems that detergent treatment does not release ribosomes attached to the outer nuclear membrane as well as to the nuclear pore complexes. However, to rule out the possibilities that the method of nuclear isolation detached the outer nuclear membrane of the nuclear envelope, and that the 18S–28S rRNA, in both treated and untreated nuclei were from the intranuclear pool of ribosomal subunits in transit to the cytoplasm, electron microscope studies were performed (Fig. 3*a* and *b*). Both treated and untreated nuclei

contain ribosomes attached to the nucleus. At the present time, it is unknown what forces affix these ribosomes to the outer membrane of the nuclear envelope. It is obvious that the ribosomes must remain attached to the protein matrix of this membrane as well as to the pore complexes, as detergent treatment dissolves more than 85% of the total lipids of the nucleus (1, 5, 7, 16, 17).

In the absence of the nuclear envelope, none of the smwRNAs were released from the nucleus. A similar amount and proportion of each molecule were recovered from treated and untreated nuclei (Table I). It is concluded that none of these molecules leach out from a nucleus deprived of its lipid components.

We have detected, between the 4S and 5S peaks, what Ro-Choi and Busch (27) designated 4.5S, and Zieve and Penman (34) designated SnH. It is not an abundant species, and in our electrophoretic runs it can be seen as two and sometimes three peaks (Fig. 2). It has been reported that this molecule consists of three subspecies (27). Nuclear 5S RNAs are of three different kinds (27). One is associated with the large ribosomal subunits whose abundance depends upon the preservation of the ribosomes studded on the outer nuclear membrane of the nuclear envelope, the amount of 60S ribosomal subunits inside the nuclei, and the size of the nucleolar pool of this molecule which remains unassembled to the 60S subunits. We have designated this molecule as 5Sb whose mobility is identical to that of the predominant cytoplasmic 5S. There is another nuclear 5S that we have designated 5Sa, which is not homodisperse with this electrophoretic technique. This molecule, moving faster than the 5Sb, has been designated 5SIII by Ro-Choi and Busch, and SnG′ by Zieve and Penman, and has been shown to be methylated (27, 33). It has a very different base sequence than the ribosomal 5S (27). The third 5S molecule that exists in the rat liver nucleus is the 5.5S which is hydrogen-bonded to the 28S rRNA. We observed it when we extracted RNA at 65°C or with high urea concentrations. It appears that this molecule is released as a monomer and a dimer, and that it is cleaved from the 32 ribosomal precursor (27, 33, 34).

Between the 5S and the other two prominent nuclear peaks, we always found less abundant molecules, designated N1 and N2. N1 is not homodisperse and is never observed in the rat liver cytoplasm. However, N2 has been observed in association with rat liver rough endoplasmic reticulum.[1] N2 has a faster mobility than N3 and might correspond to one of the UI RNA subspecies, most probably the U1a that Ro-Choi and Busch separated by chromatography on benzoylated diethylaminoethanol (DEAE)-cellulose (27).

The most prominent rat liver nuclear smwRNA is the N3 fraction. This molecule has been found and designated as SnD by Zieve and Penman (34), and U1b by Ro-Choi and Busch (27) in HeLa cells and Novikoff ascites hepatoma, respectively. A clear precursor-product relationship can be shown between this molecule and a rapidly labeled cytoplasmic species first described in HeLa cells by Elicieri (6). Another abundant smwRNA is N4, which in HeLa cells has been designated SnC, and in Novikoff cells U2. It has been sequenced in the latter cells (28). It is methylated at the 5′ terminal oligonucleotides, and it is synthesized only once in the cell cycle (28, 34). We observed the N5 fraction with a slower mobility than N4, which, according to Ro-Choi and Busch (27) is of nucleolar origin and has been designated as U3; in HeLa cells it has been named SnA. Much less abundant, but nevertheless, well-defined molecules are always observed with mobilities slower than that of N5. We have designated them as N6, 7, and 8, and they are very prominent in rat liver rough endoplasmic reticulum.[1] The presence of these molecules in detergent-treated nuclei is an indication that they do not arise from cytoplasmic contaminants.

