Volume 3 no.7 July 1976 Nucleic Acids Research

Diversity of sequences in total and polyadenylated nuclear RNA from Drosophila cells.

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Received 2 June, 1976

ABSTRACT

Complementary DNA was synthesized using polyadenylated nuclear RNA of cultured Drosophila cells as template. The kinetics of hybridization of this cDNA with nuclear RNA indicated that the complexity of this RNA population is five to ten times greater than that of cytoplasmic mRNA. The same difference in the fraction of DNA represented was obtained when nuclear and cytoplasmic RNA were hybridized with labeled unique sequence DNA. The fraction of the DNA sequences represented in total nuclear or polyadenylated nuclear RNA is much higher than that represented in cytoplasmic RNA.

INTRODUCTION

It is generally conceded that the production of messenger RNA in animal cells results from two post-transcriptional processes. One is the processing of high molecular weight nuclear precursors and the other is the transport to the cytoplasm of only a limited fraction of the nuclear RNA sequences. Although definitive proof of the former pathway is lacking, it is supported by several lines of evidence. In mammalian cells at least, nuclear RNA molecules are considerably larger than mRNA[1-4]; both HnRNA transcription and mRNA production display the same sensitivities to drug inhibition[1,5,6].

Early hybridization experiments restricted to repetitive sequences suggested that nuclear RNA sequences are some five-fold more complex than cytoplasmic sequences[8,9], suggesting that post-transcriptional events might restrict the appearance of some gene transcripts on the cytoplasm. More recent experiments using RNA excess hybridization with unique sequence DNA of sea urchins[9,10] and cDNA/excess RNA hybridization with mouse Friend cell nucleic acids[11] indicate that the sequence complexity of nuclear RNA is some 5-10 times higher than that of cytoplasmic RNA.

In the present communication, we present the results of experiments using both the aforementioned experimental approaches applied to nuclear RNA of cultured Drosophila cells. The genome of this insect has a

sequence complexity of about 5% of that of mammals. Nuclear polyadenylated RNA is larger than the corresponding cytoplasmic species but only by a factor of two to three[12,13,14]. Earlier hybridization experiments employing total RNA indicated that as much as 20% of the single-copy DNA is expressed in Drosophila[15,16]. In the present communication we demonstrate that most of the complexity is accounted for by nuclear RNA.

MATERIALS AND METHODS

Preparation of Nuclear RNA

For a typical preparation, 1000 ml of a suspension culture of Drosophila cells, Schneider's line 2[17], was collected at 2000 rpm for 20 min at 4°C. The cells were resuspended in 40 ml of 10 mM NaCl, 10 mM Tris-HCl, pH 8.5, 3 mM $MgCl_2$ containing 25 mg/ml of polyvinyl sulfate, 10^{-3} M spermine and 1% diethylpryocarbonate. NP40 (Shell Oil Co.) was added to the suspension to a concentration of 0.5% and cells were lysed in a Dounce homogenizer. Nuclei were removed by centrifugation at 3500 rpm for 5 min at 0°C. The nuclear pellet was washed twice with 10 mM NaCl, 10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$. Microscopic examination of the preparation revealed nuclei free of cytoplasmic tags. Nuclei were resuspended in 20-30 ml of 0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris-HCl, pH 7.4 (NTE), containing 0.5% sodium dodecyl sulfate and shaken at room temperature with a mixture of phenol-chloroform (1:1) equilibrated in NTE buffer and then re-extracted with chloroform containing 1% isoamylalcohol. After four extractions the aqueous phase layer was removed and precipitated with two volumes of absolute alcohol overnight at -20°C.

The nucleic acid precipitate was recovered by centrifugation at 6000 rpm for 60 min at -5°C and resuspended in a solution containing 0.01 M Tris-HCl, pH 7.4, 0.1 M NaCl, 5 mM $MgCl_2$. DNAse I was added to a concentration of 50 µg/ml. The mixture was vortexed for 2 min at room temperature and then incubated for 20 min at 37°C. One-twentieth volume of 10% SDS was added and the mixture extracted as previously described. The nucleic acids were precipitated as before and DNA fragments were removed by chromatography on Sephadex G-100 equilibrated in 0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, 0.5% sodium dodecyl sulfate. RNA eluting in the void volume was pooled and precipitated with two volumes of ethanol. This RNA constituted the total nuclear RNA preparation.

