Messenger RNA Processing and Nuclear Structure: Isolation of Nuclear Ribonucleoprotein Particles Containing β-Globin Messenger RNA Precursors

THORU PEDERSON and NICHOLAS G. DAVIS
Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545. Mr. Davis's present address is The Rockefeller University, New York 10021.

ABSTRACT To explore the relationships between transcription, messenger RNA (mRNA) processing, and nuclear structure, ribonucleoprotein particles containing heterogeneous nuclear RNA (hnRNP) have been purified from globin-producing mouse Friend erythroleukemia cells. These nuclear hnRNP particles sediment at 50S–200S and contain, in addition to high molecular weight hnRNA, a specific set of nuclear proteins predominated by a major component of ~38,000 mol wt. The hnRNP particles are free of histones and ribosomal structural proteins, indicating their purification from the two other major nucleoprotein components of the nucleus: chromatin and nucleolar ribosomal precursor RNP particles. The authenticity of the Friend cell hnRNP particles is demonstrated by the results of reconstruction experiments with deproteinized hnRNA, and by the resistance of the particles to dissociation during isopycnic banding in Cs_2SO_4 gradients without prior aldehyde fixation. Hybridization analysis with cloned mouse β-globin DNA demonstrates that hnRNP particles from induced Friend cells contain newly synthesized transcripts of the β-globin gene. Agarose gel electrophoresis of hnRNP particle-derived RNA denatured in glyoxal followed by "Northern" transfer to diazobenzyloxymethyl paper and hybridization with ^{32}P-labeled cloned mouse β-globin DNA reveals the presence in hnRNP of two size classes of β-globin gene transcripts, the larger of which corresponds to the pre-spliced 15S β-globin mRNA precursor previously identified in whole nuclear RNA, and the smaller of which corresponds to completely processed 9S β-globin mRNA. These results establish, for the first time, that the nuclear transcripts of a specific, well-defined eukaryotic structural gene can be isolated in an RNP particle form, and that their RNP structure persists throughout mRNA splicing.

The transcription of structural genes in the eukaryotic cell nucleus takes place at a nucleoprotein level of organization. This is true of both the templates for transcription, chromatin (reviewed in reference 36), and the products of transcription, heterogeneous nuclear RNA, which is rapidly assembled into ribonucleoprotein particles known as hnRNP (33, 34). However, the functional significance of these nucleoprotein environments for transcription and hnRNA metabolism is not understood. As a step toward defining the relationships between messenger RNA (mRNA) processing and nuclear structure, hnRNP particles have been purified from globin-producing mouse Friend erythroleukemia cells and characterized with particular reference to transcripts of the β-globin gene. The major conclusion is that both pre-spliced and spliced mRNA sequences have a ribonucleoprotein structure in the nucleus.

MATERIALS AND METHODS

Cell Culture, Radioisotopic Labeling, and Cell Fractionation

Clone 745 mouse Friend erythroleukemia cells were maintained in monolayer culture using Joklik-modified Eagle's minimum essential medium containing 20% fetal calf serum. For large-scale experiments, the desired numbers of cells were grown up in suspension culture using the same medium, induced with 2% (vol/vol) dimethyl sulfoxide (DMSO) and harvested 72–84 h later, when 70–85% of the cells were benzidine-positive. In some cases, cells were grown in the presence

of [^{14}C]thymidine (0.025 μCi/ml) for 24 h before harvest. To label hnRNA, cells were concentrated to 2–3 × 10^7 cells/ml in fresh, prewarmed medium, incubated at 37°C for 10 min with 0.08 μg actinomycin/ml to selectively inhibit ribosomal RNA synthesis (39, 40) and then for 20 min with [^{3}H]uridine at 20 μCi/ml. The cells were then poured into 5–10 vol of ice-cold balanced salt solution (14) and harvested by low-speed centrifugation. This and all subsequent steps were performed at 2°–4°C. After an additional wash, the cells were resuspended in 10 vol of "reticulocyte standard buffer" (RSB, 0.01 M NaCl, 1.5 mM $MgCl_2$, 0.01 M Tris-HCl, pH 7.0). After 3–5 min, the osmotically swollen cells were disrupted by two to five strokes of a glass Dounce homogenizer (Kontes Co., Vineland, N. J.) (clearance = ~38 μm). Cell disruption was monitored by phase-contrast microscopy and was always >95%. (Induced Friend cells are exceptionally easy to break open under these hypotonic conditions, and there is often a small fraction of cells that lyses spontaneously.) The homogenate was centrifuged at 2,000 *g* for 5 min, the red (hemoglobin-containing) cytoplasm was aspirated off, and the white pellet of nuclei was then washed several times in 10 vol of RSB until the supernatant wash was water-clear.

The washed nuclei were resuspended in RSB at 4 × 10^7 nuclei/ml and disrupted by mild sonication (one 8-s pulse at 40 W, Bronson model W185, standard microtip, Heat Systems Ultrasonics, Inc., Plainview, N. Y.). If nuclear breakage was observed to be incomplete, a second 8-s pulse was administered. The sonicated nuclei were then layered in 5- to 8-ml portions on 25 ml of 30% sucrose in RSB and centrifuged for 20 min at 5,000 rpm (4,500 *g*) in a Beckman SW27 rotor (Beckman Instruments, Spinco Div., Palo Alto, Calif.) to pellet nucleoli (28, 33) and also most of the chromatin (see Results and Table I). The opalescent band at the 0:30% sucrose interface, containing the hnRNP particles, was removed and 6-ml portions were layered on 15–45% linear sucrose gradients (28 ml) over a 4-ml 60% sucrose cushion. All sucrose solutions were made up in RSB and autoclaved before use. The gradients were centrifuged under a variety of conditions to empirically determine the maximal separation of hnRNP from the remaining chromatin. The conditions subsequently adopted for routine hnRNP isolation throughout this study were 12,000 rpm for 17 h in the SW27 rotor (see Fig. 1).

Gel Electrophoresis of Nuclear Proteins

hnRNP particles were recovered from pooled sucrose gradient fractions by centrifugation at 35,000 rpm for 14–16 h in a Spinco 60Ti rotor. The pellets of hnRNP were dissolved in 10 mM sodium phosphate buffer, pH 7.0, containing 1% SDS and 1% mercaptoethanol, dialyzed, and electrophoresed in the gel system which we have previously described in detail (7, 23). To analyze chromatin, the pellets from the hnRNP sucrose gradients (Fig. 1) were dissolved in the same buffer and processed similarly. Molecular weights were determined from the mobility of standards electrophoresed in parallel gels: bovine serum albumin (68,000), rabbit skeletal muscle actin (45,000), pancreatic DNase (31,000), and pancreatic RNase (13,700).

RNA Extraction, Denaturation, and Gradient Analysis

RNA was purified from hnRNP by digestion of the particles with Proteinase K (200 μg/ml, 60 min, 20°C) in the presence of SDS (0.5%). The digest was then extracted once with 1 vol of phenol:chloroform:isoamyl alcohol (50:49.5:0.5 vol/vol), and the aqueous phase was then re-extracted with 1 vol of chloroform: isoamyl alcohol (99:1 vol/vol). After addition of sodium acetate to 0.2 M, the RNA was precipitated by the addition of 2.5 vol of ethanol (16 h or longer, −20°C). The RNA was collected by centrifugation and dissolved in 80% (vol/vol) DMSO, denatured at 65°C for 2 min, quick-cooled to 0°C, diluted to 16% DMSO, and analyzed on 15–30% sucrose-SDS gradients as detailed in Fig. 5.

