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Complexity of Poly(A)-Containing Heterogeneous Nuclear Ribonucleic Acid from Mouse Embryoid Bodies (OTT6050)†

Per-Erik Månsson* and Stephen E. Harris

ABSTRACT: Poly(A+)-hnRNA was isolated from mouse embryoid bodies (OTT6050). We have investigated this RNA with respect to sequence complexity by using a tritiated cDNA probe to poly(A+)-hnRNA ([^{3}H]cDNA$_{hn}$). A comparison was also made with total poly(A+)-RNA from mouse embryoid bodies. Two classes of sequences were found in poly(A+)-hnRNA. They represented 50 and 46% of the sequences in the poly(A+)-hnRNA, which corresponds to 570 and 12 500 different hnRNA sequences, respectively, of an average length of 2700 nucleotides (NT) present on an average of 12 and 1 copies per cell, respectively. The total complexity in poly(A+)-hnRNA was 3.4×10^7 NT. Three classes of sequences could be detected in total poly(A+)-RNA (mainly cytoplasmic), representing 9, 61, and 25%, respectively, of the sequences in its [^{3}H]cDNA probe. This corresponds to 12 000, 620, and 16 copies per cell of poly(A+)-RNA with each class having 2, 140, and 2500 different sequences, respectively, of an average length of 1350 NT. The total complexity of the poly(A+)-RNA was 3.4×10^6 NT. This indicates that the poly(A+)-hnRNA is approximately 10 times more complex with respect to sequence information than the total poly(A+)-RNA. The vast majority of the total poly(A+)-RNA sequences are present in the poly(A+)-hnRNA but in much lower concentration. Total poly(A+)-RNA saturated 65% of the nuclear [^{3}H]cDNA$_{hn}$ probe. This suggests that there are poly(A+)-hnRNA sequences present in the nucleus which have no major detectable counterpart in the total poly(A+)-RNA [90–96% poly(A+)-containing cytoplasmic RNA]. Total poly(A+)-RNA from the more differentiated mouse neuroepithelial teratocarcinomas saturated only 37% of the nuclear [^{3}H]cDNA$_{hn}$ probe from mouse embryoid bodies compared to 65% for the total poly(A+)-RNA from embryoid bodies.

The flow of genetic information from the nucleus to the cytoplasm in eucaryotic cells involves several posttranscriptional events (Getz et al., 1975; Hough et al., 1975; Bantle & Hahn, 1976; Herman et al., 1976). These events include specific nucleolytic cleavage from what are presumed to be high molecular weight primary transcripts (Lee et al., 1971; Greenberg & Perry, 1972; Jelinek et al., 1973) and a posttranscriptional addition of a poly(A) tract at the 3′ end to the high molecular weight nuclear precursor (Darnell et al., 1971; Philipson et al., 1971).

By the use of RNA–DNA hybridization, the amount of genetic information in cytoplasmic and nuclear poly(A+)-RNA can be determined by synthesizing cDNA to the poly(A+)-RNA templates. From such studies of different tissues, poly(A+)-hnRNA seems to have a total sequence complexity which is 5–10 times greater than that found in the poly(A+)-mRNA population (Getz et al., 1975; Bantle & Hahn, 1976; Herman et al., 1976; Levy et al., 1976; Jacquet et al., 1978).

Recently, it has been shown that the genes for globin and ovalbumin contain intervening sequences (IV's) not present

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in the final mRNA (Jeffreys & Flavell, 1977; Weinstock et al., 1978; Tilghman et al., 1978; Lai et al., 1978). Evidence is also accumulating that the IV's are present in the initial transcript (Bastos & Aviv, 1977; Tilghman et al., 1978; Roop et al., 1978) and the IV's are spliced out while the ends where the IV's were are ligated together by some unknown mechanism.

We are working with simple embryoid bodies and neuroepithelial teratocarcinomas from mice (OTT6050). The embryoid bodies are ascitic tumors consisting of an outer ring of endoderm and an inner core of embryocarcinoma stem cells. The early differentiation of embryocarcinoma cells shows many similarities to the development of the mouse embryo [for a review see Martin (1975)]. Embryoid bodies resemble the embryonic portion of the 3–5 day mouse embryo (Martin, 1975; Martin et al., 1977). Embryoid body differentiation results in solid teratocarcinomas consisting mainly of neuroepithelial cells (Herman et al., 1975). Thus, the mouse teratocarcinoma system might be used to study the molecular relationship between neoplasia and differentiation and as a model system for early mammalian development.