Preparation of Nuclear and Cytoplasmic Poly(A)-Containing RNA

The cytoplasmic RNA was prepared as described[18]. Isolation of total nuclear and cytoplasmic poly(A)-containing RNA was performed by chromatography

on polyU-Sepharose, as described[18].

The poly(A)-containing RNA was precipitated with ethanol. For use in hybridization experiments the RNA was recovered by centrifugation at 8000 rpm for 20 min and resuspended in sterile water.

When labeled RNA was required, the cells were concentrated 10x and incubated in the presence of 10 μCi/ml of ^{3}H-uridine for 20 min for the labeling of nuclear RNA and for 16 hr for the labeling of cytoplasmic RNA.

Preparations of Drosophila DNA

Drosophila DNA was extracted from Schneider's cell nuclei as described before[18]. ^{3}H-labeled Drosophila melanogaster DNA was prepared by ^{3}H-thymidine labeling of D. melanogaster cell cultures. Media was removed from the cultures and replaced with new media containing ^{3}H-thymidine (10 μCi/ml). Cells were harvested 20 hr later for DNA extraction. The specific radioactivity of the DNA was 36,000 cpm/μg.

Isolation of Unique Sequence ^{3}H-DNA

^{3}H-labeled sheared Drosophila DNA was incubated in 0.12 M phosphate buffer at 60°C to a $C_o t$ of 18, before fractionation on hydroxylapatite.

Degradation of DNA by Shearing and Partial Depurination

Sheared DNA was prepared by shearing at 12,000 psi in a French pressure cell as previously described[19]. In some cases, samples were degraded by partial depurination for 10 min in 0.1 M acetate buffer (pH 4.2) at 70°C, followed by alkaline hydrolysis as described elsewhere[20]. After partial degradation, all DNA samples were stored at 4°C in 0.01 SSC until used.

Synthesis of cDNA

Nuclear and cytoplasmic cDNA probes were synthesized as described previously[18]. In both cases, cDNA synthesis was completely dependent upon the addition of oligo dT as primer. The reverse transcriptase from avian myeloblastosis virus was kindly provided by Dr. W.J. Rutter and Dr. Beard (NIH).

Hybridization Reactions

Small amounts of nuclear or cytoplasmic cDNA (500 cpm-1000 cpm) were mixed with the appropriate amount of RNA and sealed in 5 μl capillaries. The RNA's were present at concentrations ranging between 100 μg/ml to 2 mg/ml in 0.24 M phosphate buffer containing 1 mM EDTA. The capillaries were boiled for 10 min and incubated at 70°C for the time required to reach the $R_o t$ value desired. At the end of each incubation period, the content of each was ejected into 4 ml of 0.3 M NaCl, 0.03 M NaOAc-3 mM $ZnCl_2$, pH 4.5 and digested with S1 nuclease in the presence of 10 μg of bacterial DNA at 37°C for 2 hr as described by Leong et al.[21]. Each point in a curve is the aver-

age of duplicate determinations. In all experiments the hybridization at 0 time (6-12%) was subtracted as background.

Nuclear cDNA Fractionation

The nuclear cDNA was fractionated by partial hybridization with mRNA as described before[18].

Hydroxylapatite Chromatography

Hydroxylapatite chromatography was also used to monitor DNA renaturation and DNA/RNA hybridization[10,20]. DNA renaturation was carried out in 0.14 M phosphate buffer, pH 6.8 at 60°C. DNA/RNA hybridization was carried out in 0.4 M phosphate buffer at 70°C. Concentrations and sample sizes varied as described in the figure legends.

Samples in 5 ml of 0.14 M phosphate buffer were applied to 1 ml columns in BioRad HTP hydroxylapatite. Five ml plastic syringes were used as columns. An elution temperature of 60°C was maintained by an immersion heater. 0.14 M phosphate buffer was used to elute the single-stranded material and the double-stranded material was eluted with 0.5 M phosphate buffer. Both fractions were diluted to 20 ml and a final concentration of 0.125 M phosphate buffer. Nucleic acids were precipitated by addition of 400 µg of bovine serum albumin and TCA, and the radioactivity was determined.

