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Small Nuclear RNA Molecules in Nuclear Ribonucleoprotein Complexes from Mouse Erythroleukemia Cells

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ABSTRACT: Ribonucleoprotein (RNP) complexes were prepared by sonic disruption of mouse erythroleukemia (MEL) cell nuclei. After removal of nucleoli and insoluble chromatin by centrifugation, the RNP sedimented on sucrose gradients as two populations: one at 10 S and the other at 30–60 S. Each RNP fraction contained both rapidly labeled and stable nuclear RNA molecules. The bulk of the stable RNA was recovered in the 10 S RNP. No ribosomal or preribosomal RNAs were recovered from the RNP. The labeling of the fast turnover RNA was not suppressed by low concentrations of actinomycin D. Most of the rapidly labeled RNA in both RNA fractions sedimented on sucrose gradients at 8–12 S. The buoyant density of formaldehyde-fixed RNP in CsCl was ρ = 1.38 g/cm^3 (10 S) and ρ = 1.436 g/cm^3 (30–60 S). These are characteristics exhibited by nuclear RNP complexes which contain degraded hnRNP. Both the 10S and 30–60S hnRNP contained several small, stable, monodisperse RNA molecules which belong to a class of cellular RNA called snRNA. The 10S hnRNP contained snRNA species A–E. The 30–60S hnRNP contained snRNA species, A, B, D, and E. No nucleolar snRNA species were recovered in the hnRNP. The snRNAs cosedimented with the hnRNP on sucrose gradients. With the exception of snRNA species A, nearly all of the snRNAs remained associated with that portion of the 10S and 30–60S hnRNP which bound to oligo(dT)-cellulose. The recovery of snRNAs from the bound hnRNP fraction was not due to nonspecific binding of deproteinated snRNAs to the column or an artificial association of snRNAs and nuclear protein. It is proposed that several snRNAs are integral components of MEL cell hnRNP complexes.

Eucaryotic cells contain a unique class of small, monodisperse, metabolically stable RNA molecules which are referred to collectively as small nuclear RNAs (snRNAs), since they appear to be confined to the nucleus, at least during the majority of their lifetime in the cell (Busch et al., 1971; Weinberg, 1973; Ro-choi and Busch, 1974). The cellular function of most snRNAs is unknown. It has been suggested that snRNAs may be involved in the regulation of gene expression in eucaryotic cells (Kanehisha et al., 1974; Goldstein, 1976). Some of the regulation by snRNAs could occur during the posttranscriptional processing of other species of RNA in the nucleus. The observation that three species of snRNA are hydrogen-bonded to 28S rRNA in the nucleolus is consistent with the hypothesis that these nucleolar snRNAs are involved in some unknown manner in the maturation of 28S rRNA from larger precursors and in one case (5.8S snRNA) with the function of 28S rRNA in the ribosome (Prestayko et al., 1970). Recently, it has been reported that individual snRNA species can be recovered from nuclear ribonucleoprotein preparations which contain heterogeneous nuclear RNA (hnRNP; Deimel et al., 1977). This raises the interesting possibility that the snRNAs in hnRNP are involved in the processing of hnRNA and perhaps also in the maturation and transport of mRNA to the cytoplasm. Before this possibility can be considered seriously it must be demonstrated that snRNAs are native components and not contaminating elements of hnRNP. In this report, we show that several nonnucleolar snRNA species copurify on sucrose gradients with hnRNP obtained by the sonic disruption of MEL cell nuclei under conditions where the artificial associ-

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ation of snRNA and protein can be ruled out for the larger RNP complexes. Furthermore, most of the snRNAs remain associated with that fraction of the hnRNP preparation which binds to oligo(dT)-cellulose. These observations support the hypothesis that several snRNAs are real, integral components of hnRNP. A preliminary report of these results has been published elsewhere (Howard et al., 1977).

Materials and Methods

Cell Culture. MEL cells (T3-C12; Ross et al., 1972; Ikawa et al., 1973) were propagated as suspension cultures in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 μg of streptomycin/mL, and 100 units of penicillin/mL. The cell culture medium and additives were obtained from the Grand Island Biological Co. Cells were grown in plastic roller bottles (Corning) which contained 100 mL of medium. In order to obtain optimal and reproducible growth in these containers, cells were seeded at a density of 5×10^4 cells/mL and subcultured 4 days later when they had grown to 1.2×10^6 cells/mL. Under these conditions, cells were maintained in logarithmic growth. The MEL cells were routinely tested for mycoplasma contamination by the fluorescent dye method of Chen (1975) and for bacterial and fungal contamination by the broth culture method of Coriell (1973). Cells were renewed from frozen stock cultures at 3-month intervals.

Radiolabeling of Cells. For labeling of stable nucleic acids in nuclear RNP, radioactive precursors were added directly to the medium of MEL cell cultures on the third day following subculture. Cells were harvested 24 h later at which time they had grown to a density of 1.2×10^6 cells/mL and were nearing the end of the exponential phase of growth. A total of 5×10^8 cells was used in most experiments. For short-term labeling, 5×10^8 cells were first washed several times by centrifugation in fresh, serum-containing medium at room temperature. They were then suspended in 50 mL of fresh, prewarmed medium (37 °C) at a concentration of 1.0×10^7 cells/mL. Subsequent incubation was performed at 37 °C. Ten minutes after the cells were suspended in warm medium, radiolabeled precursors were added for periods of 15–30 min. The uptake of isotope was terminated by pouring the cell suspension over 2 volumes of frozen, crushed isotonic saline.

Isolation of Cell Nuclei. Labeled MEL cells were washed twice by low-speed centrifugation (600*g*) in ice-cold, isotonic saline. Nuclei were prepared from these cells essentially as described by Sarma et al. (1976). All operations were performed at 0 °C. The cell pellet was suspended in 20 mL of homogenizing buffer (0.3 M sucrose, 2 mM magnesium acetate, 3 mM $CaCl_2$, 10 mM Hepes[1] buffer, pH 7.6, and 0.1% Triton X-100), agitated briefly with a vortex mixer, and centrifuged at 900*g* for 10 min. The cells were then suspended in 40 mL of homogenizing buffer and broken with three to five strokes in a Dounce homogenizer (tight pestle). When the homogenate was observed by phase-contrast microscopy, no unbroken cells were seen and nuclei did not exhibit cytoplasmic tabs. The homogenate was then mixed with an equal volume of 2 M sucrose solution (2 M sucrose, 5 mM magnesium acetate, 10 mM Hepes buffer, pH 7.6). Twenty milliliters of this mixture was layered over 15 mL of the 2 M sucrose solution and centrifuged for 50 min at 20 000 rpm in a Beckman SW-27 rotor. The supernatant, which included cytoplasmic material at the interface between the 1 and 2 M sucrose, was carefully removed by aspiration and the nuclear pellet was processed for the recovery of the RNP particles. Nuclear yields with this method averaged 80%.