TABLE I

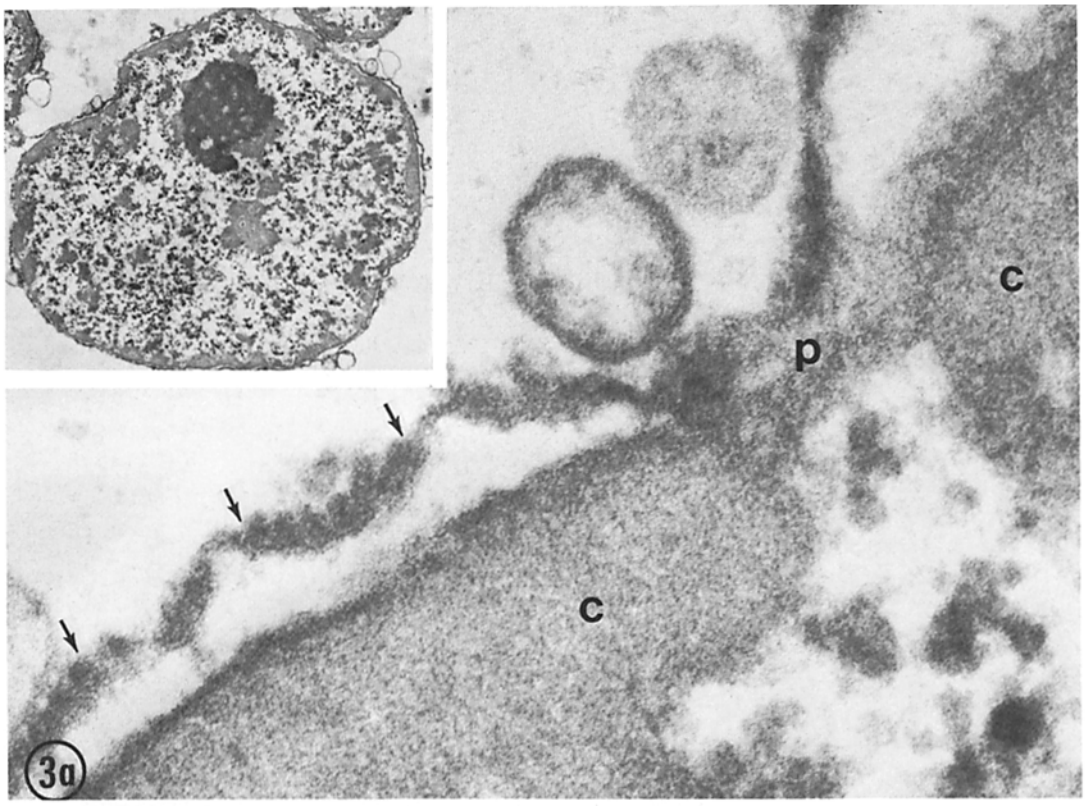
Estimated PG × 10^{-3}/Nucleus of smwRNAs

	N8,7,6*	N5*	N4‡	N3‡	N2,1‡	5Sb*	5Sa*	4.5S*
Nuclei	7.5	7.2	27.6	51.4	23.9	28.8	19.1	14.2
Triton-treated nuclei	9.0	9.8	29.4	51.3	27.0	38.3	22.2	21.6

The amount of each smwRNA species was estimated from Triton-treated and untreated rat liver nuclei as explained in Materials and Methods.

* Range of estimation ±15%.