This report compares the sequence complexity of total poly(A+)-RNA from embryoid bodies and teratocarcinomas with the sequence complexity of poly(A+)-hnRNA from embryoid bodies by using cDNA techniques. Homologous and heterologous hybridizations were performed to elucidate the sequence complexity and the sequence relationship between total poly(A+)-RNA and poly(A+)-hnRNA.

Material and Methods

Materials. [^{3}H]Poly(deoxythymidylic acid) and S1 nuclease were purchased from Miles. [^{3}H]Deoxycytidine triphosphate was purchased from New England Nuclear. Poly(U)–Sepharose and Sephadex G-50 were purchased from Pharmacia.

Isolation and Storage of Embryoid Bodies and Teratocarcinomas. Simple embryoid bodies [as defined by size ~80 μm (30–140 μm)] (EBs) and teratocarcinomas (0.2–0.8 cm in diameter) were removed from the peritoneal cavity of 129 SL mice by washing with phosphate-buffered saline (PBS) and sedimenting the embryoid bodies by gravity. After washing three times with PBS, we stored the embryoid bodies at −70 °C in PBS made 10% with respect to dimethyl sulfoxide. Solid teratocarcinomas were frozen immediately on dry ice and then stored at −70 °C.

Isolation of Nuclei. Nuclei were isolated essentially as described by Sippel et al. (1977). Embryoid bodies isolated as described above were homogenized in 10 volumes of 2.5% citric acid and 0.14% Triton X-100 by using a Dounce homogenizer with a loose-fitting pestle. The homogenate was centrifuged at 1000*g* for 10 min. The nuclear pellet was washed once with the homogenization solution, followed by centrifugation. The nuclear pellet was resuspended and homogenized in 0.25 M sucrose containing 2.5% citric acid, followed by centrifugation as above. The pellet was washed again in 0.25 M sucrose–citric acid as above. After resuspending the pellet in 0.25 M sucrose and 2.5% citric acid, we layered the nuclei on top of 0.88 M sucrose in 2.5% citric acid and centrifuged them for 10 min at 1000*g*. The centrifugation through the 0.88 M sucrose was repeated twice. The purity of the nuclei was checked in a phase-contrast microscope. Nuclei were stored at −70 °C.

Preparation of hnRNA. The embryoid body nuclei isolated from 40 to 50 mL of gravity-packed EBs were homogenized in 40 mL of phenol–chloroform (1:1) and 40 mL of saline–EDTA (0.075 M NaCl and 0.025 M EDTA, pH 8.0) and 4 mL of 10% $NaDodSO_4$. After shaking for 10 min at room temperature, we placed the mixture on ice for 20 min and then centrifuged the mixture at 10 K for 10 min. The aqueous phase was reextracted once with phenol–chloroform and once with chloroform. Nucleic acids were precipitated with two volumes of EtOH after making the aqueous phase 0.2 M with respect to NaCl. The precipitate was stored at −20 °C for 2 h. The precipitate was collected by centrifugation at 10 K for 15 min and was dissolved in 0.1 M NaCl, 0.01 M $MgCl_2$, and 0.01 M Tris (pH 7.4) to a final concentration of 400 μg/mL, and the dissolved precipitate was then treated with RNase-free DNase (50 μg/mL) for 10 min at 37 °C. Labeled DNA was added to monitor the DNase treatment. The reaction mixture was made 3 mM with respect to EDTA and 0.2 M NaCl and precipitated with two volumes of EtOH and stored at −20 °C for 2 h. The precipitate was collected by centrifugation as above, dissolved in 0.5 mL of 0.5% $NaDodSO_4$ and 3 mM EDTA, and treated with proteinase K (33 μg/mL) for 30 min at 37 °C. The sample was then applied to a Sephadex G-50 column equilibrated with 0.5% $NaDodSO_4$ and 3 mM EDTA. The void fraction was collected and extracted three times with chloroform. The RNA from the final aqueous phase, made 0.2 M NaCl, was precipitated overnight with two volumes of EtOH.