In some experiments, poly(A)$^+$ nuclear or cytoplasmic RNA was extracted and used for purposes of comparison. Nuclei in RSB were incubated with 50 μg pancreatic DNase/ml for 30 min at 4°C, followed by digestion with Proteinase K and phenol:chloroform extraction as detailed above. Cytoplasmic RNA was obtained by making the cytoplasmic fraction 0.1 M in NaCl, 0.5% in SDS, and 10 mM in EDTA, followed by phenol:chloroform extraction. After ethanol precipitation, the nuclear and cytoplasmic RNAs were chromatographed on oligo(dT)-cellulose as detailed previously (22). The RNA which was eluted from the column by low ionic strength buffer (10 mM Tris-HCl, pH 7.5) was made 0.2 M in sodium acetate, reprecipitated with ethanol, and used as "poly(A)$^+$" nuclear or cytoplasmic RNA (see Fig. 8).

Hybridization of hnRNP Particle-derived RNA with Cloned β-Globin DNA

The bacteriophage λgtWES recombinant mouse DNA clone MβG-2 (47) was grown in *E. coli* DP50 *sup*F under P2 containment. Phage was recovered from cleared lysates by polyethylene glycol extraction, followed by CsCl gradient sedimentation of the aqueous phase and then CsCl isopycnic banding. Pronase was added to the purified phage (1 mg/ml) and the mixture was dialyzed 1–2 h at 37°C. The DNA was extracted with phenol, followed by a second extraction with phenol:chloroform (1:1) followed by a final extraction with chloroform alone. The DNA was ethanol-precipitated from the last aqueous phase, digested with Hind III (2 U/μg DNA, 37°C, 2 h) and electrophoresed on 1.0% agarose slab gels. The desired 1.05 kilobase (kb) fragment (see Fig. 6) was then recovered from the gel (46). The DNA was heat-denatured and aliquots containing 0.5 μg were trapped on nitrocellulose filters. The filters were annealed with ^{3}H-labeled RNA from hnRNP particles (see Fig. 7) at RNA concentrations of between 80 and 320 μg/ml. The hybridization reactions contained, in 1.0 ml, 0.6 M NaCl, 0.06 M sodium citrate, 40% (vol/vol) deionized formamide, and 0.02% each (wt/vol) of polyvinylpyrolidone, Ficoll, and bovine serum albumin. Reactions were carried out at 42°C for 44 h. In addition to a filter containing cloned β-globin DNA, each hybridization reaction contained a duplicate blank nitrocellulose filter as a monitor for nonspecific binding, and a third filter containing 0.5 μg of total (uncloned) mouse DNA, prepared from cultured L cells. At the end of the hybridization, the set of three filters was removed from each reaction and washed for 5 min at 20°C in two 100-ml changes of 6 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate), followed by three 100-ml washes (5 min each) in 2 × SSC. Each set of filters was then digested with 50 μg/ml of pancreatic RNase in 2 × SSC (total volume = 50 ml) for 90 min at 37°C with gentle agitation. The RNase-digested filters were washed three times with 250 ml of 2 × SSC containing 0.5% SDS, four times with 250 ml of 70% ethanol, air-dried at 37°C, and counted in a toluene-based scintillation cocktail. All samples were counted for a period of time so that the 2σ counting error was ±5% or less. Hybrids were scored as RNase-resistant radioactivity corrected for counts on blank filters.

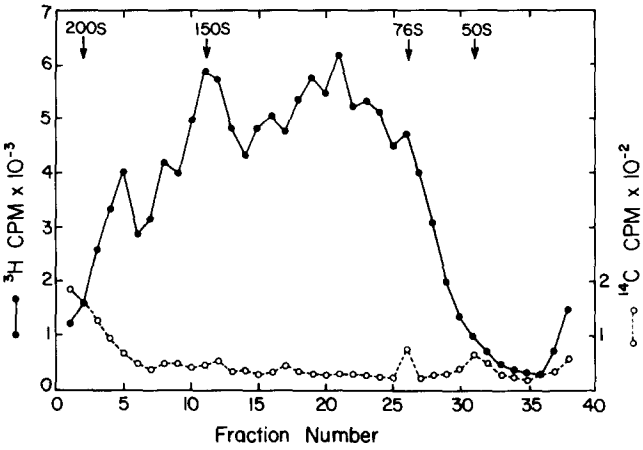

FIGURE 1 Sucrose gradient separation of hnRNP particles from chromatin. hnRNP particles were isolated from Friend cells that had been labeled for 24 h with [^{14}C]thymidine and then pulse labeled for 20 min with [^{3}H]uridine. The hnRNP particles were centrifuged in a 28-ml 15–45% sucrose gradient over a 4-ml 60% sucrose cushion in a Beckman SW27 rotor for 17 h at 12,000 rpm (4°C); the direction of sedimentation is from right to left. The gradient was collected in 1-ml fractions and the amount of TCA-insoluble ^{14}C and ^{3}H radioactivity in each fraction was determined by liquid scintillation counting. ●, [^{3}H]uridine; ○, [^{14}C]thymidine.

Northern Blot Hybridization

Ethanol-precipitated RNA was dissolved in 1 M deionized glyoxal, 50% DMSO, 10 mM sodium phosphate buffer, pH 7.0, at a concentration of 750 μg/ml and denatured at 50°C for 1 h. Samples containing 5–30 μg of RNA were electrophoresed in a 1% agarose slab gel containing 10 mM sodium phosphate buffer, pH 7.0. Electrophoresis was at 40 V for 4 h (20°–23°C). The gel was stained with ethidium bromide to visualize RNA size markers, washed, and the RNA was then transferred from the gel onto diazobenzyloxymethyl (DBM) paper following the procedure described by Alwine et al. (1) as later modified (2). After pre-hybridization with salmon sperm DNA, the transfer was hybridized as described (2) with the 1.05 kb Hind III β-globin DNA fragment (Fig. 6) which had been labeled by nick translation with [α-^{32}P]dCTP (41). The probe sp act was 1–2 × 10^7 cpm/μg. After hybridization, the paper was washed as described

(2) and autoradiographed with x-ray film using a Dupont "Lightning Plus" intensification screen (DuPont Instruments, Wilmington, Del.).