‡ Range of estimation ±5%.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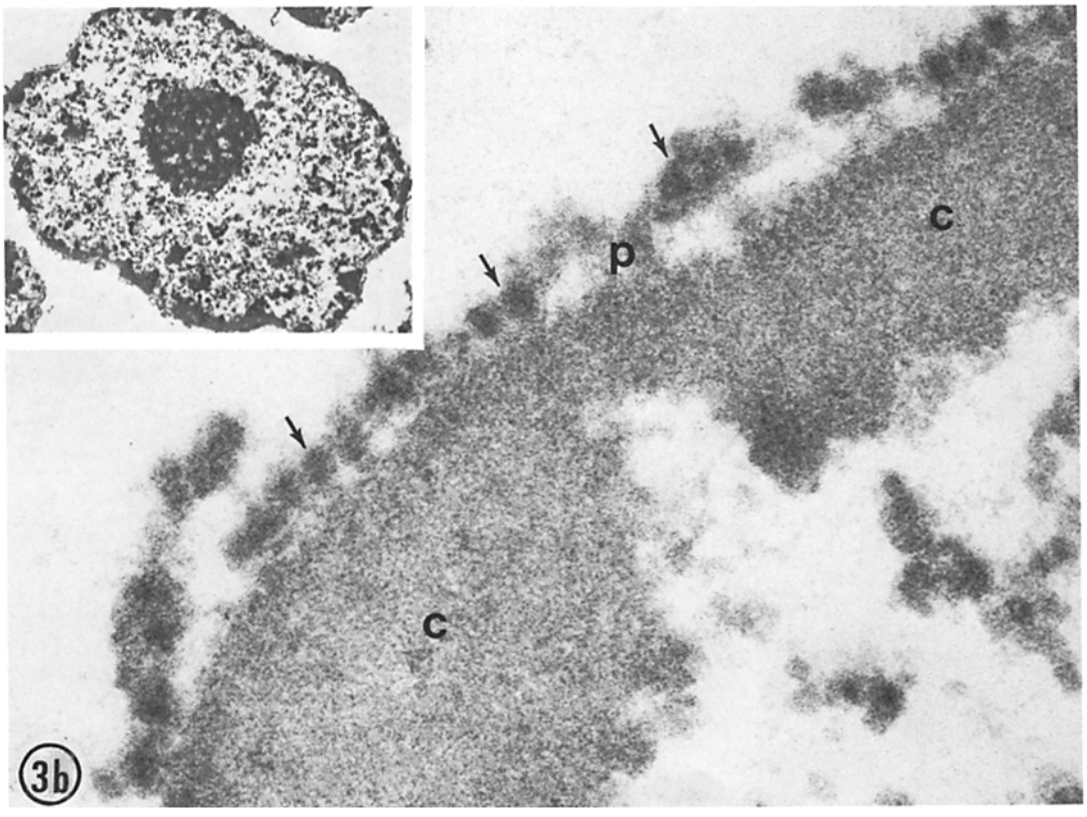
c
p
c
3b

SmwRNA in Achromatinic Nuclei and Nucleoplasm

A nuclear structure deprived of chromatin has been isolated and described in the preceding publication (22). Fig. 4 shows the polyacrylamide gel electrophoresis of smwRNAs extracted from total nuclei and from achromatinic nuclei. Fig. 5 shows the densitometric tracings of smwRNA molecules that remain in the achromatinic nuclei and those that have been released after chromatin extraction and purification through a 30–68% sucrose gradient.

The amounts of smwRNA attached and released from achromatinic nuclei are shown in Table II. A small amount of the major molecules is released when nuclei are deprived of their chromatin. The 4.5S, 5Sa, N1, N2, N3, and N4 molecules, which can be considered to be nonnucleolar molecules, remain attached to the sponge-like network that we described in the preceding paper (22). Furthermore, the 4.5S molecule, which has been claimed to be limited in location to the chromatin or nucleoplasm (27), remains attached to the nuclei after HSB-DNase-PMSC treatment and centrifugation throughout the sucrose gradient, a procedure which removes both chromatin and nucleoplasm. This is also true for the 5Sa. In addition, N3, the most abundant smwRNA, is hardly released from the nuclear skeleton. It has been claimed that this molecule is released as RNP particles during a brief warming of the nucleus (34). Inasmuch as endogenous proteolytic activity releases RNP complexes containing HnRNA from the nuclear skeleton, it is not unreasonable to assume that this activity is also responsible for the release of N3 molecules as RNP particles from HeLa cell nuclei.