Isolation of Poly(A+)-Containing hnRNA. Poly(U)-Sepharose chromatography, essentially according to Molloy et al. (1974), was used to isolate poly(A)-containing hnRNA [poly(A)-hnRNA]. Total nRNA samples were applied to the column in 0.4 M NaCl, 0.01 M Tris (pH 7.4), 0.01 M EDTA, and 0.2% $NaDodSO_4$. hnRNA molecules containing internal oligo(A) sequences but no poly(A+) sequence at the 3′ end were eluted off the column with 0.01 M Tris (pH 7.4), 0.01 M EDTA, and 25% formamide with 0.2% $NaDodSO_4$. Poly(A)-containing hnRNA molecules were eluted with 90% formamide in the Tris, EDTA, and $NaDodSO_4$ buffer as above. The efficiency of the fractionation of the RNA was monitored by RNase A + T_1 (400 μg/mL) digestion of hnRNA eluted with 25 and 90% formamide, respectively. The RNase digests were analyzed on polyacrylamide gels, and the results showed that at least 90% of the poly(A) tracts were eluted with 90% formamide (data not shown).

Preparation of Total Poly(A+)-RNA. Gravity-packed EBs were homogenized in 10 volumes of phenol–chloroform plus 10 volumes of a buffer containing 0.075 M NaCl, 0.025 M EDTA, pH 8.3, and 1% sodium dodecyl sulfate ($NaDodSO_4$). The procedure for extraction of hnRNA was followed until the first precipitation with ethanol. The precipitate was collected by centrifugation at 10000*g* for 20 min and redissolved in 10 mM Tris-HCl, pH 7.6, 5 mM EDTA, and 1% $NaDodSO_4$ to a concentration of 2–3 mg/mL. The nucleic acid extract was incubated with proteinase K (50 μg/mL), and following incubation the nucleic acids were extracted three times with chloroform. The aqueous phase was adjusted to 0.2 M NaCl and nucleic acids were precipitated with two volumes of EtOH overnight at −20 °C. The precipitate was collected as mentioned above and dissolved in 10 mM Tris-HCl, pH 7.6, 5 mM EDTA, and 1% $NaDodSO_4$ to a concentration ≤1 mg/mL. Poly(A+)-RNA was separated from the total nucleic acid extract by affinity chromatography on oligo(dT)-cellulose. The total nucleic acid extract in Tris, EDTA, and $NaDodSO_4$ as mentioned above was adjusted to 0.5 M NaCl and applied to the column. Oligo(dT)-bound material was eluted with 10 mM Tris-HCl, pH 7.6, 5 mM EDTA, and 1% $NaDodSO_4$. Fractions showing absorbance at 254 nm were combined, diluted fivefold with low-salt elution buffer, and heat denatured for 10 min at 68 °C. Salt was then

readjusted to 0.5 M NaCl, and the sample was applied to the oligo(dT)-cellulose column a second time. Twice oligo-(dT)-bound poly(A+)-RNA fractions were combined and precipitated overnight with two volumes of ethanol. The poly(A+)-RNA was collected by centrifugation as mentioned above, dissolved in distilled H_2O, and stored at −70 °C.

Synthesis of [^{3}H]DNA Complementary to Poly(A+)-Containing RNA. The reaction was carried out in a volume of 100–150 μL. The final reaction mixture contained 50 mM Tris (pH 8.0), 50 mM DTT, 6 mM $MgCl_2$, 60 mM KCl, 36 μg of actinomycin D/mL, 20 μM [^{3}H]dCTP, and 200 μM each of dATP, dGTP, and dTTP. Oligo(dT)$_{12-18}$ (1.5 μg) was added as a primer and 5–10 μg of RNA was added as template. AMV reverse transcriptase was used at a concentration of 550 units/mL. The reaction mixture was incubated for 30 min at 37 °C and terminated by addition of EDTA and $NaDodSO_4$ to 10 mM and 1.5%, respectively. Denatured calf thymus DNA (120 μg) was added as a carrier before placing the entire sample on a Sephadex G-50 column (80 × 1.5 cm) previously equilibrated with 0.1 M NaCl and 3 mM EDTA. The void volume fraction was collected and precipitated overnight with two volumes of EtOH after addition of more denatured calf thymus DNA as carrier. The precipitate was dissolved in 0.2 mL of 0.1 M NaOH and 3 mM EDTA and incubated at 60 °C for 30 min. The solution was then neutralized with 5 N HCl. The [^{3}H]cDNA was sized on a alkaline 5–20% sucrose gradient, and fractions corresponding to 350–1500 nucleotides (NT) were pooled and precipitated overnight with two volumes of EtOH (Monahan et al., 1976a). The precipitate was dissolved in distilled water and stored at −70 °C.