RESULTS

Preparation of Total and Polyadenylated Nuclear RNA

Schneider's cells were labeled for 20 min with ^{3}H-uridine and the RNA extracted from the nuclei. Polyadenylated nuclear RNA was prepared by chromatography on polyU-Sepharose. The results shown in Table I indicate that

TABLE 1: Efficiency of PolyU-Sepharose

RNA Fraction	% Total cpm	% Total cpm Rebound
Unbound	90	--
Bound	10	94

Total nuclear RNA, labeled with ^{3}H-uridine, was fractionated on polyU-Sepharose. The bound material was precipitated with ethanol at -20°C, resuspended in polyU-Sepharose loading buffer and reapplied to the column.

about 10% of the total nuclear RNA population contains poly(A); when this RNA was recycled, 94% of it bound to polyU-Sepharose. This figure compares well with data reported by others[2,3,11].

The mean size of the nuclear RNA molecules was determined by sucrose gradient centrifugation. Fig. 1A shows that pulse-labeled nuclear RNA from

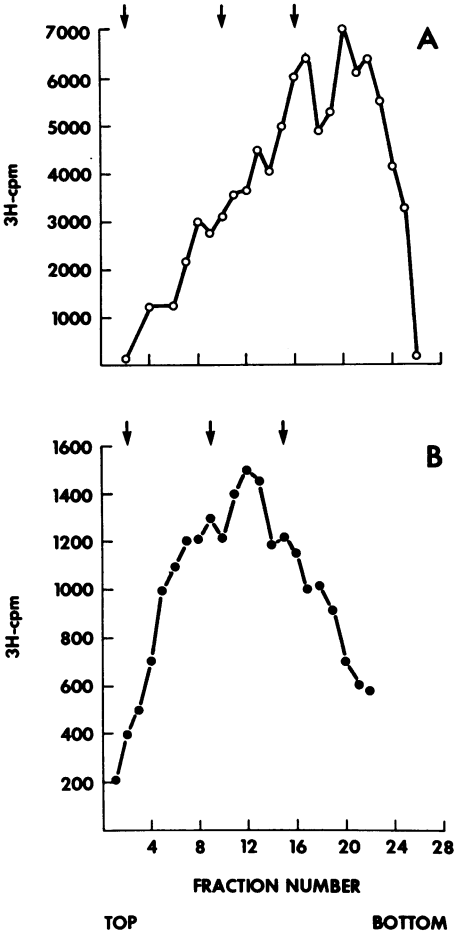

FIGURE 1. Size distribution of total and polyadenylated nuclear RNA from Schneider's cells. Total and polyadenylated nuclear RNA were prepared from cells labeled in vivo for 20 min with ^{3}H-uridine (5 µCi/ml) as described in Methods. An aliquot of each RNA sample was analyzed on a 10-30% sucrose gradient in 0.1 M NaCl, 10 mM Tris HCl, pH 7.4, 1 mM EDTA, containing 0.5% SDS. Centrifugation was in a Beckman SW41 rotor for 6 hr at 40,000 rpm (A) or for 6 hr at 38,000 rpm (B) at 24°C. A) (O-O) Total nuclear RNA, B) (●-●) Polyadenylated nuclear RNA. Reading from left to right, the arrows designate the positions of 5S, 19S and 26S Drosophila ribosomal RNA's run on a parallel gradient.

Schneider's cells displays a very broad size distribution with a mean sedimentation value of about 26S. Polyadenylated nuclear RNA also displays a broad sedimentation distribution with a mean value of about 20S (Fig. 1B). These results are consistent with those of Lengyel and Penman[12] and Lamb and Laird[14], and indicate that Drosophila nuclear RNA is smaller than that of mammals. Pulse-labeled polyadenylated RNA molecules in the nucleus are about 2-3 times as large as the average cytoplasmic polyadenylated molecule[12-14].