Not all rat liver nuclei survive the HSB-DNase-PMSC treatment. Therefore, it is obvious that

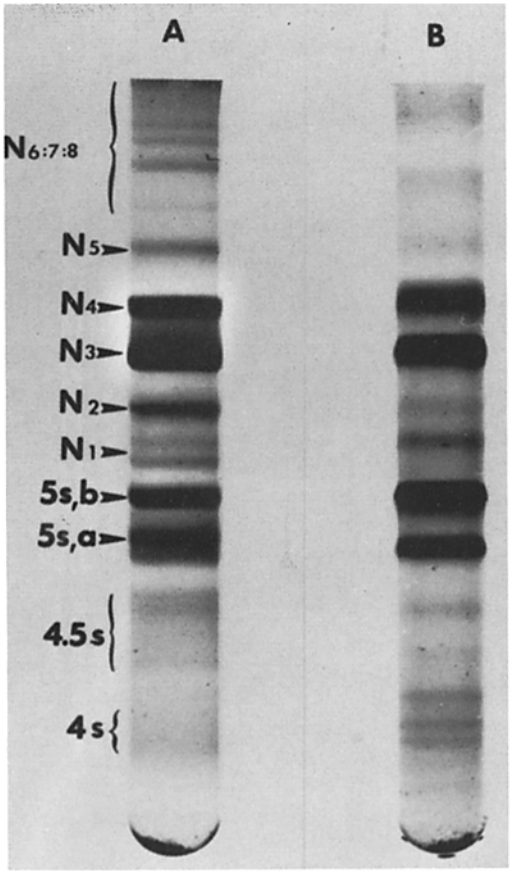

FIGURE 4 Polyacrylamide gel electrophoretic pattern of smwRNAs extracted from total nuclei (*A*) and achromatinic nuclei (*B*). RNA sedimenting more slowly than 18S was used for electrophoresis analysis. The samples were denatured as explained in Materials and Methods and treated as explained in Fig. 2. Direction of migration from top to bottom. The gels were stained with methylene blue as explained in Materials and Methods.

FIGURE 3 (*a*) Rat liver nuclei were isolated and washed twice with TMS as explained in Materials and Methods. A portion of the nucleus is shown. × 210,000. Inset shows a whole nucleus. × 9,000. The outer and inner membranes of the nuclear envelope are well preserved. Ribosomes (arrows) are studded on the outer membrane and near the nuclear pore (*P*). The inner membrane of the nuclear envelope is clearly shown on the surface of the chromatin (*C*). (*b*) Rat liver nuclei were isolated, washed twice with TMS, and treated with 1% Triton X-100 as explained in Materials and Methods. A portion of the nucleus is shown. × 210,000. Inset shows a whole nucleus. × 9,000. The nuclear envelope has disintegrated. Both the outer and inner membranes have disappeared almost completely, but ribosomes (arrows) still remain in a row adjacent to a stripped nucleus. (*P*) indicates nuclear pore area and (*C*) indicates chromatin.