RESULTS

Isolation of hnRNP Particles from Friend Cell Nuclei

We have previously developed methods for purifying hnRNP particles from a number of different eukaryotic cells (15, 23, 33, 34). These methods are based upon the gentle disruption of nuclei by controlled, brief sonication to release hnRNP, which is normally anchored to elements of the nuclear structure (33). The released hnRNP particles are then separated from nucleoli and chromatin by successive sucrose gradient fractionations (6, 7, 33, 34). In the present investigation, these methods have been employed for the purification of hnRNP particles from globin-synthesizing mouse erythroleukemia cells. Induced cells were labeled for 24 h with [^{14}C]thymidine and hnRNA was then selectively pulse labeled with [^{3}H]uridine in the presence of a low dose of actinomycin (40). Table I shows the distribution of hnRNA ([^{3}H]uridine) and DNA ([^{14}C]thymidine) during cell and nuclear fractionation. It can be seen that 100% of the initial hnRNA and DNA is recovered in the first nuclear pellet, while the subsequent washed nuclear fraction is found to contain 86% of the initial hnRNA and 82% of the initial DNA ("2nd nuclear pellet," Table I). After the mild sonication step (see Materials and Methods), all of the nuclear hnRNA and DNA is recovered. Sedimentation of the nuclear sonicate on 30% sucrose at 4,500 *g* produces a tightly packed, opalescent band at the 0:30% sucrose interface; this band contains 70% of the initial hnRNA, but only 12% of the DNA (Table I).

In the initial isolation method developed for HeLa cell hnRNP particles (33), the 4,500 *g* sedimentation step was designed to selectively pellet nucleoli through the 30% sucrose, as initially shown by Maggio et al. (28). With HeLa cells, this nucleolar pellet contains only ~5% of the chromatin, with 84% remaining at the 0:30% sucrose interface along with the hnRNP particles and other small nuclear elements (see Table I in reference 7). The same general distribution of chromatin during the 4,500 *g* 30% sucrose step is observed with rat liver nuclei (34) and nuclei from the slime mold *Dictyostelium* (35); in both cases the great majority of the chromatin remains at the 0:30% sucrose interface. However, as can be seen in Table I, the result for induced Friend cell nuclei is different. In this case, only 12% of the chromatin remains in the 0:30% sucrose band, rather than the usual values of 85–90% obtained with the aforementioned cell systems. This may be because of the highly condensed state of chromatin in the fully induced Friend erythroleukemia cell nucleus. Thus, the 4,500 *g* 30% sucrose sedimentation step, originally designed to deplete the nuclear sonicate of specifically nucleoli, also serves in the case of the induced Friend cell to remove the majority of the chromatin as well (Table I).

To further purify the hnRNP particles from the small amount of remaining chromatin, the 0:30% sucrose band (Table I) was removed and sedimented on 15–30% linear sucrose gradients as previously described for HeLa cell hnRNP particles (33). This resulted in a partial separation of hnRNP from the remaining chromatin, as monitored by the gradient distribution of [^{14}C]DNA and [^{3}H]hnRNA radioactivity and by gel electrophoresis of proteins from across the gradients, which revealed some overlap of hnRNP particles with histones (data not shown). Through empirical trials of several different sucrose gradient sedimentation conditions, it was found that maximal separation of hnRNP particles from the chromatin was obtained by centrifugation of 6-ml aliquots of the 0:30% sucrose band (Table I) on a 28-ml linear 15–45% sucrose gradient over a 4-ml 60% sucrose cushion in a Spinco SW27 rotor for 17 h at 12,000 rpm. Fig. 1 shows the results of this gradient fractionation. As is the case for other eukaryotic systems (15, 23, 33, 34), Friend cell hnRNP particles display a heterodisperse sedimentation profile between ~50S and 200S. The sedimentation behavior of Friend cell hnRNP (Fig. 1) is dependent upon the integrity of the RNA, as shown by the marked reduction in S values observed if particles are subjected to mild pancreatic RNase digestion (0.1 μg/ml, 15 min, 4°C) before gradient analysis (data not shown). It can also be seen in Fig. 1 that, in contrast to the heterodisperse hnRNP which is mainly confined to the 50S–200S region of the gradient, the chromatin (as [^{14}C]DNA) is present only as a flat background across the gradient. As shown in Table II, 77% of the chromatin present in the 0:30% sucrose band (12% of the total chromatin, Table I) fractionates in the gradient pellet and 60% sucrose cushion, while 83% of the hnRNP particles remains in the gradient.

hnRNP Proteins

Fractions 6–30 from gradients such as that illustrated in Fig. 1 were pooled and the particles pelleted by ultracentrifugation. The pellets from the preparative hnRNP gradients were also recovered for analysis of chromatin proteins. Analysis of these two nuclear fractions by SDS-polyacrylamide gel electropho-

TABLE I

Distribution of hnRNA and DNA during Cell Fractionation

	[^{3}H]hnRNA	[^{14}C]DNA
	% of initial cpm	
1st Nuclear pellet	100	100
Cytoplasm	21	3
2nd Nuclear pellet	86	82
Sonicated nuclei	87	81
0:30% Sucrose band	70	12

Friend cells were grown for 24 h in the presence of [^{14}C]thymidine (0.025 μCi/ml) and then hnRNA was selectively pulse labeled with [^{3}H]uridine as detailed in Materials and Methods. Cells were washed and homogenized as described in Materials and Methods, aliquots of the homogenate were withdrawn, and the amounts of 10% TCA-precipitable ^{3}H and ^{14}C radioactivity were determined. These data were taken as "100%" initial values for comparison with comparable data from subsequent fractions (see Table). The data shown are the averages of duplicate determinations in two separate experiments.

TABLE II

Sucrose Gradient Fractionation of hnRNP and Chromatin

	[^{3}H]hnRNP	[^{14}C]chromatin
	% of initial cpm	
15–45% Gradient	83	23
60% Sucrose cushion	6	16
Pellet	11	61

Cells were labeled and fractionated as in Table I. The 0:30% sucrose band was layered on 15–45% sucrose gradients as detailed in Materials and Methods and centrifuged as in Fig. 1. The amounts of 10% TCA-precipitable ^{3}H and ^{14}C radioactivity throughout the gradient, cushion, and pellet were determined. The pellet material was first solubilized in 2% SDS before withdrawing aliquots for radioactivity determinations. The values shown in the table are expressed as percentages of recovered radioactivity, which was consistently between 80 and 90% of the initial ^{3}H and ^{14}C loaded on the gradients.

resis revealed major differences in their protein constituents. Histones dominate the chromatin fraction as expected (Fig. 2 *B*), but are present in only very low concentrations in the hnRNP particles (Fig. 2 *A*). The Friend cell hnRNP proteins are complex but, as in all the other eukaryotic cells previously investigated (5, 15, 18, 33, 34), there is a major component of ~38,000 mol wt ("p38"). The hnRNP contains very little protein of <38,000 mol wt, indicating the absence of nucleolar ribosomal precursor particles (37), which contain proteins of mainly 15,000–55,000 daltons (25). The proteins characteristic of the hnRNP particles (Fig. 2 *A*) are present at low levels in the chromatin (Fig. 2 *B*). This could represent incomplete separation of free hnRNP particles from chromatin by gradient sedimentation (Fig. 1 and Table II). However, it is also known that hnRNP particle assembly occurs as a very early post-transcriptional event on nascent hnRNA transcripts (E. Wieben and T. Pederson, unpublished results), which could explain the presence of a small amount of hnRNP proteins in the chromatin fraction.