Standard Hybridization Reaction. Purification of ovalbumin mRNA and synthesis and characterization of its ^{3}H-labeled cDNA have been described elsewhere (Monahan et al., 1976a). The [^{3}H]cDNA$_{ov}$ was at least 95% pure.

Isolation and characterization of the hen oviduct RNA used in the standard hybridization reaction have been described in detail elsewhere (Harris et al., 1975; Chan et al., 1977). The hen oviduct RNA was found to contain 0.65–0.67% ovalbumin mRNA.

The hybridization between hen oviduct RNA and [^{3}H]-cDNA$_{ov}$ was performed as described in the following paragraph for poly(A+)-RNA with [^{3}H]cDNA.

Hybridization of Poly(A+)-RNA with [^{3}H]cDNA. The RNA hybridizations were carried out in a final volume of 50 μL in 0.6 M NaCl, 2 mM EDTA, and 10 mM Tris (pH 7.6). [^{3}H]cDNA was added to give approximately 3000 cpm per hybridization point. Calf thymus DNA concentration was 5–10 μg/mL. Poly(A+)-RNA was added to the reaction in a concentration range of 5×10^{-4} to 10 μg/mL. Two to four samples per experiment were assayed with no added RNA for determination of the total Cl_3AcOH precipitable counts prior to S_1 nuclease treatment and radioactivity remaining after S_1 nuclease treatment. Background was 3–5%. Samples were placed in a boiling water bath for 10 min before being incubated at 68 °C for various times. Incubation time at 68 °C varied from 0.5 to 72 h. The S_1 nuclease treatment was performed as previously described (Harris et al., 1975). The hybridization data was analyzed by hand, by assuming 1.5 log units of reaction represents one component, and by computer. The computer program has been described elsewhere (Monahan et al., 1977).

Results

Characterization of Poly(A+)-RNA from Mouse Embryoid Bodies. Total and nuclear poly(A+)-RNA were sized on

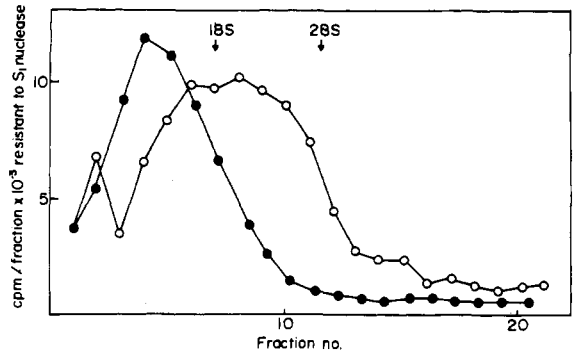

FIGURE 1: Number-average molecular size of (○) poly(A+)-hnRNA and (●) total poly(A+)-RNA from mouse embryoid bodies. Samples were analyzed on linear 5–20% sucrose gradients in 70% formamide. Gradients were run at 20 °C for 20 h at 39 K. An aliquot from each gradient fraction was hybridized to [^{3}H]poly(dT), and the amount of hybrid was determined by S_1 nuclease treatment.

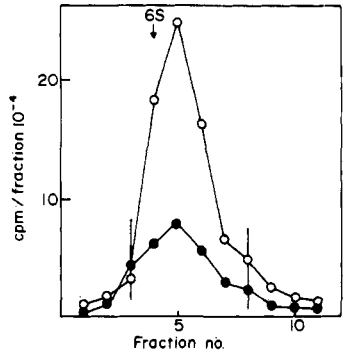

FIGURE 2: cDNA from total poly(A+)-RNA (○) and poly(A+)-hnRNA (●) analyzed on alkaline 5–20% sucrose gradients. Gradients were run at +5 °C for 18 h at 38 K. Sheared DNA was used as markers. The bars show the fractions that were pooled and used for the hybridization experiments.