Preparation of Nuclear HnRNP Particles. Nuclear hnRNP particles were prepared by a modification of the method of Louis and Sekeris (1976). Nuclei from 5×10^8 labeled cells were suspended in 10 mL of TKM-sucrose buffer (50 mM Tris-HCl, pH 7.6, 25 mM KCl, 10 mM $MgCl_2$, and 0.25 M sucrose) by very gentle agitation with a rubber policeman. This and all subsequent operations were performed at 0 °C. An equal volume of TKM-sucrose buffer which contained 100 mM EDTA was then added to make the final concentration of EDTA 50 mM. The nuclei were gently homogenized in this solution for 10 min in a Dounce homogenizer with a loose-fitting pestle. After centrifugation for 5 min at 600*g*, the nuclei were resuspended in 10 mL of STM 8.0 buffer (0.14 M NaCl, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.0) and sonicated at 40–50 W with a Branson sonifier fitted with a micro tip. Nuclei were subjected to six 10-s sonications alternated with 3-min cooling intervals. Approximately 95% of the nuclei was disrupted by this treatment and many nucleoli were visible when the sonicate was viewed by phase-contrast microscopy. The sonicate was centrifuged at 15 000*g* and then 5-mL aliquots were layered over 30-mL, 15–30%, linear sucrose gradients in STM 8.0 buffer. Following centrifugation at 20 500 rpm for 16 h in a Beckman SW-27 rotor, the gradients were fractionated on an ISCO apparatus fitted with a flow cell detector, a fraction collector, and a chart recorder. Gradients were scanned for absorbance at 254 nM and for soluble and Cl_3-AcOH-precipitable radioactivity to reveal the location of labeled RNP fractions.

Affulose. Chromatography of nuclear hnRNP fractions on oligo(dT)-cellulose was performed essentially as described by Lindberg and Sundquist (1974) and Kumar and Pederson (1975). Oligo(dT)-cellulose (Collaborative Research) was suspended in loading buffer (0.25 M NaCl, 0.01 M sodium phosphate, pH 7.0). A 7 × 100 mm column was poured and washed with loading buffer. Nuclear hnRNP fractions were recovered from sucrose gradients, dialyzed for 16–24 h at 0 °C against loading buffer, concentrated by centrifugation in Millipore centriflo membrane cones, and then applied to the column in a volume of 5–6 mL. The flow rate of the column was adjusted to 0.25 mL/min and 1-mL fractions were collected. After 20 mL had been collected subsequent to application of the sample to the column, bound hnRNP was eluted with 50% formamide in 0.01 M sodium phosphate, pH 7.0. Aliquots of each column fraction were assayed for total radioactivity in a scintillation counter. Peak fractions were pooled and dialyzed to remove salt or formamide.

Extraction of RNA from Nuclear hnRNP Fractions Obtained from Sucrose Gradients and Affinity Columns. RNA was extracted with $NaDodSO_4$ and phenol at pH 5.0 in order to enhance recovery of snRNAs (Moriyama et al., 1969; Howard, 1973a). The hnRNP in sucrose gradient fractions was precipitated at −20 °C with 2.5 volumes of ethanol which contained 2% (w/v) potassium acetate (KOAc-ethanol). Two hundred micrograms of unlabeled, MEL cell, ribosomal RNA was present as carrier during precipitation. Bound and unbound hnRNP fractions from affinity columns were dialyzed for 16–24 h at 0 °C against 20 mM sodium acetate, pH 5.0, to remove salt or formamide and then precipitated with

[1] Abbreviations used are: RNP, ribonucleoprotein; mRNP, ribonucleoprotein which contains messenger RNA; hnRNP, ribonucleoprotein which contains heterogeneous nuclear RNA; MEL cells, mouse erythroleukemia cells; snRNA, small nuclear RNA; rRNA, ribosomal RNA; mRNA, messenger RNA; Cl_3AcOH, trichloroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

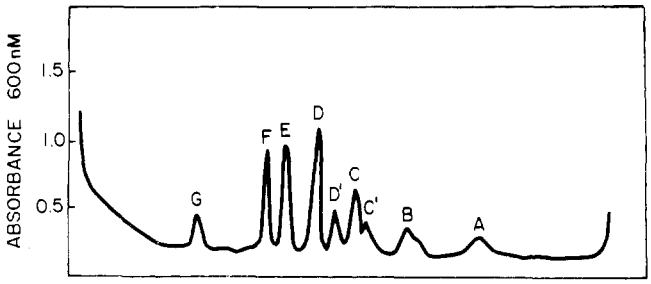

FIGURE 1: snRNAs in MEL cell nuclei. Total RNA was extracted from MEL cell nuclei with phenol and $NaDodSO_4$ at 58 °C and pH 5.0. Deproteinated RNA was centrifuged through 10–45% sucrose gradients (30 mL) which contained acetate buffer, pH 5.0. Centrifugation was at 25 000 rpm for 17 h in a Beckman SW-27 rotor. RNA which sedimented at 4–9 S was recovered and analyzed by electrophoresis in 10% polyacrylamide gels. The gels were stained with methylene blue, destained in water, and then scanned for absorbance at 600 nM. The direction of migration was from left to right.

KOAc–ethanol in the presence of carrier RNA. Precipitates were concentrated by centrifugation, washed with 70% ethanol, air-dried, and suspended in 5 mL of acetate buffer (0.01 M EDTA, 0.01 M NaCl, 0.05 M sodium acetate, 20 μg of polyvinyl sulfate/mL, pH 5.0) which contained 0.125% (w/v) $NaDodSO_4$. After brief homogenization in this buffer with a Teflon–glass homogenizer (loose pestle) at room temperature, an equal volume of redistilled phenol which was saturated with acetate buffer was added and an emulsion formed by vigorous shaking at room temperature for 20 min. Following separation of phases by centrifugation at 2000*g* for 5 min, the aqueous phase was recovered and the phenol and protein interface extracted at 58 °C with an equal volume of acetate buffer for 10 min. The homogenate was chilled on ice, 0.5 volume of chloroform–isoamyl alcohol (24:1) was added, and the phases were separated by centrifugation at 10 000*g* for 10 min. The aqueous phases from the extractions at room temperature and 58 °C were pooled and repeatedly extracted at room temperature with phenol and chloroform–isoamyl alcohol until no visible protein remained at the interface following centrifugation. RNA was precipitated from the aqueous phase with KOAc–ethanol.