The possibility arises that only some of the proteins observed in the gradient-purified hnRNP particles (Fig. 2 *A*) are bound to the hnRNA, with others being derived from cosedimenting, nonribonucleoprotein nuclear structures. This is ruled out by the fact that prior treatment of the pooled gradient fractions with pancreatic RNase (500 μg/ml, 30 min, 37°C) followed by collection of any RNase-resistant structures by high speed sedimentation results in a total elimination of all the protein components normally observed (not shown). This is not caused by proteolysis during the RNase digestion, because hnRNP mock-digested at 37°C without RNase retains its usual protein complement.

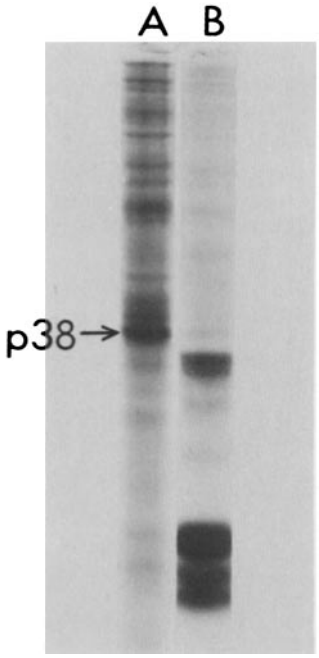

FIGURE 2 Distinct proteins of hnRNP and chromatin. hnRNP was recovered by high-speed centrifugation from fractions 6–30 of gradients such as shown in Fig. 1, and chromatin was obtained as the gradient pellet. Samples were dissolved in SDS, electrophoresed, and stained with Coomassie Blue as detailed previously (7, 23). (*A*) hnRNP proteins. (*B*) Chromatin proteins. The molecular weight of the protein labeled "p38" in gel *A* was determined on three separate hnRNP samples as detailed in Materials and Methods, and the average value was 37,866 daltons (standard deviation = ±986 daltons).

Authenticity and Stability of hnRNP

To investigate the possibility that hnRNP particles are formed as the result of nonspecific RNA:protein interactions during nuclear fractionation, which had been ruled out in earlier studies on hnRNP isolated from other cells (15, 33, 49), reconstruction experiments were performed in which deproteinized, ^{3}H-labeled Friend cell hnRNA was added to nuclei before their disruption. After isolation of the 0:30% sucrose band (Table I), which contained 88% of the added [^{3}H]hnRNA, this fraction was subjected to controlled pancreatic RNase digestion under conditions in which previous studies had defined a significant differential in the digestion kinetics of naked hnRNA vs. hnRNP. As shown in Fig. 3, the added [^{3}H]hnRNA was digested much more rapidly than endogenous hnRNP (see legend for details), indicating that the addition of a naked hnRNA probe to nuclei does not result in the formation of material that is as nuclease-protected as the endogenous hnRNP particles. Similar results are obtained if the naked hnRNA probe is added to nuclei after sonication, and in both cases the digestion of the probe is very similar to that of naked RNA assayed in buffer alone. In an occasional experiment, the added probe was observed to digest with slightly slower kinetics than naked hnRNA assayed in buffer alone, but still much faster than the endogenous hnRNP (as in Fig. 3), indicating its possible interaction with a small amount of nuclear protein. The conclusion being drawn therefore is not that hnRNA-protein associations never occur during nuclear fractionation, but rather that they cannot be a major factor in the formation of the observed hnRNP particles. These nuclease protection

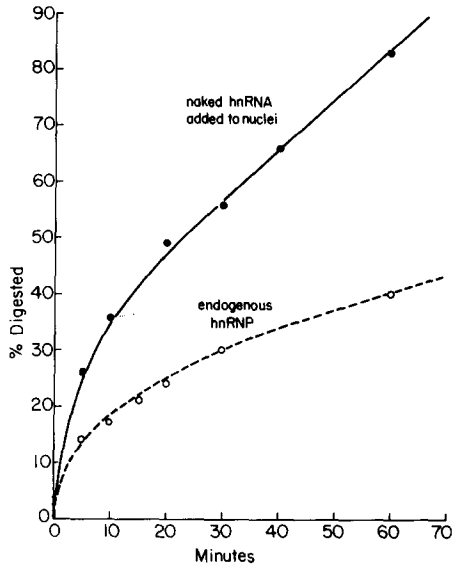

FIGURE 3 Reconstruction experiment. ^{3}H-labeled deproteinized hnRNA was added to 10^8 isolated Friend cell nuclei in a final volume of 2.5 ml of RSB. The endogenous (unlabeled) hnRNA concentration was 11 μg/ml and the endogenous hnRNA:exogenous ^{3}H-hnRNA probe mass ratio was ~100:1. After nuclear fractionation, the hnRNP fraction was digested with 0.1 μg pancreatic RNase/ml at 4°C, and aliquots were removed at the indicated times and the digestion quenched by the immediate addition of mercaptoethanol (final concentration = 0.75 mM, which is a 100,000-fold molar excess over the pancreatic RNase). The amounts of [^{3}H]hnRNA probe remaining TCA-insoluble were measured (●). In parallel, a separate ^{3}H-labeled endogenous hnRNP preparation (○) was digested under identical conditions, including the same enzyme:endogenous hnRNA ratio as in the reconstruction experiment.

studies confirm the results of earlier reconstruction experiments in which nonspecific hnRNA:protein interactions were ruled out using a velocity sedimentation analysis (15, 33).

As shown in Fig. 4, the gradient-purified hnRNP particles (Fig. 1) are resistant to dissociation when banded in a preformed Cs_2SO_4 density gradient, even without prior fixation. This rather remarkable stability of hnRNP, which was initially reported by Wilt et al. (49) in a study of sea urchin embryo hnRNP, has emerged as a characteristic attribute of these nuclear particles (*e.g.* for HeLa cell hnRNP see reference 11), and also serves as a useful diagnostic property for hnRNP, because ribosomal particles are stripped almost completely free of protein in these gradients. However, the resistance of hnRNP to dissociation in Cs_2SO_4 as shown in Fig. 4 is relative, not absolute, for if particles are mixed with Cs_2SO_4 of density 1.50 g/cm^3 and then banded in a self-generated gradient, the [^{3}H]-uridine radioactivity is observed as a single homogenous peak at 1.66 g/cm^3, which is the density of naked RNA in these gradients (data not shown). In the case of preformed Cs_2SO_4 gradients (Fig. 4), the hnRNP apparently bands at its isopycnic density of 1.33 g/cm^3 before it reaches a gradient position where the Cs_2SO_4 ion activity is high enough to promote its dissociation. The observed buoyant density of 1.33 g/cm^3 is very similar to that previously reported for HeLa cell and sea urchin embryo hnRNP (11, 49), and is estimated to reflect a protein:RNA mass ratio of ~4:1 (80% protein).

