Relationship between Nuclear and Cytoplasmic RNA in *Drosophila* Cells†

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ABSTRACT: Polyadenylated RNA was isolated from nuclei of cultured *Drosophila* cells, Schneider's line 2, and used as a template to synthesize a complementary DNA probe. Hybridization experiments were performed to study the relationship between nuclear and cytoplasmic RNA. About two-thirds of the nuclear polyadenylated RNA sequences exist in the cytoplasm. Experiments with fractionated cDNA probes demonstrated that RNA sequences that are frequent in the nucleus are also abundant in the cytoplasm. These findings are consistent with a precursor–product relationship in which some polyadenylated molecules in the nucleus are destined for the cytoplasm while other sequences are polyadenylated but not transferred.

It appears that mammalian messenger RNA is derived by processing of high molecular weight precursors in the cell nucleus (Darnell et al., 1973), although definitive proof of this pathway is lacking. The HnRNA[1] population turns over very rapidly within the nucleus and only a small part of it ever reaches the cytoplasm (Scherrer et al., 1970). However, the actual proportion of the HnRNA that becomes mRNA has not been firmly established for any mammalian cell. Part of the evidence for a precursor–product relationship between HnRNA and mRNA is based on the presence of 3′-poly(A) in HnRNA as well as mRNA. Furthermore, the proportion of poly(A) in HnRNA is much lower than in mRNA and only the smallest size class contains as much poly(A) by weight as cytoplasmic mRNA (Derman and Darnell, 1974). Thus, it seems likely that polyadenylation is a late event in processing of HnRNA, which immediately precedes entry of mRNA into the cytoplasm (Adesnik et al., 1972; Perry et al., 1974). In contrast to this complex situation in higher eucaryotes, the relationship between nuclear and cytoplasmic RNA in *Dictyostelium discoideum* is well established. In this case, the HnRNA is only 30% larger than mRNA and the evidence for a precursor–product relationship is more persuasive (Lodish et al., 1973).

The situation in other cells of intermediate stages of evolutionary advancement has not yet been investigated in detail. It has recently been demonstrated that 10% of the nuclear RNA in *Drosophila* is polyadenylated. Hybridization experiments using DNA complementary to the polyadenylated nuclear RNA indicate that it is five times more complex than the cytoplasmic polyadenylated RNA (Levy W., Johnson, and McCarthy, unpublished results).

In the present communication, we are concerned with the relationship between nuclear and cytoplasmic RNA in *Drosophila* cells. By hybridization of a cDNA probe representing nuclear polyadenylated molecules with an excess of mRNA, we show that about two-thirds of the polyadenylated HnRNA sequences are represented in the cytoplasm. On the other hand, all the polyadenylated sequences of the cytoplasm appear to be represented in the population of nuclear RNA molecules. Using fractionated cDNA probes representing high and low frequency polyadenylated sequences in both mRNA and HnRNA, we have asked whether the most abundant polyadenylated HnRNA becomes the most abundant cytoplasmic RNA.

Materials and Methods

Preparation of Nuclear RNA. Nuclear RNA was prepared by the method of Getz et al. (1975). For a typical preparation, 1000 ml of a suspension culture of *Drosophila,* Schneider's line 2 (Schneider, 1972), was collected by centrifugation 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 polyvinyl sulfate, 10^{-3} M spermine, and 1% diethyl pyrocarbonate. 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 prepared 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 4% isoamyl alcohol. After four extractions the aqueous phase layer was removed and precipitated with 2 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 incubated for 20 min at 37 °C. One-twentieth volume of 10% sodium dodecyl sulfate 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 sul-

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[1] Abbreviations used: mRNA, messenger RNA; HnRNA, heterogeneous nuclear RNA; cDNA, complementary DNA; poly(A), poly(adenylic acid); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NTE buffer, 0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris-HCl, pH 7.4; poly(U), poly(uridylic acid).