Polyacrylamide Gel Electrophoresis of snRNA. Total RNA isolated from nuclear hnRNP fractions was dissolved in electrophoresis buffer (0.04 M Tris–HOAc, pH 7.2, 20 mM sodium acetate, 2 mM EDTA) which contained 10% glycerol and 0.1% bromophenol blue just prior to loading on 5 × 115 mm, cylindrical, 10% polyacrylamide gels (9.75% monomer, 0.25% bisacrylamide). Gels were polymerized in electrophoresis buffer with 0.2% (w/v) ammonium persulfate and 0.25% (v/v) Temed (*N,N,N′,N′*-tetramethylenediamine). All chemicals for electrophoresis were obtained from Bio-Rad. The gels were prerun at 5 mA/gel for 30 min before application of the samples. Both the upper and lower buffer chambers contained electrophoresis buffer. For most analytical runs, 40 μg of RNA was applied to each gel. In some experiments, 100 μg of RNA was applied to gels which had a 1-cm stacking gel (5% acrylamide) polymerized on top of the 10% gel. Electrophoresis was performed at 5–7 mA/gel at 0 °C until the dye marker had reached the bottom of the gels (6–7 h). The gels were removed from the tubes, frozen, and sliced into 1-mm sections with razor blades. Each slice was placed in a scintillation vial with 5 mL of PPO–POPOP–toluene cocktail (LSC complete, Yorktown Research) which contained 9% (v/v) NCS solubilizer (Amersham; Mayol and Sinsheimer, 1970). After storage at room temperature for 16 h, the radioactivity in each slice was determined with a scintillation counter. The resolution of snRNAs on the 10% polyacrylamide gels was not changed or improved when electrophoresis was performed at room temperature in the presence of $NaDodSO_4$.

Isopycnic Banding of Nuclear hnRNP Particles in CsCl. Labeled hnRNP fractions from sucrose gradients or oligo(dT)-cellulose affinity columns were dialyzed against 10 mM sodium phosphate buffer, pH 7.2, to remove sucrose, salt, or formamide. The hnRNP was then fixed by dialysis at 4 °C against 4% (v/v) formaldehyde (Samarina et al., 1968). The fixed hnRNP suspension was then divided and one-half was made 1.3 g/cm^3 in CsCl and the other 1.65 g/cm^3 in CsCl. Linear gradients (5 mL) were formed with these solutions. Gradients were centrifuged at 35 000 rpm for 20 h in a Beckman SW-41 rotor and then fractionated into 0.1-mL fractions. The density of every fifth fraction was determined from refractive-index measurements. The radioactivity in each fraction was determined by measuring soluble or Cl_3AcOH-precipitable radioactivity in a liquid scintillation counter. An average of 85% of the applied, Cl_3AcOH-precipitable radioactivity was recovered from each gradient.

Results

snRNAs in T3-C12 MEL Cells. MEL cell nuclei were prepared in the presence of Triton X-100, and total RNA was extracted with hot phenol and $NaDodSO_4$ at pH 5.0. After centrifugation through 10–45% sucrose gradients, 4–9S RNA was recovered and analyzed by electrophoresis on 10% polyacrylamide gels. An electrophoretogram of MEL cell snRNAs is shown in Figure 1. Nine peaks were resolved in the 10% gels and these were designated with a nomenclature used previously in this laboratory (Howard and Stubblefield, 1972; Howard, 1973a,b). Peak A had the greatest electrophoretic mobility and peak G had the lowest mobility in these gels. When compared to the electrophoretic migration pattern of snRNAs which are extracted with similar methods in other laboratories, snRNA peaks A through G could be tentatively identified as follows: A, nuclear 4S RNA; B, stable 4.5S RNA; C and C′, nuclear 5S RNA; D′, 5.8S RNA; D and E, uridine-rich snRNAs found associated with chromatin and free in the nucleoplasm; F and G, snRNAs found in the nucleolus. The following experiments were designed to determine if any of the snRNAs could be recovered from nuclear hnRNP fractions.

Properties of RNP Particles Recovered from MEL Cell Nuclei. Log-phase MEL cells were labeled with [^{14}C]uridine for 24 h. The cells were then washed several times with fresh medium, concentrated tenfold, and labeled for 15 min at 37 °C with [^{3}H]uridine. In this manner, both stable and rapidly labeled RNA molecules were recovered in the same preparation. The short-term label was terminated by pouring the cells over frozen, isotonic saline. Nuclei were isolated in the presence of Triton X-100, washed with EDTA, and then broken by gentle sonication in STM 8.0 buffer. The sonicate was clarified by several cycles of centrifugation at 15 000*g* and then centrifuged through 15–30% sucrose gradients which contained STM 8.0 buffer. The ^{14}C and ^{3}H radioactivity profiles in the gradient are shown in Figure 2. It can be seen that rapidly labeled, [^{3}H]RNA was recovered in two major peaks at approximately 10 and at 30 S (estimated by comparison to the sedimentation of rabbit reticulocyte ribosomal subunits). The pattern of short-term labeling was not changed when cells were labeled for 15 min in the presence of a low concentration of actinomycin D (0.05 μg/mL) which inhibited the labeling of rRNA and rRNA precursors in the MEL cells (data not shown). Rapidly labeled RNA was recovered in all gradient fractions, and it was determined that a small amount of ra-

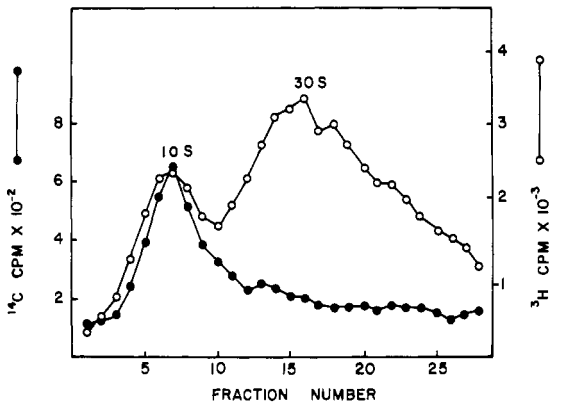

FIGURE 2: Velocity sedimentation analysis of presumptive RNP obtained by sonic disruption of MEL cell nuclei. Log-phase MEL cells were labeled with [^{14}C]uridine (Schwarz/Mann, 58 mCi/mM, 0.4 μCi/mL) for 24 h. Cells were harvested by centrifugation, washed in culture medium, and suspended in prewarmed (37 °C), serum-supplemented culture medium at a concentration of 10^7 cells/mL. Subsequent incubation was at 37 °C. The cells were then labeled for 15 min with [^{3}H]uridine (Schwarz/Mann, 27 Ci/mM, 10 μCi/mL). Labeling was terminated by pouring the cells over frozen saline. Nuclei were prepared with Triton X-100, washed with EDTA, and then disrupted by sonication in STM 8.0 buffer. Nucleoli and insoluble chromatin were removed from the sonicate by two cycles of centrifugation at 15 000g. The sonicate was then layered over 15–30% linear sucrose gradients (30 mL) which contained STM 8.0 buffer. The gradients were centrifuged at 20 500 rpm in a Beckman SW-27 rotor for 16 h: (●–●) ^{14}C radioactivity; (○–○) ^{3}H radioactivity. The direction of sedimentation was from left to right.

dioactivity was pelleted under these conditions of sedimentation. The last fraction (bottom) of the gradient contained material which sedimented at approximately 60 S. Stable, ^{14}C-labeled RNA was recovered primarily from the 10S region of the gradient, but some stable RNA was also recovered from the 30–60S region of the gradient.