5–20% linear sucrose gradients in 70% formamide at 20 °C (Monahan et al., 1976a,b). [^{3}H]Poly(dT) was used as a probe to detect poly(A+)-RNA in the gradients. The results show that the number-average molecular size is 2700 and 1350 NT for nuclear and total poly(A+)-RNA, respectively (Figure 1).

As judged by annealing to poly(U)-Sepharose, 10–15% of the nRNA consisted of poly(A+)-hnRNA. The poly(A) content of the poly(A+)-hnRNA was found to be 2–3% by using [^{3}H]poly(dT) as a probe and known amounts of poly(A) as standard(s) (Monahan et al., 1976a,b). On the other hand, the poly(A) content of the total poly(A+)-RNA, isolated by oligo(dT)-cellulose, was 3–5%. The size of the poly(A) tracts was estimated to be 138 and 65 NT on the poly(A+)-hnRNA and total poly(A+)-RNA, respectively, by using polyacrylamide gel analysis and hybridization with [^{3}H]poly(dT) across the gel.

Synthesis of [^{3}H]cDNA. Total poly(A+)-RNA and poly(A+)-hnRNA were used as templates for the AMV reverse transcriptase reaction. The reaction in both cases was at least 95% dependent of the presence of oligo(dT)$_{12-18}$. With poly(A+)-hnRNA as template, the yield of cDNA was 8–12% of the template by mass compared to 30–40% with total poly(A+)-RNA as template. Similar results have been reported by others (Herman et al., 1976; Jacquet et al., 1978). The [^{3}H]cDNA was sized on alkaline 5–20% sucrose gradients with sheared DNA as marker (Figure 2). The average size was the same for both [^{3}H]cDNAs and estimated to be 900 NT. The relatively low efficiency of transcription of poly-(A+)-hnRNA raises the question of whether all classes of sequences in the template are represented in the [^{3}H]cDNA. Thus, all calculations for poly(A+)-hnRNA complexity might

Table I: Sequence Complexity in Poly(A+)-RNA from Mouse Embryoid Bodies

	fraction of total	$C_{r_0}t_{1/2}$ obsd (Ms)	$C_{r_0}t_{1/2}$ isolated[a] (Ms)	sequence complexity[b] NT × 10^{-6}	no. of different sequences[c]	copies per cell[d]
total poly(A+)-RNA	0.09	0.033	0.003	0.0019	1–2	12000
	0.61	0.50	0.30	0.185	140	620
	0.25	0.22	5.50	3.40	2500	16
poly(A+)-hnRNA	0.50	5.00	2.50	1.50	570	12
	0.46	120.00	55.00	31.80	12500	1

[a] $C_{r_0}t_{1/2}$ observed time fraction of total [also corrected for rRNA content in total poly(A+)-RNA preparation]. [b] [($C_{r_0}t_{1/2}$ isolated)/($C_{r_0}t_{1/2}$ standard)] × 1850 ($mRNA_{ov}$ = 1850 NT). [c] Number average size for poly(A+)-hnRNA = 2700 NT; for poly(A+)-RNA = 1350 NT. [d] By assumption of 1.2 × 10^{-7} μg of cytoplasmic poly(A)-RNA/cell and 2.0 × 10^{-8} μg of nRNA/cell (Jacquet et al., 1978), copies per cell = [(μg of RNA)(fraction of total)(6.0 × 10^{23})]/[(average-size RNA)(330)(number of sequences)].

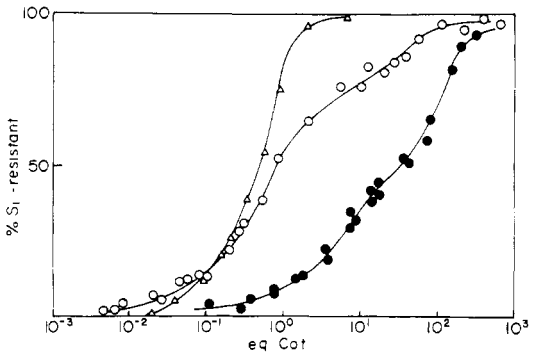

FIGURE 3: Homologous hybridizations. (△) Hen oviduct RNA containing 0.67% $mRNA_{ov}$ hybridized to [^{3}H]$cDNA_{ov}$; (○) total poly(A+)-RNA and (●) poly(A+)-hnRNA with their respective [^{3}H]cDNAs. Hybridizations were performed as described under Material and Methods. Background (5%) was subtracted before calculating the amount of material resistance to S_1 nuclease. The data represent three preparations of total poly(A+)-RNA and two preparations of poly(A+)-hnRNA.