The mean sizes of the unlabeled, i.e., steady-state total and polyadenylated nuclear RNA were found to be 18S and 14S respectively. We do not know to what extent this is the result of degradation by endogenous RNases during preparation or whether it reflects in vivo processing. In either event, the small size of the RNA's employed is advantageous in terms of its role as template for the reverse transcriptase. Moreover, the kinetics of hybridization reactions driven by nuclear and cytoplasmic RNA can be directly compared without correction of rates for different size molecules.

Diversity of Sequences in Total Nuclear RNA from Schneider's Cells

Two experimental approaches are available to measure the complexity of a given RNA population. The first one involves hybridization in RNA excess with labeled purified unique sequence DNA (see for example[10,15,20]).

Unique sequences were isolated from radio-labeled Drosophila DNA by standard methods and checked for purity by renaturation kinetics (Fig. 2). Aliquots were then incubated with an excess of total nuclear or cytoplasmic RNA. Fig. 3 shows an experiment in which nuclear and cytoplasmic RNA's are incubated with the radio-labeled unique DNA. Nuclear RNA reacts to about 23% while only about 2-3% of unique DNA hybridizes to cytoplasmic RNA. Background zero time reaction levels of about 2% were consistently obtained.

As further proof that unique sequences are responsible for the hybridization levels measured, the ^{3}H-labeled DNA which reacted with cytoplasmic RNA was recovered and allowed to renature with unlabeled total homologous DNA. Data in Fig. 2 show clearly that such DNA renatures with kinetics characteristic of unique sequences. DNA in the absence of RNA shows no appreciable reaction. Alkali treatment of the RNA eliminates all reaction, proving that the RNA preparations are free of contaminating DNA and that reaction is exclusively one of DNA/RNA hybrid formation.

We conclude that the fraction of the genome represented by nuclear RNA is about five to ten times higher than that represented by cytoplasmic RNA.

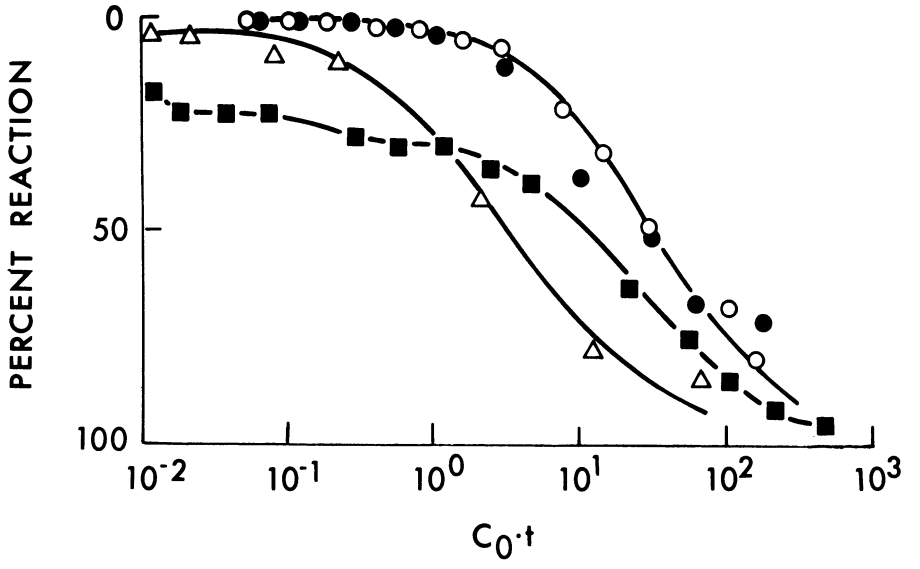

FIGURE 2. Renaturation kinetics of total and unique Drosophila melanogaster DNA. The figure shows reference renaturation kinetics for total Drosophila DNA (■---■) and B. subtilis DNA (Δ---Δ). In addition the kinetics are shown for purified unique sequence DNA (O---O) and unique sequence DNA recovered after hybridization with cytoplasmic RNA to a $R_o t$ value of 5000 (●---●). In each case the reactions were driven by a gross excess of total Drosophila DNA.