Experiments were performed to demonstrate that the labeled RNA was recovered in the form of RNP particles. Putative RNP was prepared from MEL cells which had been incubated with [^{3}H]uridine for 15 min. Labeled material was recovered from the 10S (fractions 5–8, Figure 2) and 30–60S (fractions 13–30, Figure 2) regions of sucrose gradients, fixed with formaldehyde, and centrifuged to equilibrium in preformed CsCl gradients. It can be seen in Figure 3 that the 10S material exhibited a peak buoyant density of 1.38 g/cm^3 and that the 30–60S material had a higher buoyant density at 1.436 g/cm^3. These values are within the range reported for hnRNP in other laboratories (Georgiev and Samarina, 1971; Martin and McCarthy, 1972; Pederson, 1974; Quinlan et al., 1977) and are lower than the densities reported for ribosomes and ribosomal subunits (1.53–1.58 g/cm^3; Irwin et al., 1975). When MEL cells were labeled for 24 h with [^{3}H]uridine, the buoyant density of the putative 10S RNP was also 1.38 g/cm^3. These experiments indicated that both long- and short-term labeled RNA were recovered in RNP complexes which exhibited buoyant densities characteristic of hnRNP. Recovery of Cl_3AcOH-precipitable radioactivity from all CsCl gradient fractions averaged 85%, which suggests that the bulk of labeled RNA was recovered in RNP.

Nuclear RNP was prepared from cells which had been incubated with [^{3}H]uridine for 15 min. The particles were sedimented through 15–30% sucrose gradients and RNA was extracted at pH 5.0 and 58 °C from the 10S and 30–60S fractions, respectively, in the presence of unlabeled, MEL cell ribosomal RNA. Total RNA from each fraction was subjected to velocity sedimentation through 10–45% sucrose gradients. The sedimentation pattern of the RNA is shown in Figure 4.

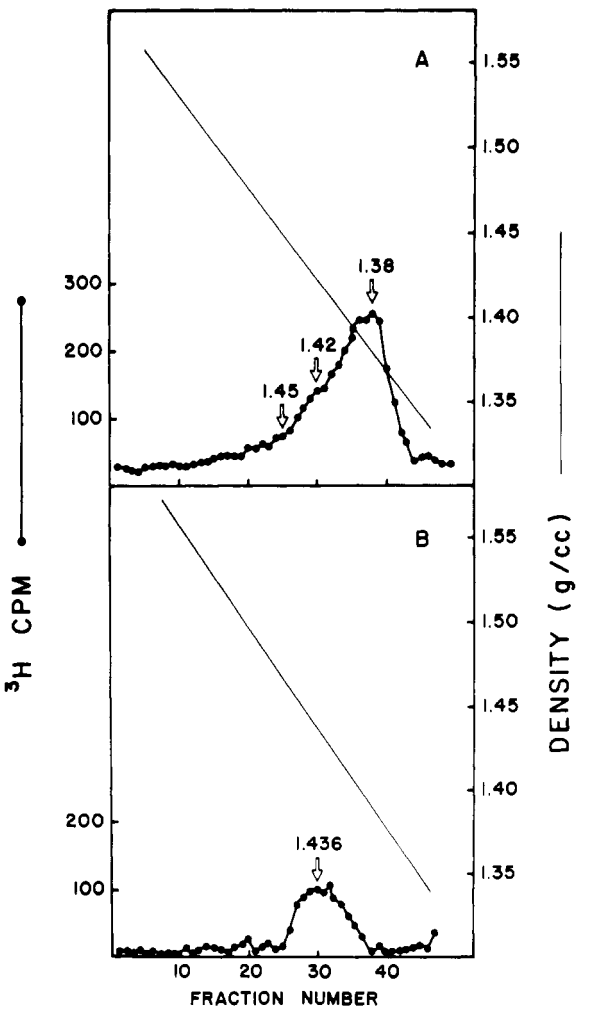

FIGURE 3: Buoyant density of presumptive RNP in CsCl. MEL cells were harvested, resuspended at a concentration of 10^7 cells/mL, and labeled with [^{3}H]uridine (10 μCi/mL) for 15 min. ^{3}H-labeled, presumptive RNP was prepared as described in the legend to Figure 2. Labeled material was recovered from the 10S and 30–60S regions of 15–30% sucrose gradients and fixed by dialysis against phosphate-buffered formaldehyde. The fixed material was then centrifuged in preformed CsCl gradients at 35 000 rpm for 20 h in a Beckman SW-41 rotor: (—) density (g/cm^3); (●–●) Cl_3-AcOH-insoluble radioactivity.

The peak fraction of the rapidly labeled RNA from the 30–60S RNP was slightly larger (about 10–12S) than the RNA from the 10S RNP (about 8 S). A small fraction of the RNA extracted from 30–60S RNP sedimented between 10 and 40 S in the sucrose gradients. These patterns are typical of degraded hnRNA (Samarina et al., 1968; Pederson, 1974; Beyer et al., 1977). Rat liver ribonuclease inhibitor was not used in this study. No labeled RNAs with the sedimentation properties of rRNA or rRNA precursors were recovered from the RNP particles. Furthermore, short-term labeling of the RNA shown in Figure 4 was not reduced by low concentrations (0.05 μg/mL) of actinomycin D (data not shown). Since intact rRNAs were recovered from MEL cell nuclei which had not been prewashed with EDTA, it was concluded that the 8–12S RNA recovered from the rapidly labeled RNP was not degraded rRNA but rather degraded hnRNA which was produced by nuclease digestion of larger, labile hnRNA molecules during the preparation of the RNP.

An experiment was performed to test for the presence of DNA in the hnRNP preparations. Log-phase MEL cells were labeled with [^{3}H]thymidine for two cell generations during growth from 5×10^5 to 2×10^6 cells/mL. Nuclei from 5×10^8

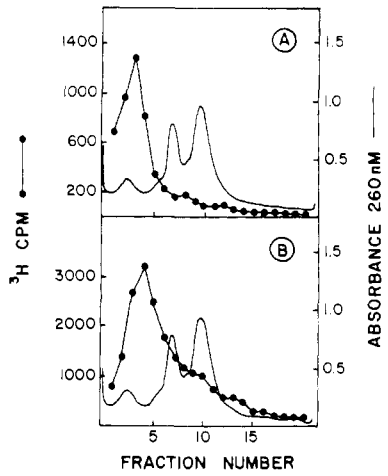

FIGURE 4: Velocity sedimentation analysis of rapidly labeled RNA in 10S and 30-60S nuclear RNP. MEL cells were concentrated and labeled with [^{3}H]uridine (10 μCi/mL) for 15 min. RNP was prepared from sonic-disrupted nuclei and centrifuged through sucrose gradients as described in the legend to Figure 2. Both 10S and 30-60S gradient fractions were precipitated with ethanol in the presence of unlabeled, MEL cell, ribosomal RNA. Total RNA was then extracted with phenol and Na-DodSO$_4$ at pH 5.0 and 58 °C. Deproteinated RNA was centrifuged in 10-45% linear sucrose gradients (30 mL) which contained acetate buffer, pH 5.0. The gradients were centrifuged at 25 000 rpm in a Beckman SW-27 rotor for 17 h. Gradients were fractionated and scanned for absorbance at 260 nM. The total radioactivity in each gradient fraction was also determined. (A) Sedimentation pattern of the [^{3}H]uridine-labeled RNA and the unlabeled carrier RNA from the 10S RNP fraction. (B) Sedimentation pattern of the [^{3}H]uridine-labeled RNA and unlabeled carrier RNA from the 30-60S RNP fraction: (●-●) ^{3}H radioactivity; (—) absorbance at 260 nM. The unlabeled carrier RNA exhibited three peaks at approximately (left to right) 4-9, 18, and 28S.