Detection of Specific mRNA Sequences in hnRNP Particles

The fact that the isolated particles contain rapidly labeled RNA that is refractory to the selective inhibition of ribosomal RNA synthesis by a low concentration actinomycin (Fig. 1) identifies these particles as containing hnRNA. This is confirmed by sucrose gradient analysis of the hnRNP-derived RNA after thermal denaturation in DMSO (Fig. 5), which demonstrates this RNA to have the sedimentation behavior

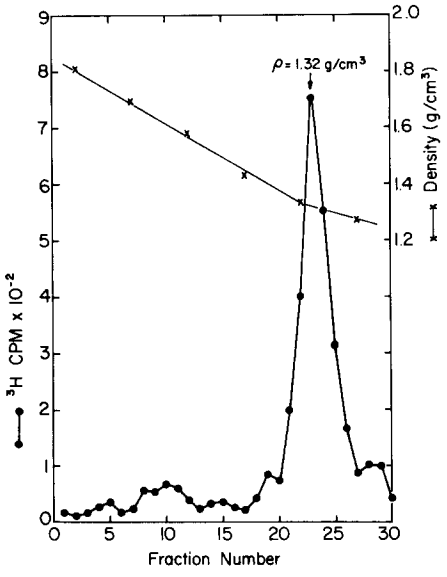

FIGURE 4 Stability of hnRNP particles in Cs_2SO_4. 0.50 ml of hnRNP was taken from fraction 20 of a preparative gradient such as shown in Fig. 1 and layered on a preformed 4.5 ml Cs_2SO_4 gradient having an initial density range of 1.25–1.75 g/cm^3 (38). The gradient was centrifuged in a Beckman SW50.1 rotor at 35,000 rpm for 65.5 h (19°C). ●, TCA-insoluble ^{3}H radioactivity; ×, density.

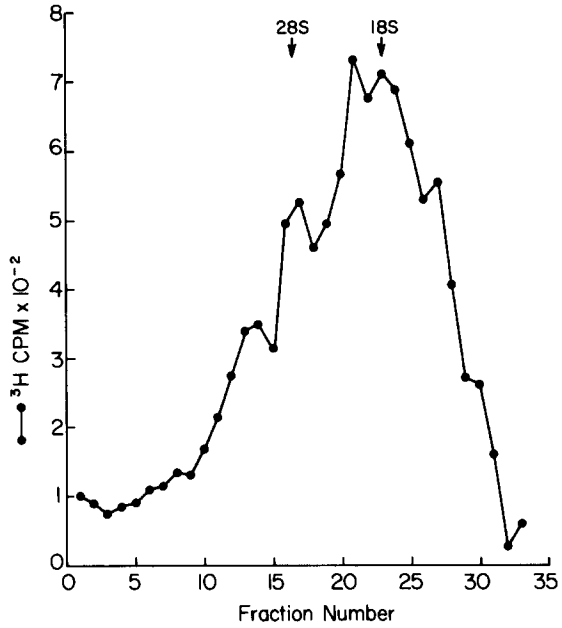

FIGURE 5 Size of hnRNP particle-derived RNA after denaturation in DMSO. RNA was extracted from [^{3}H]uridine-labeled hnRNP and thermally denatured in DMSO as detailed in Materials and Methods. A 0.50-ml sample was layered on a 17 ml 15–30% sucrose-SDS gradient and centrifuged in an SW27.1 rotor at 23,500 rpm for 14.25 h (20°C). The gradients contained 0.1 M NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 7.0, and 0.5% SDS. ●, TCA-precipitable ^{3}H radioactivity. Arrows denote the positions of 18S and 28S ribosomal RNA markers run in a parallel gradient.

characteristic of mammalian hnRNA after disruption of intermolecular aggregates. Although it is never possible to know how much endonucleolytic degradation has occurred in such hnRNA preparations relative to their native size in vivo (because the size of gene-specific RNA sequences is not visualized), the sedimentation profile in Fig. 5 does establish that Friend cell hnRNP particles can be isolated without gross degradation of the hnRNA. (Evidence for the presence of covalently intact globin gene transcripts in these particles will be presented in the following section.)

To examine the presence of specific gene transcripts in the particles, [^{3}H]uridine pulse-labeled hnRNP was isolated and displayed on sucrose gradients as in Fig. 1. Pooled gradient fractions of hnRNP were deproteinized and the labeled hnRNA was hybridized with a mouse β-globin gene probe. As shown in Fig. 6, restriction endonuclease Hind III digestion of the bacteriophage λ-cloned mouse DNA fragment MβG-2 (47) produces a 1.05 kb fragment carrying the 5′ half of the β-globin gene (see legend to Fig. 6 for details). Fig. 7 shows the results of hybridizing pulse-labeled hnRNA retrieved from pooled hnRNP gradient fractions with this β-globin gene-specific probe. The heterodisperse sedimentation behavior of the β-globin sequences indicates that they are present in a range of high molecular weight hnRNP particles. However, the distribution of β-globin sequences is different from that of the bulk hnRNP, with globin sequences being somewhat more concentrated in fractions 18–30 (~60S–120S), which contain 52% of the β-globin sequences but only ~25% of the total hnRNP. Hybridization of parallel gradient fractions with total mouse DNA revealed a pattern that closely followed the total hnRNP profile (not shown), indicating that, in contrast to β-globin sequences, repetitive DNA sequence transcripts are present at

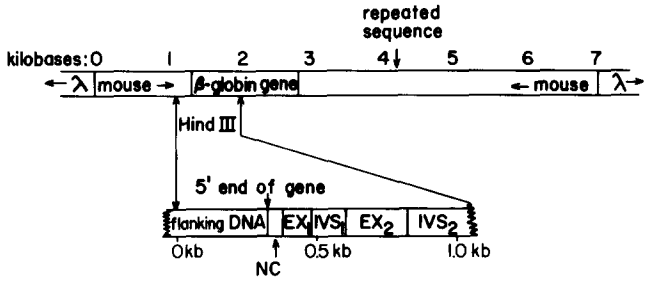

FIGURE 6 Isolation of β-globin DNA probe. The recombinant phage λgtWES·MβG-2 carries a 7 kb insert of mouse DNA containing the β-globin$_{major}$ gene (47). Hind III digestion of λgtWES·MβG-2 yields a 1.05 kg fragment containing the 5′ half of the β-globin gene, including a 52 nucleotide 5′ noncoding leader sequence (*NC*), the two most 5′-ward coding regions of the gene (EX_1 and EX_2, respectively), the entire smaller intervening sequence (IVS_1) and approximately one-third of the larger intervening sequence (IVS_2). The arrow labeled "repeated sequence" indicates the approximate location of a transcribed repetitive DNA sequence that hybridizes extensively with Friend cell hnRNA unless first removed by Hind III digestion (N. G. Davis and T. Pederson, unpublished results). The 1.05 kb Hind III fragment was used as a hybridization probe for β-globin gene transcripts in hnRNP (Figs. 7 and 8).

a rather uniform level throughout the different hnRNP size classes.

These data show that newly synthesized transcripts of the β-globin gene are present in high molecular weight hnRNP particles. The covalent integrity of these globin RNA sequences in hnRNP is now considered.