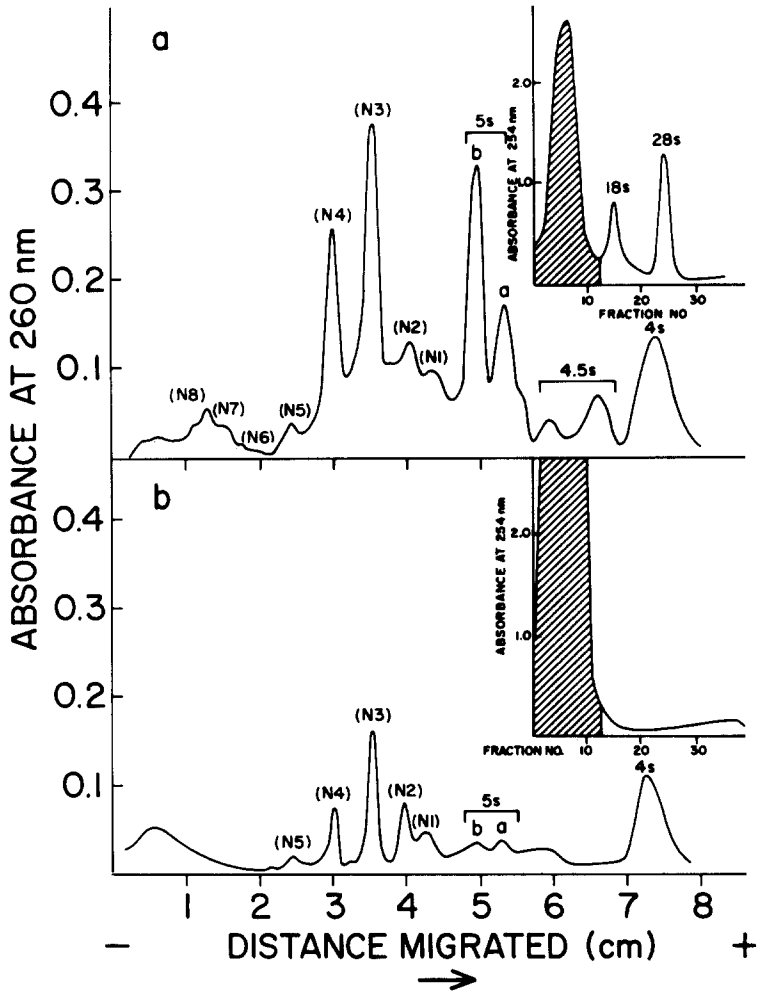

FIGURE 5 RNAs were extracted by phenol-chloroform from achromatinic nuclei (*a*) and from the nuclear structures that sediment on top of the 30–68% sucrose gradient (*b*) as explained in Materials and Methods, and in Miller and Pogo (22). Inset shows the sucrose gradient sedimentation. RNA sedimenting more slowly than 18S (shown by shaded area) was denatured and electrophoresis was performed as explained in Fig. 2.

what was released might well be smwRNA molecules still attached to fragments of nuclear skeletons sedimenting on top of the sucrose gradient (22). The quantity of molecules which remain attached to the achromatinic nuclei agrees very well with the amount of rapidly labeled RNA which remains attached to the same structure. These recoveries are also consistent with the number of nuclei that survive the treatment. It is concluded that the major localization of these molecules is limited to the nuclear skeleton or the spongelike network that we described in the preceding publication (22). It follows that the so-called "released molecules" or nucleoplasmic molecules arise from either disrupted or digested nuclear skeletons. This might be true for other cell types.

In order to examine the possibility that the HSB-DNase-PMSC treatment may cause spurious attachment of smwRNA to the nuclear skeleton, rat liver nuclei were disrupted in a nitrogen cavitation bomb. This procedure does not produce the release of RNP complexes containing rapidly labeled RNA (7). Fig. 6 shows the densitometric pattern of released and attached molecules, and Table III shows their amounts. No significant

TABLE II

Estimated PG × 10^{-3} of Attached and Released smwRNAs per Achromatinic Nuclei

	N8,7,6*	N5*	N4‡	N3‡	N2,1*	5Sb‡	5Sa‡	4.5S*
Achromatinic nuclei (*a*)	8.8	3.5	20.9 (83%)	37.7 (80%)	25.2 (73%)	27.4	19.8	13.5
Released (*b*)	–	–	4.3 (17%)	9.4 (20%)	9.2 (27%)	–	–	–

The amount of each smwRNA species present in achromatinic nuclei (*a*) was estimated as explained in Materials and Methods. (*b*) the amount of smwRNAs which were in the nuclear structures sedimenting on top of the sucrose gradient were estimated by a different procedure inasmuch as a large proportion of the UV absorption material ran off of the gel. It was calculated by relating the area of each peak to the corresponding area in (*a*). This calculation was possible because the smwRNAs in both fractions were resuspended in equal volumes of the buffer solution, and the same volumes were applied to the gels. Notice that these estimations do not include the RNAs present in those nuclear structures sedimenting between the 20 top fractions and the region of the sucrose gradient where achromatinic nuclei sediment. They represent 10% of the total nuclear RNA.

* Range of estimation ±15%.

‡ Range of estimation ±5%.

difference exists in the amount of the major molecules released when nuclei are disrupted and fractionated in this manner versus the HSB-DNase-PMSC method. Furthermore, the pattern distribution and amounts of smwRNA which remain with the bulk of the nuclear structures, the 16,000 *g* pellet, are similar to those that are observed in achromatinic nuclei. The same is true if nuclei are disrupted in low salt buffers, but by compression and decompression in a French pressure cell. The latter is a strong mechanical procedure which produces the release of ~50% of the HnRNA as RNP particles (7, 8).