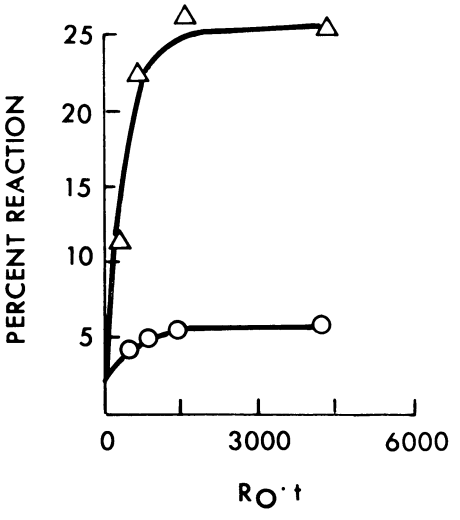

FIGURE 3. Hybridization of nuclear and cytoplasmic RNA with labeled unique sequence Drosophila DNA. 1.6 μg/ml ^{3}H-labeled unique DNA was incubated with 7 mg/ml nuclear (Δ-Δ) or 2 mg/ml cytoplasmic (O-O) Schneider's cell RNA. All samples were incubated at 70°C in 0.4 M phosphate buffer. At appropriate times, samples were removed, diluted with 5 ml of 0.14 M phosphate buffer and frozen for subsequent fractionation on hydroxylapatite columns.

Kinetics of Hybridization of cDNA Complementary to Nuclear Polyadenylated RNA with DNA and its Template

The second experimental approach involves hybridization of a cDNA complementary to polyadenylated nuclear RNA with an excess of template RNA[11,12,22]. For this purpose cDNA was prepared using polyadenylated nuclear RNA as template. Synthesis of the cDNA was totally dependent upon the addition of oligo dT as primer, showing that nuclear sequences adjacent to polyadenylate were the template.

About 15% of the polyadenylated RNA present in the cytoplasm of Schneider's cells is transcribed from repetitive DNA sequences, while the majority of the RNA molecules are transcribed from unique DNA sequences[18]. In order to determine the proportion of the nuclear polyadenylated RNA molecules derived from unique and repetitive DNA, the nuclear cDNA probe was annealed with an excess of unlabeled Drosophila DNA.

Reproducibility was checked by using three different cDNA preparations synthesized with different batches of polyadenylated RNA as a template and three different Drosophila DNA preparations (Fig. 4). Some 10-20% of the

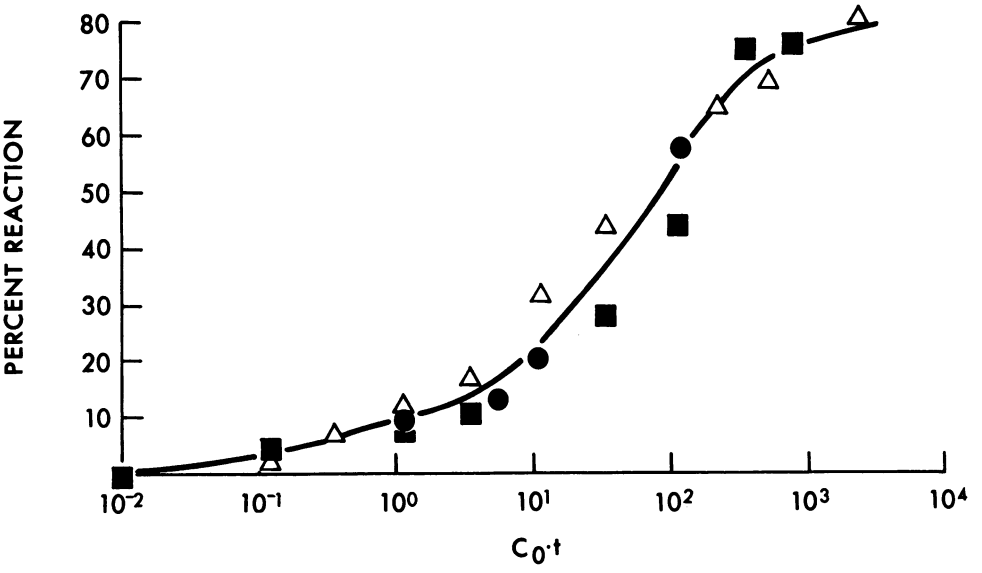

FIGURE 4. Renaturation of cDNA made using polyadenylated nuclear RNA of Schneider's cells as a template with an excess of unlabeled DNA. 5 µl capillaries, containing 800 cpm of cDNA and Drosophila DNA at a concentration of 1 mg/ml were incubated in 0.24 M phosphate buffer at 70°C for different lengths of time. Samples were chilled to 0°C, diluted with 4 ml of S1 buffer and digested with S1 nuclease. Three experiments are shown, performed with different cDNA and Drosophila DNA preparations (■, ●, Δ).