Identification of Covalently Intact β-Globin mRNA Precursors in hnRNP

Unlabeled hnRNP was isolated from induced cells as usual and the RNA was deproteinized and subjected to agarose gel electrophoresis after thermal denaturation in the presence of glyoxal. The RNA was then transferred covalently onto diazobenzyloxymethyl paper (1, 2) and hybridized with the 1.05 kb Hind III β-globin DNA fragment which had been ^{32}P-labeled by nick translation. To calibrate the gel positions of the known mouse globin RNA species, total Friend cell poly(A)$^+$ cytoplasmic and nuclear RNA were electrophoresed and analyzed in parallel. Lanes *A* and *B* of Fig. 8 show the typical results obtained with the standards of poly(A)$^+$ cytoplasmic RNA (lane *A*) and poly(A)$^+$ nuclear RNA (lane *B*). The cytoplasmic RNA contains a single component reacting with the β-globin probe. Based upon the electrophoretic migration of RNA standards of known molecular weight, the cytoplasmic species reacting with globin DNA in Fig. 8 *A* is estimated to have a mol wt of 280,000. For convenience, this cytoplasmic globin RNA will be hereafter referred to as "9S." As shown in lane *B*, the poly(A)$^+$ nuclear RNA contains two β-globin sequence components, one of which reproducibly comigrates with the cytoplasmic 9S globin mRNA and a second, larger species which has a measured mol wt of 600,000, corresponding to the "15S" mouse β-globin mRNA precursor previously described (42). The 9S component in the nuclear RNA (Fig. 8, lane *B*) does not appear to be caused by polyribosomal contamination, because washing the nuclei in 10 mM EDTA before RNA extraction does not alter the ratio of 15S to 9S globin RNA in the Northern blots (data not shown). It therefore appears that there is a true nuclear stage of 9S globin sequences, which corroborates an earlier conclusion based on

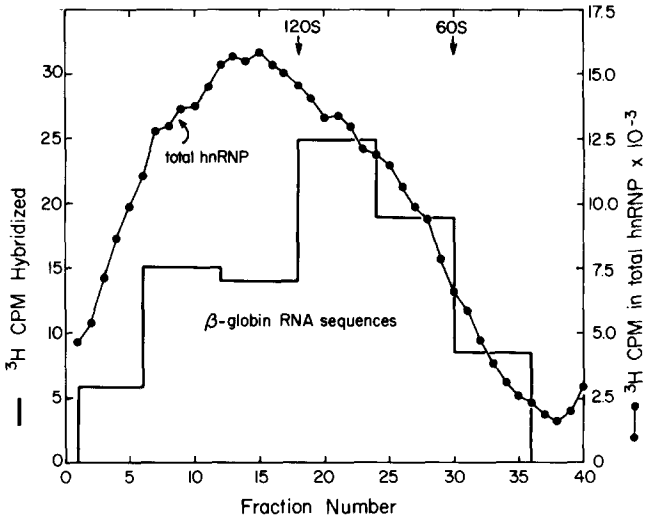

FIGURE 7 Detection of newly synthesized β-globin gene transcripts in Friend cell hnRNP particles. [^{3}H]uridine pulse-labeled hnRNP was isolated as detailed in Materials and Methods and centrifuged in 15–45% sucrose gradients as in Fig. 1. RNA was extracted from pooled gradient fractions as shown and hybridized to nitrocellulose filters containing cloned mouse β-globin DNA (see Fig. 6). Details of the reaction conditions and assay of hybrid formation are given in Materials and Methods. ●, TCA-precipitable ^{3}H radioactivity in total hnRNP, monitored in small aliquots of each gradient fraction. The histogram shows the total amount of β-globin RNA sequence present in each pool of gradient fractions. The amount of RNase-resistant ^{3}H radioactivity observed on blank filters was 0–3 cpm.

labeling kinetics (4).

As can be seen in lane *C* of Fig. 8, the RNA recovered from the purified hnRNP particles contains both the 15S and 9S globin sequences, and in approximately the same relative proportions as in the total nuclear RNA (lane *B*). These results therefore demonstrate that 15S pre-spliced β-globin mRNA precursors (19, 44, 48) have an RNP structure in the Friend cell nucleus, and that these mRNA precursors can be isolated as covalently intact RNA molecules in RNP particles.

DISCUSSION

It is now firmly established that hnRNA exists in an RNP form in the eukaryotic cell nucleus. This fact was first suggested by light microscope studies of nascent RNA on the lateral loops of amphibian oocyte lampbrush chromosomes (9, 16), and has been more dramatically demonstrated by the electron microscope identification of hnRNA:RNP particles *in situ* (29, 32, 45) and on unfolded chromatin fibers spread from nuclei or cells by detergent lysis (8, 26, 27, 31). Initial attempts to extract these hnRNP particles from nuclei resulted in the isolation of degraded, 40S RNP complexes (43), because of the action of endogenous nucleases. While these early degraded RNP preparations were of value for some purposes, such as enumerating the minimal set of proteins present in these so-called "core" particles (5, 18, 30), the fact remains that the 40S RNPs contain highly degraded hnRNA fragments and therefore cannot be regarded as native structures.

We have developed an alternative approach for purifying hnRNP which leads to the isolation of larger particles that contain high molecular weight hnRNA (7, 23, 33). In addition, it has been possible to demonstrate that these latter hnRNP preparations contain specific proteins bound to defined nucleotide sequences, such as poly(A) (15, 20, 22), and that they

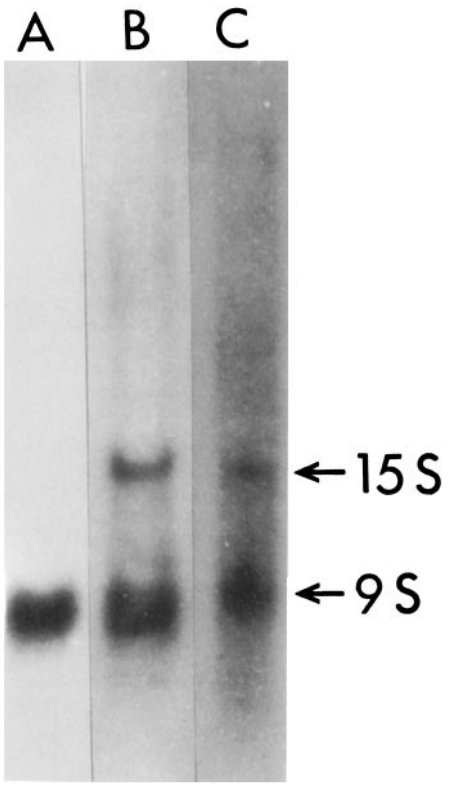

FIGURE 8 Northern blot hybridization of β-globin gene transcripts in hnRNP. RNA was recovered from nuclei, cytoplasm, or purified hnRNP particles by Proteinase K-phenol deproteinization and electrophoresed in agarose gels after denaturation in glyoxal (see Materials and Methods). After transfer to DBM paper, the RNA was hybridized with nick-translated [^{32}P]β-globin DNA (see Fig. 6). Lane *A*: total poly(A)$^+$ cytoplasmic RNA; lane *B*: total poly(A)$^+$ nuclear RNA; lane *C*: RNA from purified hnRNP particles. See text for description of molecular weight calibrations of the 15S and 9S β-globin RNA components.