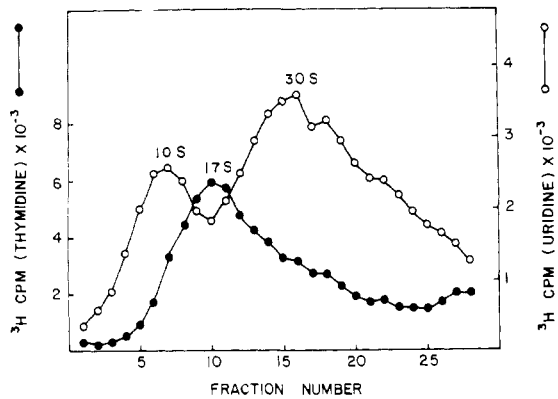

FIGURE 5: Recovery of [^{3}H]thymidine-labeled material in MEL cell nuclear RNP preparations. Log-phase MEL cells were incubated with [^{3}H]thymidine Schwarz/Mann (62 Ci/mM, 5 μCi/mL) for two cell generations (24 h). Nuclear RNP was prepared from sonicated nuclei and centrifuged in 15-30% linear sucrose gradients (30 mL) which contained STM-8.0 buffer as described in the legend to Figure 2. hnRNP which had been labeled for 15 min with [^{3}H]uridine was centrifuged in parallel gradients: (●-●) sedimentation profile of [^{3}H]thymidine-labeled material; (○-○) reference sedimentation profile of [^{3}H]uridine-labeled hnRNP.

cells were disrupted by sonication, and the clarified sonicate was centrifuged through 15-30% sucrose gradients. [^{3}H]-Thymidine-labeled material sedimented as a single peak at approximately 17 S (Figure 5). This 17S, thymidine-labeled peak probably represented soluble chromatin produced during sonic disruption of the nuclei (Kimmel et al., 1976). Since the bulk of the chromatin did not cosediment with the 10S or 30-60S hnRNP, it is likely that the hnRNP and the chromatin existed as separate entities in these preparations; i.e., the labeled RNA was probably not bound to the chromatin. However, this does not preclude the possibility that the hnRNP was stripped from chromatin during sonication (Faiferman and Pogo, 1975).

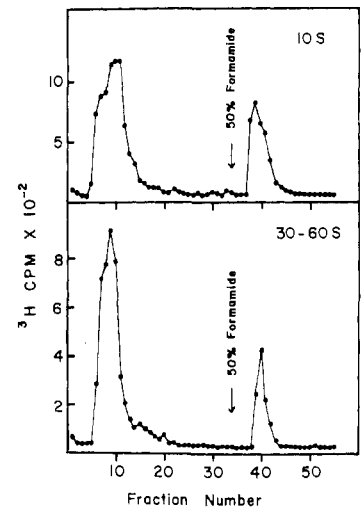

FIGURE 6: Oligo(dT)-cellulose chromatography of 10S and 30-60S hnRNP. Log-phase MEL cells were labeled with [^{3}H]uridine (5 μCi/mL) for 24 h. hnRNP was prepared from sonic-disrupted nuclei and centrifuged in 15-30% linear sucrose gradients (30 mL) which contained STM-8.0 buffer. The 10S and 30-60S regions of the gradients were recovered and dialyzed against 0.25 M NaCl and 0.01 M sodium phosphate, pH 7.0. After concentration to 5 mL in Amicon Centriflo membrane cones, the dialysates were loaded on oligo(dT)-cellulose columns which were subsequently washed with 25-30 mL of the phosphate-buffered, 0.25 M NaCl. Bound hnRNP was eluted from the column with 50% formamide, 0.01 M sodium phosphate, pH 7.0: (●-●) ^{3}H radioactivity.

snRNAs in Nuclear RNP Preparations. MEL cells were exposed to [^{3}H]uridine for 24 h before harvest in order to label all snRNAs. Nuclear hnRNP particles were prepared and centrifuged through sucrose gradients. Both 10S and 30-60S hnRNP were recovered, and a portion of the 10S fraction was precipitated with ethanol in the presence of unlabeled carrier RNA. The remaining 10S hnRNP and all of the 30-60S hnRNP were dialyzed against 0.25 M NaCl, 10 mM sodium phosphate (pH 7.0) and chromatographed on oligo(dT)-cellulose. Flow-through (unbound) fractions were collected, and bound particles were eluted with phosphate buffered, 50% formamide (Figure 6). Approximately 95% of the applied radioactivity was recovered from the column in these experiments. In four experiments, the bound fraction of 10S hnRNP represented 27.8 ± 4.6% of the radioactivity which was recovered from the oligo(dT)-cellulose column and the bound fraction of 30-60S hnRNP represented 21 ± 4% of the recovered counts. Column fractions were dialyzed against 20 mM sodium acetate (pH 5.0) to remove salt and formamide and then precipitated with ethanol in the presence of unlabeled carrier RNA. Total RNA was extracted from all ethanol precipitates and analyzed for the presence of labeled snRNAs by electrophoresis in 10% polyacrylamide gels (Figure 7). Five prominent snRNA peaks were recovered from unchromatographed 10S hnRNP (Figure 7A). These were species A-E. It should be noted that the three snRNAs which are thought to be exclusively nucleolar in location (D′, F, and G; Howard, 1973b) were absent or present in very small amounts (D′) in the 10S hnRNP. This suggested that the removal of nucleoli during the 15 000g centrifugation of the nuclear sonicate was efficient and that these hnRNP preparations were not contaminated with significant amounts of nucleolar snRNP. The 10S hnRNP which did not bind to oligo(dT)-cellulose con-