DISCUSSION

In this report we have examined the smwRNA species found in rat liver nuclei with and without chromatin, as well as with and without nuclear envelope and nucleoplasm. The most important conclusion drawn from this study is that stable smwRNAs are localized exclusively in the nuclear skeleton. These RNA molecules behave like rapidly labeled RNA, i.e., they remain attached to the nuclear skeleton when nuclei are deprived of nucleoplasm and chromatin. Therefore, this skeleton is formed by a protein matrix in which HnRNA and smwRNA are attached. With the information presently available, it is not possible to know whether both species are in the same or different RNP complexes. Thus, when rat liver nuclei are disrupted by a drastic mechanical procedure, such as compression and decompression in a French pressure cell, a method which is known to produce RNP complexes containing HnRNA (7, 8), only a small amount of smwRNA appears associated with, or co-sediments with, the RNP complexes. The majority sediments on top of the sucrose gradient (unpublished results). Therefore, there are two possibilities. One is that HnRNA and smwRNAs are attached to the nuclear skeleton but are not in the same RNP complexes. The other is that there is a core structure formed by the association of smwRNAs with proteins which is covered by HnRNA.

The observation that smwRNAs are located exclusively in the nuclear skeleton differs from what has been reported by other investigators (20, 27, 33, 34). We believe that a well-preserved nuclear skeleton and, most important, inhibition of proteolysis, are what prevent the release of these smwRNAs. The presence of some smwRNA molecules in the nucleoplasm or associated with chromatin might well be due to the action of endogenous proteolytic activity. At the present time, it is unknown whether the smwRNA species contribute to the architecture of the nuclear skeleton. The possibility exists that some of them are structural components of this skeleton and that others are, like HnRNA, attached to the skeleton. The latter assumption is supported by the observation that RNase treatment not only releases HnRNA but reduces the density of achromatinic nuclei (22).

Among all the smwRNA molecules that we observed, only 5Sb and N5 are related to ribosomes. The 5Sb is attached to the 60S ribosomal subunit. The N5 is somehow involved in ribosomal processing and, therefore, has a specific localization in the nucleolus (27). The remainder must be components of the spongelike network that we described in the preceding paper (22). At the present time, however, it is not possible to know