contain different domains of nucleoprotein structure in which some RNA regions are less complexed with protein than others (e.g., double-stranded regions [10, 11, 13]). In some cases, these same domains can also be demonstrated in hnRNP in vivo (12). All of these facts suggest, but of course do not prove, that the isolated hnRNP particles represent native structures. However, it has not been possible in our previous studies (10–13, 15, 20–22, 24, 33, 34) to examine these particles in terms of specific, intact mRNA precursor sequences, because of the lack of gene-specific probes in the particular cellular systems being used, e.g., HeLa cells. In the present investigation, we have embarked upon the isolation of high molecular weight hnRNP particles from DMSO-induced mouse erythroleukemia cells that are synthesizing substantial quantities of globin mRNA, as a step toward the analysis of defined gene transcripts in hnRNP. The results of this study demonstrate that Friend cell hnRNP particles can be isolated in good purity by slight modifications of our original methods. The physical and biochemical properties of the Friend cell particles—sedimentation behavior, buoyant density, protein content, and nuclease sensitivity (Figs. 1–4)—all recapitulate those described previously for HeLa cell hnRNP (11, 23, 33). More importantly, the present results establish that the purified Friend cell particles contain high molecular weight, rapidly labeled hnRNA (Fig. 5), including covalently intact nuclear precursors of β-globin mRNA (Figs. 7 and 8).

In any isolation procedure for nucleoprotein, it is essential to show that the isolated material does not arise through nonspecific nucleic acid: protein interactions during cell fractionation. Indeed, the original studies of eukaryotic cytoplasmic mRNP particles were compromised by just such artifacts, which were subsequently brought to light through specific reconstruction studies and more critical experimental work (3, 17). These later studies revealed that cytoplasmic extracts of mammalian cells contain a large concentration of RNA-binding proteins that rapidly associate nonspecifically with added exogenous RNAs (3, 17). The existence of these RNA-binding proteins does not of course eliminate the possibility that there is really such a thing as cytoplasmic mRNP (nor does it prove that there is), but it does argue for caution in its isolation and characterization. In contrast to the results for cytoplasmic fractions, reconstruction experiments involving the addition of naked RNA to nuclei or nuclear extracts have consistently failed to generate nonspecific RNP complexes (15, 33, 49, and the present study). In these experiments the amounts of added probe RNA are very low relative to the endogenous nuclear protein mass (15, and see legend to Fig. 3), a situation that should be optimal for detecting nonspecific interactions of proteins with the probe. Thus, the consistent negative outcome of these nuclear reconstruction experiments argues against the existence of a soluble pool of the major hnRNA-binding proteins in the cell nucleus. Apparently the hnRNP proteins are delivered from the cytoplasm to the nuclear interior at the correct stoichiometry to support the ongoing tempo of transcription and hnRNP assembly. This situation constitutes an intriguing case of intracellular coordination that is richly deserving of further study.

The present identification of pulse-labeled transcripts of the β-globin gene in Friend cell hnRNP constitutes the first demonstration of a specific, well-defined mRNA sequence in high molecular weight hnRNP particles. The more important point, however, is that covalently intact, 15S β-globin mRNA precursor can be isolated as hnRNP, which immediately opens the door to the use of these hnRNP particles to address mRNA processing, especially splicing. This represents a small, but finite step in the continuing exploration of nuclear RNP particles as both a conceptual framework and a technical vehicle for investigating mRNA processing at the subcellular level of biological organization.

We thank Arthur Skoultchi for providing us with a starter culture of Friend cells, Philip Leder for λgtWES·MβG-2, Marcel Gut for synthesizing 1-[(*m*-nitrobenzyloxy)methyl] pyridinium chloride, and Eileen Falvey for expert assistance with the Northern hybridizations.

This work was supported by National Institutes of Health grants GM 21595, GM 23914, and CA 12708. Central instrument facilities were supported in part by the Mimi Aaron Greenberg Cancer Research Fund.

A partial account of this work was presented at the Second International Symposium on RNA in Development, 25–30 April 1980, in Peking, China.

This is paper 12 in a series entited "Ribonucleoprotein Organization of Eukaryotic RNA." The 11th paper in the series is reference 13.

Received for publication 28 March 1980, and in revised form 17 June 1980.