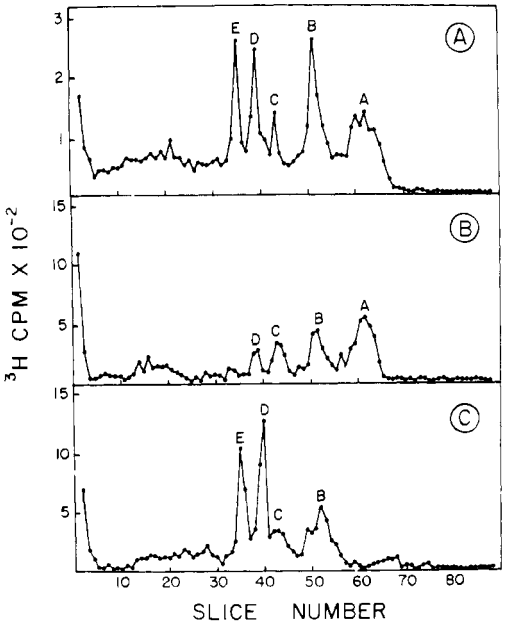

FIGURE 7: snRNAs in 10S hnRNP before and after chromatography on oligo(dT)-cellulose. Log-phase MEL cells were labeled with [^{3}H]uridine (5 μCi/mL) for 24 h. 10S hnRNP was recovered from 15–30% sucrose gradients, and a portion of this fraction was precipitated with ethanol in the presence of unlabeled carrier RNA. The remaining 10S hnRNP was chromatographed on oligo(dT)-cellulose. Bound and unbound 10S hnRNP were dialyzed to remove salt or formamide and were then precipitated with ethanol in the presence of unlabeled carrier RNA. Total RNA was extracted from all precipitates with phenol and $NaDodSO_4$ at 58 °C and pH 5.0. Labeled RNA was analyzed by electrophoresis in 10% polyacrylamide gels: (A) labeled snRNAs in unchromatographed 10S hnRNP; (B) labeled snRNAs in unbound 10S hnRNP; (C) labeled snRNAs in bound hnRNP.

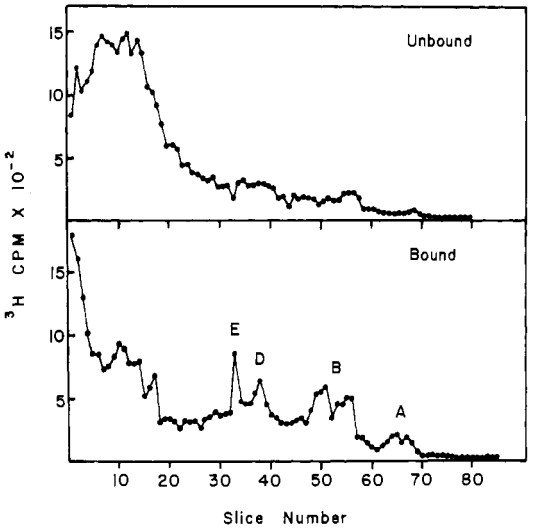

FIGURE 8: snRNAs in 30–60S hnRNP which was chromatographed on oligo(dT)-cellulose. hnRNP was prepared from MEL cells which had been labeled with [^{3}H]uridine (5 μCi/mL) for 24 h. The 30–60S hnRNP was recovered from sucrose gradients and chromatographed on oligo(dT)-cellulose. RNA was extracted from bound and unbound column fractions which had been precipitated with ethanol in the presence of unlabeled carrier RNA. Total RNA was subjected to electrophoresis in 10% polyacrylamide gels. The electrophoretic profiles of labeled snRNAs in unbound and bound 30–60S hnRNP are shown in the upper and lower panels of this figure, respectively.

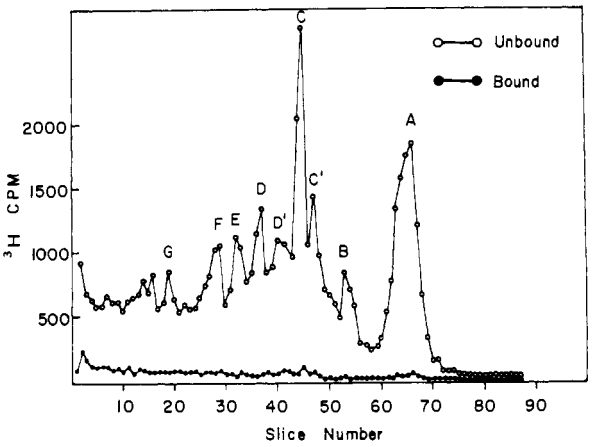

FIGURE 9: Recovery of snRNAs in bound and unbound fractions following chromatography of deproteinated 4–9S RNA on oligo(dT)-cellulose. Log-phase MEL cells were labeled with [^{3}H]uridine (5 μCi/mL) for 24 h. Total RNA was extracted from purified nuclei with phenol and $NaDodSO_4$ at 58 °C and pH 5.0. [^{3}H]Uridine-labeled, 4–9S RNA was prepared as described in the legend to Figure 1. The 4–9S RNA was dialyzed against 0.25 M NaCl and 0.01 M sodium phosphate, pH 7.0, and then chromatographed on oligo(dT)-cellulose in the same buffer. Bound radioactivity was eluted with 50% formamide. Bound and unbound column fractions were dialyzed and then precipitated with ethanol in the presence of unlabeled carrier RNA. RNA was extracted from the precipitates with phenol and $NaDodSO_4$ at 58 °C and pH 5.0 and then analyzed by electrophoresis in 10% polyacrylamide gels: (O–O) electrophoretic profile of [^{3}H]uridine-labeled RNA which did not bind to oligo(dT)-cellulose; (●–●) electrophoretic profile of [^{3}H]uridine-labeled RNA which bound to oligo(dT)-cellulose.

tained snRNAs B, D, and E and some poorly resolved material with the electrophoretic mobility of C (Figure 7B). It can be seen in Figure 7C that all of species E and the majority of species D were recovered from the 10S hnRNP which bound to oligo(dT)-cellulose. Because of an anticipated low recovery of snRNAs in the 30–60S hnRNP, all of this material was chromatographed on oligo(dT)-cellulose. The electrophoretic spectrum of snRNAs in both bound and unbound 30–60S hnRNP is shown in Figure 8. A large, diffuse peak of label was recovered in the top quarter of the gel which contained RNA from unbound 30–60S hnRNP. However, no recognizable snRNAs which corresponded to the peaks shown in Figure 1 were recovered in this preparation. In contrast, several snRNAs were recovered from the oligo(dT)-cellulose-bound fraction of 30–60S hnRNP. These were single peaks at A, D, and E and a double peak in the B region of the gels.

Total long-term labeled RNA was extracted from bound and unbound column fractions of both 10S and 30–60S hnRNP and centrifuged through 10–45% sucrose gradients. The sedimentation patterns of these RNAs were similar to that exhibited by the short-term labeled RNA which was derived from these particles; i.e., most of the labeled RNA sedimented at 8–12 as shown in Figure 4. Thus, the average size of the rapidly labeled and stable RNA in these particles was approximately the same. Bound and unbound hnRNP which were recovered from the oligo(dT)-cellulose column had buoyant densities in the range of 1.38–1.42 g/cm^3 in CsCl, which indicated that the integrity and composition of the hnRNP were not altered by affinity chromatography.

The possibility was considered that the snRNAs which bound to oligo(dT)-cellulose were protein-free contaminants of the hnRNP preparations. To test this, deproteinated, MEL cell snRNA which was labeled with [^{3}H]uridine was chromatographed on oligo(dT)-cellulose. It can be seen in Figure 9 that all of the snRNAs were recovered in the unbound fraction. About 9% of the radioactivity which was applied to the column bound to the oligo(dT)-cellulose. However, no snRNAs were found in this fraction.