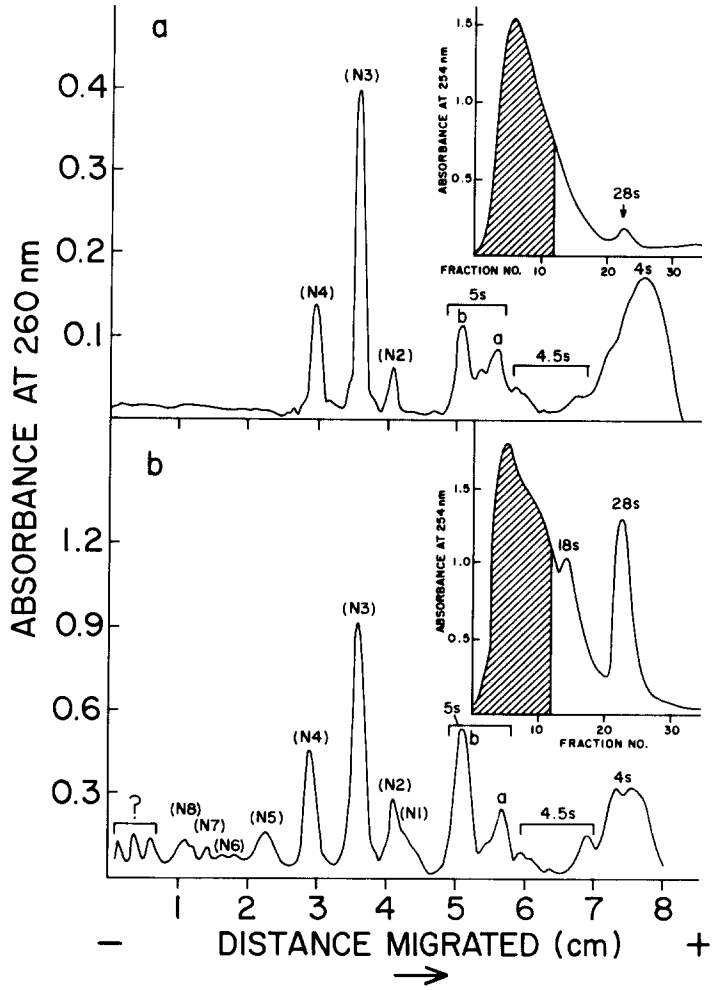

FIGURE 6 RNA was extracted from the supernatant fraction (*a*) and the 16,000 *g* pellet (*b*) of rat liver nuclei was disrupted by treatment in a nitrogen cavitation bomb and fractionated by differential centrifugation as explained in Materials and Methods. Inset shows the sucrose gradient sedimentation. RNA sedimenting more slowly than 18S (shown by shaded area) was denatured and electrophoresis was performed as explained in Fig. 2.

whether some of these molecules are located in the nuclear envelope. In any case, nuclear protein matrix and the protein matrix of the inner membrane of the nuclear envelope are linked and might contain similar components. These assumptions are supported by the EM study of Triton-treated nuclei and by the similarities between RNA species present in treated and untreated nuclei. The protein matrix of the inner and outer membranes of the nuclear envelope, the fibrous lamina, the pore complexes, and the spongelike network that we previously described most probably belong to the same nuclear structure, i.e., the nuclear skeleton. It is worth mentioning that except for the 4.5S, 5Sa, and N1, all the other molecules are present in membranes of the rat liver endoplasmic reticulum. The most prominent are the N2, N7, and N8, and none of these are associated with either free polysomes or ribosomes, or exist free in the soluble cytoplasmic fraction.[1]

In addition to containing unique molecules, i.e. 5Sa, N1, and 4.5S, the nuclear skeleton contains several prominent smwRNAs, the N2, N3, and N4, which also exist in endoplasmic reticulum but not in polysomes. The question that remains to be answered is whether they are also attached to a cytoplasmic skeleton. Such a skeleton has been

TABLE III

Estimated PG × 10^{-3}/Nucleus of smwRNAs in 16,000 g Pellet and Nucleoplasmic Fractions of Nuclei Disrupted with Nitrogen Cavitation Bomb

	N8,7,6*	N5*	N4‡	N3‡	N2,1‡	5Sb‡	5Sa‡	4.5S*
16,000 *g* pellet	5.5	5.5	20.2 (85.6%)	50.2 (81%)	18.0 (90%)	31.5 (88%)	18.1 (77%)	14.6
Nucleoplasm	–	–	3.4 (14.4%)	11.6 (19%)	2.0 (10%)	4.4 (12%)	5.3 (23%)	–

The amount of each smwRNA species present in the bulk of nuclear components (16,000 *g* pellet) and in the supernate (nucleoplasm) was estimated as explained in Materials and Methods.

* Range of estimation ±15%.

‡ Range of estimation ±5%.

inferred from our previous work as well as from the work of others (3, 8, 18, 30, 31). More recently, it has been visualized in HeLa cells (19). In any case, both skeletons differ in the proportion of these smwRNA molecules. Thus, rat liver nuclei are enriched in N2, N3, and N4, and rat liver endoplasmic reticulum in N2, N7, and N8.[1] Some of these species could be related to the shuttling RNA found in amoeba by Goldstein and Ko (12).

Finally, another finding in our studies is that similar smwRNAs are observed in a tissue in which cells, unlike HeLa cells, divide at a low rate and are not malignant. Nevertheless, these two cell types differ in the amounts and proportion of some of their major molecules. For example, the amount of N5 molecules in HeLa cells is as much as twice the amount (estimated number of molecules per nucleus) found in rat liver. On the other hand, N3 and N4, the more abundant molecules in both cell types, are present ~40% less in rat liver than in HeLa cells. The significance of this difference will be known when properties and functions of these molecules are well established.

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