REFERENCES

1. Alwine, J. C., D. J. Kemp, and G. R. Stark. 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA

probes. *Proc. Natl. Acad. Sci. U. S. A.* 74:5350–5354.
2. Alwine, J. C., D. J. Kemp, B. A. Parker, J. Reiser, J. Renart, G. R. Stark, and G. M. Wahl. 1980. Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxymethyl-paper. *Methods Enzymol.* 68:220–242.
3. Baltimore, D., and A. S. Huang. 1970. Interaction of HeLa cell proteins with RNA. *J. Mol. Biol.* 47:263–273.
4. Bastos, R. N., and H. Aviv. 1977. Globin RNA presursor molecules: Biosynthesis and processing in erythroid cells. *Cell.*11:641–650.
5. Beyer, A. L., M. E. Christensen, B. W. Walker, and W. M. LeStourgeon. 1977. Identification and characterization of the packaging proteins of core 40S hnRNP particles. *Cell.* 11:127–138.
6. Bhorjee, J. S., and T. Pederson. 1972. Non-histone chromosomal proteins in synchronized HeLa cells. *Proc. Natl. Acad. Sci. U. S. A.* 69:3345–3349.
7. Bhorjee, J. S., and T. Pederson. 1973. Chromatin: Its isolation from cultured mammalian cells with particular reference to contamination by nuclear ribonucleoprotein particles. *Biochemistry.* 12:2766–2773.
8. Busby, S., and A. Bakken. 1979. A quantitative electron microscopic analysis of transcription in sea urchin embryos. *Chromosoma (Berl.).* 71:249–262.
9. Callan, H. G., and L. Lloyd. 1960. Lampbrush chromosomes of crested newts *Triturus cristatus* (Laurenti). *Phil. Trans. Roy. Soc. London Ser. B.* 243:135–219.
10. Calvet, J. P., and T. Pederson. 1977. Secondary structure of heterogeneous nuclear RNA: Two classes of double-stranded RNA in native ribonucleoprotein. *Proc. Natl. Acad. Sci. U. S. A.* 74:3705–3709.
11. Calvet, J. P., and T. Pederson. 1978. Nucleoprotein organization of inverted repeat DNA transcripts in heterogeneous nuclear RNA-ribonucleoprotein particles from HeLa cells. *J. Mol. Biol.* 122:361–378.
12. Calvet, J. P., and T. Pederson. 1979. Heterogeneous nuclear RNA double-stranded regions probed in living HeLa cells by cross-linking with the psoralen derivative aminomethyltrioxsalen. *Proc. Natl. Acad. Sci. U. S. A.* 76:755–759.
13. Calvet, J. P., and T. Pederson. 1979. Photochemical cross-linking of secondary structure in HeLa cell heterogeneous nuclear RNA in situ. *Nucleic Acids Res.* 6:1993–2001.
14. Earle, W. R. 1943. Production of malignancy *in vitro.* Part IV. The mouse fibroblast cultures and changes seen in the living cells. *J. Natl. Cancer Inst.* 4:165–212.
15. Firtel, R. A., and T. Pederson. 1975. Ribonucleoprotein particles containing heterogeneous nuclear RNA in the cellular slime mold, *Dictyostelium discoideum. Proc. Natl. Acad. Sci. U. S. A.* 72:301–305.
16. Gall, J. G. 1955. On the submicroscopic structure of chromosomes. *Brookhaven Symp. Biol.* 8:17–32.
17. Girard, M. and D. Baltimore. 1966. The effect of HeLa cell cytoplasm on the rate of sedimentation of RNA. *Proc. Natl. Acad. Sci. U. S. A.* 56:999–1002.
18. Karn, J., G. Vidali, L. C. Boffa, and V. G. Allfrey. 1977. Characterization of the non-histone nuclear proteins associated with rapidly labeled heterogeneous nuclear RNA. *J. Biol. Chem.* 252:7307–7322.
19. Kinniburgh, A. J., J. E. Mertz, and J. Ross. 1978. The precursor of mouse β-globin messenger RNA contains two intervening RNA sequences. *Cell.* 14:681–693.
20. Kish, V., and T. Pederson. 1975. Ribonucleoprotein organization of polyadenylate sequences in HeLa cell heterogeneous nuclear RNA. *J. Mol. Biol.* 95:227–238.
21. Kish, V., and T. Pederson. 1976. Poly(A)-rich ribonucleoprotein complexes from HeLa cell messenger RNA. *J. Biol. Chem.* 251:5888–5894.
22. Kish, V., and T. Pederson. 1977. Heterogeneous nuclear RNA secondary structure: Oligo(U) sequences base-paired with poly(A) and their possible role as binding sites for heterogeneous nuclear RNA-specific proteins. *Proc. Natl. Acad. Sci. U. S. A.* 74:1426–1430.
23. Kish, V., and T. Pederson. 1978. Isolation and characterization of ribonucleoprotein particles containing heterogeneous nuclear RNA. *Methods Cell Biol.* 17:377–399.
24. Kumar, A., and T. Pederson. 1975.Comparison of proteins bound to heterogeneous nuclear RNA and messenger RNA in HeLa cells. *J. Mol. Biol.* 96:353–365.
25. Kumar, A., and J. R. Warner. 1972. Characterization of ribosomal precursor particles from HeLa cell nucleoli. *J. Mol. Biol.* 63:233–246.
26. Laird, C. D., and W. Y. Chooi. 1976. Morphology of transcription units in *Drosophila melanogaster. Chromosoma (Berl.).* 58:193–218.
27. Laird, C. D., L. E. Wilkinson, V. E. Foe and W. Y. Chooi. 1976. Analysis of chromatin-associated fiber arrays. *Chromosoma (Berl.).* 58:169–192.
28. Maggio, R., P. Siekevitz, and G. E. Palade. 1963. Studies on isolated nuclei. II. Isolation and chemical characterization of nucleolar and nucleoplasmic subfractions. *J. Cell Biol.* 18:293–312.
29. Malcolm, D. B., and J. Sommerville. 1974. The structure of chromosome-derived ribonucleoprotein in oocytes of *Triturus cristatus carnifex* (Laurenti). *Chromosoma (Berl.).* 48: 137–158.
30. Martin, T., P. Billings, A. Levey, S. Ozarslan, T. Quinlan, H. Swift, and L. Urbas. 1973. Some properties of RNA:protein complexes from the nucleus of eukaryotic cells. *Cold Spring Harbor Symp. Quant. Biol.* 38:921–932.
31. McKnight, S. L., and O. L. Miller, Jr. 1979. Post-replicative nonribosomal transcription units in *D. melanogaster* embryos. *Cell.* 17:551–563.
32. Monneron, A., and W. Bernhard. 1969. Fine structural organization of the interphase nucleus in some mammalian cells. *J. Ultrastruct. Res.* 27:266–288.
33. Pederson, T. 1974. Proteins associated with heterogeneous nuclear RNA in eukaryotic cells. *J. Mol. Biol.* 83:163–183.
34. Pederson, T. 1974. Gene activation in eukaryotes: Are nuclear acidic proteins the cause or the effect? *Proc. Natl. Acad. Sci. U. S. A.* 71:617–621.
35. Pederson, T. 1977. Isolation and characterization of chromatin from the cellular slime mold, *Dictyostelium discoideum. Biochemistry.* 16:2771–2777.
36. Pederson, T. 1978. Chromatin structure and gene transcription. Nucleosomes permit a new synthesis. *Int. Rev. Cytol.* 55:1–22.
37. Pederson, T., and A. Kumar. 1971. Relationship between protein synthesis and ribosome assembly in HeLa cells. *J. Mol. Biol.* 61:655–668.
38. Pederson, T., and J. S. Bhorjee. 1979. Evidence for a role of RNA in eukaryotic chromosome structure. Metabolically stable, small nuclear RNA species are covalently linked to chromosomal DNA in HeLa cells. *J. Mol. Biol.* 128:451–480.
39. Perry, R. P. 1962. The cellular sites of synthesis of ribosomal and 4S RNA. *Proc. Natl. Acad. Sci. U. S. A.* 48:2179–2196.
40. Perry, R. P., T-Y. Cheng, J. J. Freed, J. R. Greenberg, D. E. Kelley, and K. D. Tartof. 1970. Evolution of the transcription unit of ribosomal RNA. *Proc. Natl. Acad. Sci. U. S. A.* 65:609–616.
41. Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* 113:237–251.
42. Ross, J., and D. A. Knecht. 1978. Precursors of alpha and beta globin messenger RNAs. *J. Mol. Biol.* 119:1–20.
43. Samarina, O. P., E. M. Lukanidin, J. Molnar, and G. P. Georgiev. 1968. Structural organization of nuclear complexes containing DNA-like RNA. *J. Mol. Biol.* 33:251–263.
44. Smith, K., and J. B. Lingrel. 1978. Sequence organization of the β-globin mRNA precursor. *Nucleic Acids Res.* 5:3295–3301.
45. Stevens, B. J., and H. Swift. 1966. RNA transport from nucleus to cytoplasm in *Chironomus* salivary glands. *J. Cell Biol.* 36:55–77.
46. Thuring, R. W. J., J. P. M. Sanders, and P. Borst. 1975. A freeze-squeeze method for recovering long DNA from agarose gels. *Anal. Biochem.* 66:213–220.
47. Tilghman, S. M., D. C. Tiemeier, F. Polsky, M. H. Edgell, J. G. Seidman, A. Leder, L. W. Enquist, B. Norman, and P. Leder. 1977. Cloning specific segments of the mammalian genome: Bacteriophage λ containing mouse globin and surrounding gene sequences. *Proc. Natl. Acad. Sci. U. S. A.* 74:4406–4410.
48. Tilghman, S. M., P. J. Curtis, D. C. Tiemeier, P. Leder, and C. Weissmann. 1978. The intervening sequence of a mouse β-globin gene is transcribed within the 15S β-globin mRNA precursor. *Proc. Natl. Acad. Sci. U. S. A.* 75:1309–1313.
49. Wilt, F., M. Anderson, and E. Ekenberg. 1973. Centrifugation of nuclear ribonucleoprotein particles of sea urchin embryos in cesium sulfate. *Biochemistry.* 12:959–966.