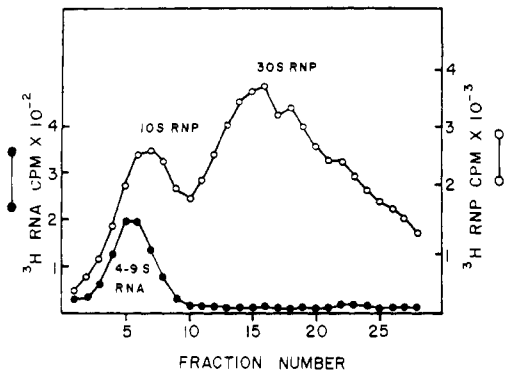

FIGURE 10: Recovery of added sn[^{3}H]RNA in nuclear hnRNP preparations. [^{3}H]Uridine-labeled, deproteinated, 4–9S nuclear RNA was prepared as described in the legend to Figure 1. The labeled RNA (1.1×10^6 dpm; 10^5 dpm/μg) was mixed with 4×10^8 MEL cell nuclei just prior to sonication in STM-8.0 buffer. Nuclear RNP was prepared as described previously and centrifuged in 15–30% linear sucrose gradients (30 mL) which contained STM-8.0 buffer. Centrifugation was at 20 500 rpm for 16 h in a Beckman SW-27 rotor. hnRNP which had been labeled with [^{3}H]uridine for 15 min was centrifuged in parallel gradients: (●–●) sedimentation profile of [^{3}H]uridine-labeled, 4–9S RNA; (○–○) reference sedimentation profile of [^{3}H]uridine-labeled hnRNP.

snRNAs might become artifically associated with nuclear proteins during the preparation of nuclear hnRNP. To test if this occurs, [^{3}H]uridine-labeled, whole nuclear snRNAs were mixed with unlabeled MEL cells during nuclear isolation in one experiment and with unlabeled nuclei just prior to sonic disruption in another experiment. Nuclear hnRNP was prepared as described previously. Labeled snRNAs which were added to unlabeled cells were not recovered in the nuclear hnRNP preparation. However, labeled snRNAs which were present during sonication sedimented at about 4–9 S in 15–30% sucrose gradients (Figure 10). This is not surprising, since deproteinated snRNAs are in the 4–9S size range and would be recovered from this region of the gradient in any event. Nevertheless, it is clear that labeled, deproteinated snRNAs might be recovered in our 10S hnRNP preparations. However, the previous experiment clearly shows that the snRNAs would not bind to oligo(dT)-cellulose unless they were associated with RNP. It is important to note that the sedimentation coefficient of the snRNA was not increased in the presence of unlabeled, nuclear protein. It is clearly shown in Figure 10 that radioactive snRNAs which were present during the sonic disruption of unlabeled nuclei did not cosediment with the 30–60S hnRNP. Therefore, it is unlikely that the recovery of snRNAs from 30–60S hnRNP and the binding of the snRNA to oligo(dT)-cellulose were due to the artificial association of snRNA and proteins during the preparation of nuclear hnRNP.

Discussion

This study presents evidence that MEL cell snRNA species B, D, and E are integral components of nuclear hnRNP. The observations which support this conclusion are: (1) several nonnucleolar snRNA species (A–E) copurified on sucrose gradients with 10S and 30–60S RNP particles which exhibited properties characteristic of hnRNP; (2) with the exception of snRNA species A in the 10S hnRNP, nearly all of the snRNAs remained associated with that portion of each hnRNP fraction which bound to oligo(dT)-cellulose; (3) the recovery of snRNAs from the hnRNP which was retained on oligo(dT)-cellulose was not due to nonspecific binding of deproteinated snRNAs to the column or to the formation of spurious snRNA–protein complexes during the preparation of hnRNP. Therefore, the continued association of snRNAs and hnRNP during sucrose gradient centrifugation and subsequent oligo(dT)-cellulose chromatography suggest that the snRNA–hnRNP complex is a bona fide nuclear entity and not an artificial product of the experimental manipulations.

The association of snRNAs with hnRNP which binds to oligo(dT)-cellulose could be attributable to two factors. The hnRNA may contain 3′-terminal poly(A) tracts, some of which are available for hybrid formation with oligo(dT) in the presence of high salt (Lindberg and Sundquist, 1974; Pederson, 1974; Kumar and Pederson, 1975). Since snRNAs contain no poly(A) or oligo(A) sequences (Ro-Choi and Busch, 1974), their binding to the column could be substantially dependent on an association with an RNP complex which contains poly(A)$^+$ hnRNA. Preliminary data in our laboratory suggest that both 10S and 30–60S hnRNP may contain poly(A). However, it is possible that the retention of snRNAs on oligo(dT)-cellulose may also depend on the interaction of protein components in the hnRNP with the cellulose matrix. Such binding is reportedly minimized in the presence of 0.25 M NaCl (Lindberg and Sundquist, 1975). In any case, it is not possible at this time to assess the relative contributions of poly(A) sequences and protein interactions to the recovery of snRNAs from column-bound RNP. However, regardless of the mechanism of binding, the copurification of snRNAs and hnRNP on oligo(dT)-cellulose supports the proposition that snRNAs and hnRNA are recovered in the same RNP complex.

The nuclear RNP in this study was designated hnRNP because it contained rapidly labeled RNA which was sensitive to low concentrations of actinomycin D and exhibited a buoyant density in CsCl of 1.38–1.436 g/cm^3 (Penman et al., 1968; Georgiev and Samarina, 1971; Pederson, 1974; Irwin et al., 1975). The presence of small (8–12 S), partially degraded hnRNA in both the 10S and 30–60S particles is characteristic of hnRNP prepared in the absence of ribonuclease inhibitors (Samarina et al., 1968; Beyer et al., 1977). The buoyant density of the small hnRNP (1.38 g/cm^3) raises the possibility that this particle was somewhat larger than estimated. RNP of this buoyant density which contained 4–8S RNA molecules would also contain approximately 78% protein by weight and might be expected to exhibit a sedimentation coefficient greater than 10 S (Samarina et al., 1973; Quinlan et al., 1977). Since the *s* value designations were rough approximations, this may well be the case. However, this does not influence the major conclusions derived from this study.