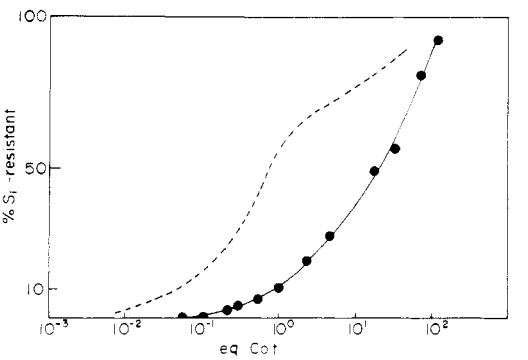

FIGURE 4: Heterologous hybridizations. (●) Poly(A+)-hnRNA hybridized to [^{3}H]$cDNA_{cyto\text{-}EB}$ prepared from total poly(A+)-RNA. (---) Homologous hybridization for total poly(A+)-RNA to its cDNA ([^{3}H]$cDNA_{cyto\text{-}EB}$) included as a reference. The experiment was performed as described under Material and Methods and in the legend to Figure 3.

be underestimations if there was selective transcription of the poly(A+)-hnRNA. However, hybridization data gave no reason to believe that this is the case.

Homologous Hybridization Reactions. The kinetic standard used was RNA prepared from hen oviducts and containing 0.67% mRNA (Harris et al., 1975; Chan et al., 1977). This oviduct RNA was hybridized to [^{3}H]$cDNA_{ov}$ which was made from 95% pure ovalbumin mRNA. The reaction was approximately pseudo first order and covered less than 2 log units. An ideal pseudo-first-order reaction has a range of about 1.5 log units, but the kinetics of a DNA–RNA hybridization are affected by variations in the size distribution of the cDNA molecules (Young et al., 1974). Larger cDNA molecules will hybridize faster than smaller ones; therefore, a slight deviation is to be expected for the unfractionated [^{3}H]$cDNA_{ov}$ ([^{3}H]$cDNA_{ov}$ has a size of 900 NT but much broader size range than cDNAs to RNAs from EBs). From Figure 3, it can be seen that pure [^{3}H]$cDNA_{ov}$ reacts with our hen oviduct RNA with a $C_{r_0}t_{1/2}$ of 4.5 × 10^{-1}.[1] This $C_{r_0}t_{1/2}$ is essentially the same value reported previously for this oviduct RNA preparation (Harris et al., 1975; Chan et al., 1977). Since this preparation contains 0.67% $mRNA_{ov}$, we calculate that pure $mRNA_{ov}$ of 1850 NT would react with a $C_{r_0}t_{1/2}$ of 3 × 10^{-3} to this pure [^{3}H]$cDNA_{ov}$ probe.

The hybridization between total poly(A+)-RNA and its [^{3}H]cDNA ([^{3}H]$cDNA_{cyto}$) covered 4.5 log units, indicating complex kinetics (Figure 3). The data can be resolved into three classes of sequences representing 9, 61, and 25%, respectively, of the sequences in the [^{3}H]$cDNA_{cyto}$. The total complexity was found to be 3.4 × 10^6 NT (Table I).

[1] In units of moles liter^{-1} s.

The homologous reaction of poly(A+)-hnRNA with its [^{3}H]$cDNA_{EB}$ required higher C_0t[1] values to achieve completion (Figure 3). The higher C_0t required would indicate that the sequences in poly(A+)-hnRNA are present in a lower concentration compared to the sequences in total poly(A+)-RNA. However, the overall reaction occurred over 3–3.5 log units, and thus, the homologous reaction of the poly(A+)-hnRNA is not as broad as the homologous reaction of total poly(A+)-RNA with its [^{3}H]cDNA. The data for the poly(A+)-hnRNA can best be resolved into two classes of sequences containing 50 and 46%, respectively, of the sequences present in the [^{3}H]$cDNA_{Hn}$ probe. From the rate of hybridization we can estimate a total complexity poly(A+)-hnRNA of 3.2 × 10^7 NT. Thus, the poly(A+)-hnRNA is about 10 times more complex than the total poly(A+)-RNA (mainly cytoplasmic). The hybridization data for the homologous hybridizations are summarized in Table I.