cDNA reacted rapidly with kinetics characteristic of repetitive sequences while the major reaction occurred with the kinetics of unique sequences. This indicates that the majority of the cDNA represents RNA sequences which are transcribed from non-repetitive regions of the genome.

Under conditions of large RNA excess, the rate of hybridization of RNA to complementary DNA, when measured in terms of $R_o t$ (R_o = initial concentration of RNA in moles of nucleotides per liter, T = time in seconds) is proportional to the base sequence complexity of the RNA population. It is therefore possible to estimate the base sequence complexity of an unknown RNA population by comparison of $R_o t_{1/2}$ for the reaction between the RNA and its cDNA with a $R_o t_{1/2}$ obtained with a kinetic standard of known base sequence complexity[22]. In an attempt to determine the complexity of the nuclear polyadenylated RNA population, the nuclear cDNA was hybridized with an excess of its template polyadenylated nuclear RNA (Fig. 5). Four experiments were

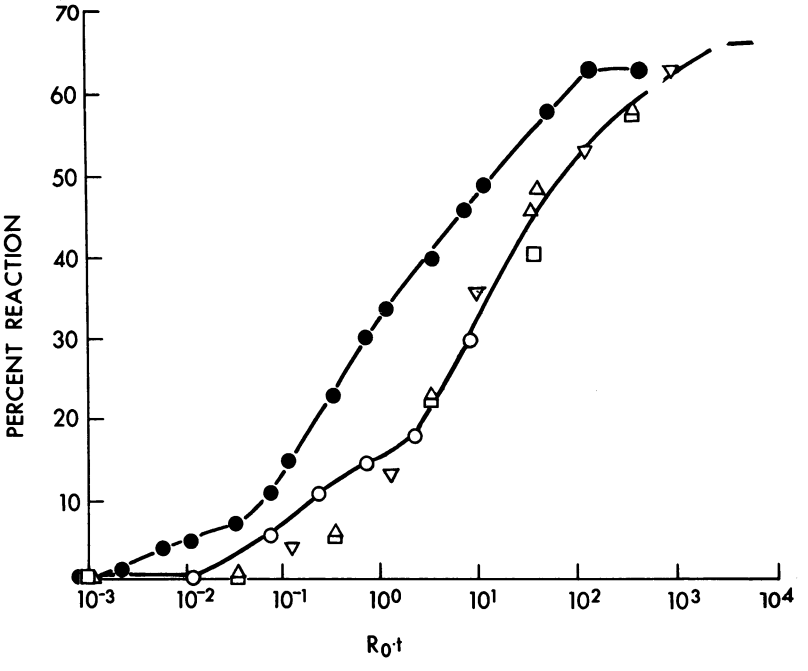

FIGURE 5. Hybridizations between nuclear and cytoplasmic cDNA probes and their corresponding templates. Five µl samples, each containing about 1000 cpm of cDNA and either nuclear or cytoplasmic polyadenylated RNA at a concentration ranging between 100 µg/ml and 1 mg/ml were annealed at 70°C in 0.24 M phosphate buffer containing 0.01% and digested with S1 nuclease. For the reaction between nuclear cDNA and nuclear polyadenylated RNA, four experiments are shown, performed with different cDNA and polyadenylated nuclear RNA preparations (○,△,□,▽). One hybridization experiment is shown between cytoplasmic cDNA and its mRNA template (●).