The identity of the snRNAs which copurified with hnRNP in sucrose gradients and during oligo(dT)-cellulose chromatography can be inferred by comparison to the electrophoretic profiles of snRNAs prepared by similar methods in other laboratories. Such assignments are tentative, since they rely solely on similarities of relative electrophoretic mobility in polyacrylamide gels. By this criterion, MEL cell snRNA species B corresponds to a 4.5S snRNA and species D and E correspond to two uridylic acid rich, nonnucleolar snRNAs (U_1 and U_2) which have been studied extensively in rat liver systems (Ro-Choi and Busch, 1974). Species A and C, which are recovered in small amounts in MEL cell hnRNP, correspond to nuclear tRNA and 5S RNA, respectively. It is of considerable interest that one or more snRNAs with the electrophoretic mobilities of B, D, and E have been found in association with nuclear RNP complexes by several investigators. Raj et al. (1975) recovered a nuclear RNP subfraction which contained U_1 (D) and U_2 (E) snRNAs from EDTA-washed Novikoff hepatoma nuclei. The RNP particles were not analyzed for the presence of hnRNA. Zieve and Penman (1976)

reported that a 40S RNP particle which contained snRNA species D (equivalent to MEL cell species D by relative electrophoretic mobility) was released from HeLa cell nuclei by treatment with micrococcal nuclease. Several snRNA species have been recovered from 30S hnRNP particles and from larger hnRNP complexes isolated in the presence of rat liver ribonuclease inhibitor (Deimel et al., 1977). The hnRNP-associated snRNAs in this study may correspond to MEL cell species B, D, E, and possibly F. Miller et al. (1978a,b) have recovered several snRNAs from a DNA-free, rat liver nuclear protein matrix which also contained hnRNA. The matrix-associated snRNAs may be equivalent to MEL species B–E. Reliable comparisons of the snRNAs described in these studies cannot be made because a common nomenclature based on standardized methods of extraction and identification does not yet exist. However, it is significant that snRNAs with similar electrophoretic mobilities are consistently found in association with nonnucleolar RNP complexes in the nucleus. This raises the possibility that the presence of snRNAs in these nuclear subfractions may have functional significance. Sekeris and Niessing (1975) have postulated that certain snRNAs are structural components of hnRNP polyparticles. Zieve and Penman (1976) have also speculated that some snRNAs are structural components in several cellular compartments which include the cell membrane and the nuclear matrix. Furthermore, Benecke and Penman (1977) have described a separate class of small RNAs (8–10 S) in HeLa cells which may be involved in nuclear structure. It is not clear at this point what the exact nature of these structural functions might be. It is conceivable that the snRNAs which copurify with hnRNP are somehow involved in the processing of hnRNA. In the nucleolus, three species of snRNA are hydrogen bounded to 28S rRNA (Prestayko et al., 1970). In our nomenclature, these correspond in electrophoretic mobility to species D′, F, and G which are not recovered in our hnRNP preparations. The association of F and G with the 28S rRNA is transient. Species D′ (5.8S snRNA) is transcribed as part of a larger precursor of 28S rRNA and is subsequently cleaved from the precursor and then hydrogen bonded to 28S rRNA (Speirs and Birnstiel, 1974). This association persists during transport of the 28S rRNA to the cytoplasm, and 5.8S RNA can be recovered from mature ribosomes. Conceivably, the nucleolar snRNAs may define sites for the binding of specific proteins or may influence the topology of the larger molecule in a manner which facilitates subsequent cleavage and maturation. In a similar fashion, the snRNAs which are recovered with hnRNP may be involved in the processing of hnRNA. In view of the observations that some snRNAs appear in the cytoplasm (Enger and Walters, 1970; Eliceiri, 1974; Zieve and Penman, 1976) and may shuttle in and out of the *Amoeba* nucleus (Goldstein and Ko, 1974), it is possible that some hnRNP-associated snRNAs are involved in the maturation and transport of mRNA from nucleus to cytoplasm. In order to define the role of snRNAs in hnRNP, it will be necessary to obtain information about the physical relationship of hnRNA, snRNA, and specific proteins in the hnRNP complex and the potential presence of snRNAs in nuclear and cytoplasmic RNP complexes which contain mRNA. In this regard, the MEL cell system, in which the synthesis of globin mRNA can be induced, may be useful for the identification of a specific mRNA in putative snRNA–mRNA–protein complexes.

Another important consideration is the identity of the nuclear site for hnRNA processing and the components of the processing complex. Some investigators envision the processing complex to be free RNP (Georgiev and Samarina, 1971), but there are data which can be interpreted to mean that the processing of nascent hnRNA, at least the initial stages of the process, occurs in close association with chromatin and perhaps also the nuclear membrane or nuclear matrix (Sommerville, 1973; Faiferman and Pogo, 1975; Augenlicht and Lipkin, 1976). When hnRNP is produced by extraction or sonication, elements of the processing complex may be released enzymatically and/or physically as free hnRNP or as hnRNP which is associated with a small amount of chromatin (Kimmel et al., 1976). In this study, DNA was recovered in our preparations and, although it did not cosediment with the 10S or 30–60S RNP, its presence means that the existence of a processing complex which contains elements of the chromatin, hnRNA, snRNA, and attached protein (including the nuclear protein skeleton) cannot be ruled out. Indeed, evidence for the existence of a nuclear complex which contains all of these elements has been presented by Miller et al. (1978a,b).

In conclusion, the experimental results obtained in this study support the contention that several snRNAs are nonartifactual, integral components of MEL cell hnRNP. This observation raises the possibility that certain snRNAs participate in the posttranscriptional processing and transport of hnRNA and mRNA.

Acknowledgments

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Gene-Sized Pieces Produced by Digestion of Linear Duplex DNA with Mung Bean Nuclease†

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ABSTRACT: The single-strand specific endonuclease from mung bean sprouts catalyzes a limited number of double-strand cleavages in linear duplex DNA from bacteriophages T_7, gh-1 and PM2. A molecular weight range of ~1.4–0.2 × 10^6 for the limit products derived from each DNA was estimated by agarose gel electrophoresis. The number of cleavages per T_7 DNA molecule at the digestion limit is 37 as determined by ^{32}P end labeling using polynucleotide kinase and [γ-^{32}P]-ATP. These cleavages are probably at unique sites, since discrete bands are seen after agarose gel electrophoresis of the products in the early stages of digestion of each DNA. Observation of discrete bands at the endonucleolytic limit is complicated by the terminally directed (exonuclease-like) activity of the enzyme and by a small number of random endonucleolytic cleavages. The unique sites are thermolabile. A 10 °C increase in temperature results in a ca. tenfold increase in the initial hydrolysis rate of T_7 DNA while the size distribution of the limit products is unchanged. Also, a change from 0.10 to 0.14 M salt concentration produces a ca. fourfold rate decrease. Double-strand cleavages in T_7 DNA arise from the preferential cleavage opposite an enzyme-inflicted nick rather than by the accumulation of nicks at potential double-strand cleavage sites. The naturally occurring nicks in T_5 DNA, which are in G + C rich sequences, are not preferred cleavage sites for mung bean nuclease unless they are first enlarged to five residue (average) gaps by *Escherichia coli* exonuclease III. The specificity of mung bean nuclease on T_7, gh-1 and PM2 DNAs is attributed to thermolabile or structural "breathing" sites which occur in the range of 1 site ~300–2100 base pairs in each of the linear duplexes, independent of the overall percent A + T. This range encompasses that of most of the known structural genes in T_7 DNA.

The so-called "single-strand specific" nucleases are a class of endonucleases which show a pronounced specificity for denatured as opposed to native DNA. Such enzymes have been isolated from many sources (for a review see Kowalski and Laskowski, 1976), but the enzymes which have been purified

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