Heterologous Hybridization Reactions. Hybridization of [^{3}H]cDNA to total poly(A+)-RNA ([^{3}H]$cDNA_{cyto\text{-}EB}$) to poly(A+)-hnRNA is presented in Figure 4. The overall hybridization kinetics was approximately 25 times slower than the kinetics of the homologous reaction with total poly(A+)-RNA. The saturation value was almost identical with that for the homologous total poly(A+)-RNA hybridization to its [^{3}H]$cDNA_{cyto\text{-}EB}$. This indicates the presence of all the total poly(A+)-RNA sequences (mainly cytoplasmic) in the nuclear poly(A+)-hnRNA population. However, the concentration of these sequences is 10–25 times lower in poly(A+)-hnRNA than in the total poly(A+)-RNA. These results would support the conclusion that total poly(A+)-RNA is at a minimum, 90–96% cytoplasmic poly(A+)-RNA in origin. The above conclusion is also supported by the physical and chemical characteristics of the total poly(A+)-RNA and the

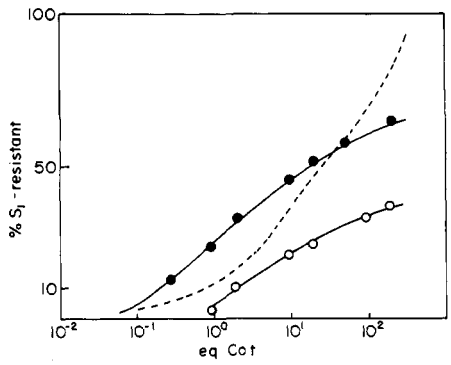

FIGURE 5: Heterologous hybridizations. (●) Hybridization between nuclear [^{3}H]cDNA$_{hn\text{-}EB}$ and total poly(A+)-RNA from embryoid bodies; (○) nuclear [^{3}H]cDNA$_{hn\text{-}EB}$ hybridized to total poly(A+)-RNA from neuroepithelial teratocarcinomas. The experiment was performed as described under Material and Methods and in the legend to Figure 3. (---) Homologous hybridization of poly(A+)-hnRNA$_{EB}$ to its cDNA ([^{3}H]cDNA$_{hn\text{-}EB}$) included as a reference.

poly(A+)-hnRNA preparations.

When total poly(A+)-RNA was hybridized to [^{3}H]cDNA$_{hn}$, the result shown in Figure 5 was achieved. The hybridization was faster than the homologous reaction of [^{3}H]cDNA$_{hn}$, but when approaching saturation, only 65% of the [^{3}H]cDNA$_{hn}$ probe could be recovered as hybrid. This indicates that as much as 30–35% of the sequences in poly(A+)-hnRNA may be nuclear specific and presumably lost during processing. For RNA sequences in the poly(A+)-hnRNA very distant from the 3′ end, it might even be more than 30–35%.

To make a comparison between different stages of differentiation, total poly(A+)-RNA was extracted from neuroepithelial-containing teratocarcinomas. This RNA was hybridized to [^{3}H]cDNA$_{hn\text{-}EB}$, and as shown in Figure 5, 37% of the nuclear [^{3}H]cDNA$_{hn\text{-}EB}$ could be driven into hybrid with total poly(A+)-RNA from tumors at C_0t values where 95% of sequences in the [^{3}H]cDNA$_{hn\text{-}EB}$ was driven into hybrid by the homologous poly(A+)-hnRNA$_{EB}$.

Discussion

Poly(A+)-RNA from the mouse teratocarcinoma system (OTT6050) was investigated with respect to sequence complexity by using cDNA probes. DNAs were synthesized with total poly(A+)-RNA and poly(A+)-hnRNA from embryoid bodies as templates. The sequence complexity was determined by RNA-excess hybridizations. Heterologous hybridization was also made with total poly(A+)-RNA from neuroepithelial teratocarcinomas to cDNA made to poly(A+)-hnRNA-EB.