performed using different cDNA and RNA preparations. The hybridization kinetics are complex, extending over four log units of $R_o t$; suggesting the presence of RNA molecules occurring at widely different frequencies in the cell [19,22]. The complexity of the nuclear polyadenylated RNA population was compared directly to that of cytoplasmic polyadenylated RNA by means of a parallel hybridization between this latter RNA and its corresponding cDNA. Analysis of the two curves of Fig. 5 can, in principle, yield a measurement of overall complexity for both nuclear and cytoplasmic polyadenylated RNA[22]. It is clear that there exists a set of components with a range of different complexities and extents of transcription in each case. Unfortunately, the hybridization reactions of nuclear and cytoplasmic cDNA do not reach 100%. This is a very common problem encountered in these type of hybridizations in which the cDNA probes do not represent a complete copy of the template. The mean sedimentation value of both nuclear and cytoplasmic cDNA's on alkaline sucrose gradients was 7S (data not shown).

A direct calculation of the total complexity for nuclear RNA is made difficult because of the inaccuracies involved in estimating the $R_o t_{1/2}$ value for the most complex class of RNA when the reaction does not reach completion. The displacement of the nuclear RNA curve from that for cytoplasmic RNA by a factor of about 5 to 10 can be explained in two ways. Conceivably a set of less complex components present in cytoplasmic RNA is drastically underrepresented in nuclear RNA, making the complexities appear different, while in fact, they are not. Alternatively, the result could indicate that each of the components in cytoplasmic RNA is less complex by a factor of 5 to 10 than the comparable set in nuclear RNA, thereby favoring the latter possibility. It should be clear that these complexity measurements relate only to the sequences immediately adjacent to 3' poly(A) in both nuclear and cytoplasmic RNA populations and represent a minimum estimate of the total sequence diversity of these RNA's.

A direct comparison of the sequences present in polyadenylated nuclear and cytoplasmic RNA was made by annealing nuclear cDNA with polyadenylated cytoplasmic RNA (Fig. 6). The initial kinetics of reaction were more rapid than with polyadenylated nuclear RNA but the reaction reached an apparent plateau. A recycling experiment was also performed to substantiate the validity of this conclusion. Single-stranded cDNA was recovered after reaction with the same polyadenylated cytoplasmic RNA to a $R_o t$ of 12. This cDNA hybridized to at least 55% with nuclear RNA but reacted only marginally with cytoplasmic RNA. From this result we conclude that polyadeny-