To obtain valid information about poly(A+)-hnRNA sequences, the isolated nuclei should contain negligible amounts of cytoplasmic contamination. As judged by phase-contrast microscopy, the nuclei seemed to be free from cytoplasmic contamination. Furthermore, nuclei isolated by the citric acid method combined with Triton X-100 treatment have been used by Sippel et al. (1977), and they reported a maximum 5% cytoplasmic contamination in the nuclei preparations. Assuming that 2% of the cytoplasmic RNA was poly(A+)-RNA and that 5% cytosol contamination occurred in the nuclei, we found that this would represent 0.1% poly(A+)-RNA and would suggest that as little as 0.03% of the nuclear poly(A+)-RNA represented cytoplasmic contamination.

Poly(A+)-hnRNA was isolated with poly(U)–Sepharose. A number of methods are available for isolation of poly(A+)-containing material, but, for large molecules, poly(U)–Sepharose chromatography seems to be more selective and with higher yields than most other methods. Similar conclusions have been made by other investigators (Herman & Penman, 1977; Molloy et al., 1974).

To isolate poly(A+)-RNA from total RNA preparations, chromatography on oligo(dT)-cellulose was used, and the number-average molecular size was found to be approximately half (1350 NT) of that found for poly(A+)-hnRNA (2700 NT). The number-average size for poly(A+)-mRNA from other studies (Ryffel & McCarthy, 1975; Levy & McCarthy, 1975; Getz et al., 1976; Jacquet et al., 1978) is similar to what we have found for total poly(A+)-RNA. This indicates that the vast majority of the poly(A+)-RNA molecules isolated by using oligo(dT)-cellulose represents cytoplasmic poly(A+)-RNA. This assumption is supported by the hybridization data. In the homologous reactions, at equivalent C_0t = 10, 65% of the total poly(A+)-RNA sequences have hybridized while only 10% of the nuclear sequences can be recovered as hybrids. If poly(A+)-hnRNA sequences contribute significantly to the homologous total poly(A+)-RNA hybridization, it most likely is only in the scarce or rare class of sequences. When total poly(A+)-RNA is hybridized to nuclear [^{3}H]cDNA$_{hn}$, 65% of the sequences in the [^{3}H]-cDNA$_{hn}$ could be saturated. Almost identical results were reported for HeLa cells by Herman et al. (1976) when hybridizing poly(A+)-mRNA HeLa to nuclear [^{3}H]-cDNA$_{hn\text{-}HeLa}$. The above results also support our assumption that the total poly(A+)-RNA represents mainly cytoplasmic poly(A+)-RNA molecules.

The poly(A+)-hnRNA from mouse embryoid bodies was found to be approximately 10 times more complex than the total poly(A+)-RNA, presumably reflecting the effect of processing of the primary nuclear transcripts into cytoplasmic transcripts. In embryocarcinoma cells grown in vitro, 90% of the hnRNA sequences corresponds to one major component present in approximately one copy per cell (Jacquet et al., 1978). In the more differentiated embryoid bodies (containing endoderm), we find two components in the poly(A+)-hnRNA, representing 12 and 1 copies per cell, respectively. This difference might represent a change in gene transcription or nuclear processing during cell differentiation due to the presence of the endoderm elements or could be due to differences in experimental procedure or data analysis.

When total poly(A+)-RNA from neuroepithelial teratocarcinomas was hybridized to nuclear [^{3}H]cDNA$_{hn\text{-}EB}$, only 35% of the sequences in the [^{3}H]cDNA$_{hn\text{-}EB}$ could be saturated compared to 65% for total poly(A+)-RNA from embryoid bodies. This indicates that as much as 50% of the sequences in total poly(A+)-RNA from teratocarcinomas may be in much lower concentration in the nuclear poly(A+)-RNA-EB transcripts than are the sequences in the embryoid body total poly(A+)-RNA population. This change in gene expression may reflect changes in the pattern of posttranscriptional processing involved in gene regulation. This may suggest that intervening sequences for one particular mRNA under one state of differentiation may, in fact, be processed cytoplasmic mRNA for a different state of differentiation. Homologous and heterologous hybridization experiments with "pure" poly(A+)-hnRNA and cytoplasmic RNA from embryoid bodies and neuroepithelial teratocarcinomas with [^{3}H]cDNA made to poly(A+)-nRNA from teratocarcinoma are presently under investigation.

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