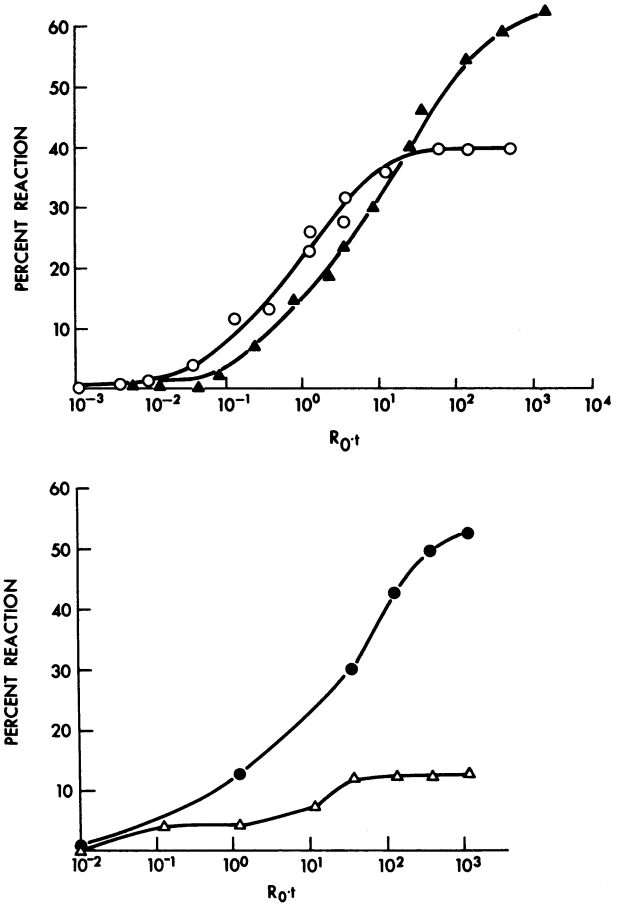

FIGURE 6. TOP: Hybridization between cDNA complementary to nuclear polyadenylated RNA and nuclear and cytoplasmic polyadenylated RNA. Hybridization reactions were performed in 5 µl capillaries as described in the legend of Fig. 3. (▲---▲) Reaction of the nuclear cDNA with nuclear polyadenylated RNA; (O---O) Reaction of the nuclear cDNA probe with cytoplasmic polyA(+) RNA. BOTTOM: Annealing between fractionated nuclear cDNA and nuclear and cytoplasmic polyadenylated RNA. Nuclear cDNA, containing 3.5 x 10^5 cpm was annealed with mRNA to a $R_o t$ of 12 and fractionated on hydroxylapatite as previously described[13]. The unreacted cDNA was recovered and hybridized with polyadenylated nuclear RNA (●---●) and polyadenylated cytoplasmic RNA (Δ---Δ).

lated nuclear RNA is indeed more complex than polyadenylated cytoplasmic RNA.

DISCUSSION

In contrast to vertebrate cells, very large RNA molecules cannot be detected in the nucleus of Drosophila cells. According to the present study

and others published recently[12-14], most nuclear RNA of Schneider's cells sediment at less than 30S. The polyadenylated fraction comprises about 10% of the total nuclear RNA in Drosophila. Its average molecular weight is about 2-3 times greater than that of cytoplasmic polyadenylated RNA[12-14]. This finding may be compared to the situation in Dictyostelium discoideum where no very large molecules of nuclear RNA are evident[23]. These data suggest that the size of HnRNA is correlated with the haploid genome size or C-value. On the other hand, if chromomeres are the units of transcription in Drosophila as has often been suggested, one might expect to isolate HnRNA molecules with a size of approximately 10^7 daltons corresponding to the average length of a chromomere. This paradox is readily explained if processing is so rapid that full length molecules fail to accumulate.

The sequence diversity of nuclear and cytoplasmic RNA has been compared in two different ways. Hybridization of cDNA in RNA excess provides a measure of the complexity of the polyadenylated fraction and RNA excess hybridization with unique DNA measures the complexity of total nuclear or cytoplasmic RNA. The strengths and limitations of each approach have been extensively discussed [20,22,25]. Analysis of cDNA hybridization kinetics for cytoplasmic polyadenylated RNA indicated the presence of some 7000 different species of RNA of average molecular 4×10^5 for a total sequence complexity of 2.8×10^9 [18]. In RNA driven reactions total cytoplasmic RNA is able to saturate some 3% of the unique sequence DNA. Taking 7.5×10^{10} daltons as the complexity of non-repetitive DNA, i.e., 75% of the total, this suggests a total sequence complexity of about 2.3×10^9. Considering the inaccuracies inherent in both approaches these two estimates are in reasonable agreement. This indicates that a large fraction of the complexity in cytoplasmic RNA can be accounted for by polyadenylated RNA.

It is possible to obtain estimates of the base sequence complexity of nuclear polyadenylated RNA from excess RNA/cDNA hybridization kinetics[11]. In the case of Drosophila RNA, the curve for nuclear polyadenylated RNA parallels that for cytoplasmic RNA and is displaced by approximately a factor of five. This provides an estimate for the complexity of nuclear polyadenylated RNA of about 1.4×10^{10}. Total nuclear RNA saturates some 23% of unique sequence DNA or a total complexity of 1.7×10^{10}. Again the two estimates are in reasonable agreement implying that most nuclear RNA sequences occur in polyadenylated form. However, this conclusion can only be substantiated by direct comparison of polyadenylated and non-polyadenylated nuclear RNA sequences. Alternatively, the lifetime of non-polyadenylated portions of the

nuclear RNA transcript may be so brief that it does not accumulate in sufficient quantities to drive hybridization of its complementary DNA strand. In general, hybridization experiments give minimum estimates of complexity particularly where heterogeneity of lifetimes exists.

ABBREVIATIONS USED: SSC, saline sodium citrate (0.15 M NaCl, 0.015 M sodium citrate).

ACKNOWLEDGEMENTS

This research was supported by NSF grant No. BMS75-19418.

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