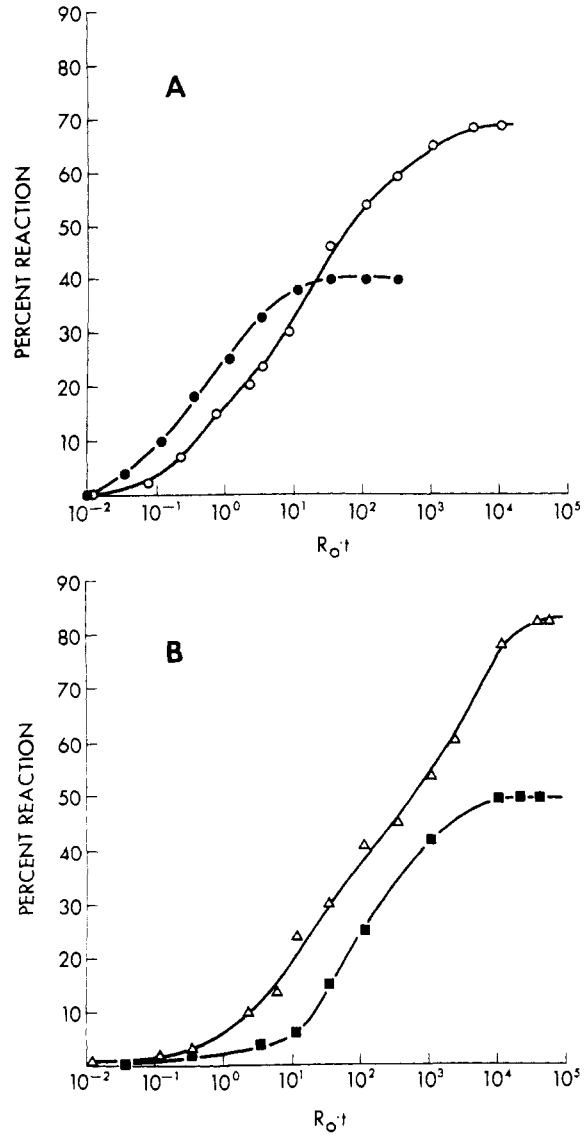

FIGURE 1: Kinetics of hybridization of cDNA complementary to nuclear polyadenylated RNA with nuclear and cytoplasmic polyadenylated RNA or total nuclear and cytoplasmic RNA. The reactions were performed in 5-μl capillaries as described in Materials and Methods. The experimental points represent the mean of four determinations. (A) Reaction with nuclear polyadenylated RNA (○) and cytoplasmic polyadenylated RNA (●). (B) Reaction with total nuclear RNA (△) and total cytoplasmic RNA (■).

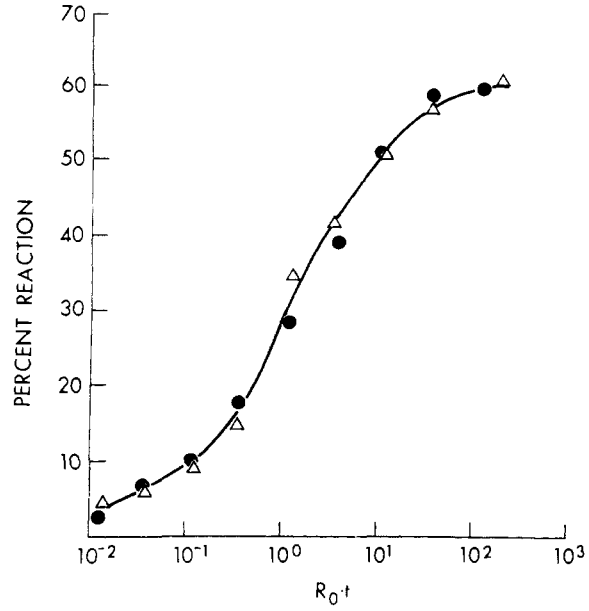

FIGURE 2: Kinetics of hybridization of fractionated nuclear cDNA. Nuclear cDNA (3.5×10^5 cpm) was annealed with cytoplasmic polyadenylated RNA to a R_0t value of 12 and fractionated on hydroxylapatite as described in Materials and Methods. The previously hybridized cDNA was recovered and hybridized with polyadenylated nuclear RNA (●) or cytoplasmic polyadenylated RNA (△).

fate. RNA eluting in the void volume was pooled and precipitated with 2 volumes of ethanol. This RNA constituted the total nuclear RNA preparation.

Preparation of Nuclear and Cytoplasmic Poly(A) Containing RNA. Cytoplasmic RNA was prepared as described before (Levy W. and McCarthy, 1975). Isolation of total nuclear and cytoplasmic poly(A) containing RNA was performed by chromatography on poly(U)-Sepharose, as described elsewhere (Levy W. and McCarthy, 1975).

The poly(A) containing RNA eluted from the column was precipitated with ethanol. For use in hybridization experiments, the RNA was recovered by centrifugation at 8000 rpm for 20 min at −5 °C and resuspended in sterile water.

When labeled RNA was required, the cells were concentrated tenfold in Schneider's medium and incubated at 25 °C in the presence of 10 μCi/ml of [^{3}H]uridine for 20 min for the labeling of nuclear RNA and for 16 h for the labeling of cytoplasmic RNA.

Synthesis of cDNA. The reverse transcriptase from avian myeloblastosis virus was kindly provided by Dr. W. J. Rutter and Dr. Beard (NIH). Nuclear and cytoplasmic cDNA probes were synthesized as described previously (Levy W. and McCarthy, 1975). In both cases synthesis of cDNA was totally dependent upon the presence of oligo(dT) as primer.

Hybridization Reactions. Small amounts of nuclear or cytoplasmic cDNA (500–1000 cpm) were mixed with the appropriate amount of RNA and sealed in 5-μl capillaries. The RNAs were present at concentrations ranging between 100 μg/ml and 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 desired value of R_0t. At the end of each incubation period, the content of each capillary was ejected into 4 ml of 0.3 M NaCl, 0.03 M NaAc–3 mM $ZnCl_2$, pH 4.5, and digested with S1 nuclease in the presence of 10 μg of single-stranded bacterial DNA at 37 °C for 2 h as described by Leong et al. (1972). Each point in a curve is the average of duplicate determinations. In all experiments the hybridization at zero time (3–8%) was subtracted as background. Thermal denaturation profiles of nuclear and cytoplasmic cDNA/DNA duplexes were determined as described previously (Levy W. and McCarthy, 1975).

Preparation of Drosophila DNA. Drosophila DNA was extracted from Schneider's cell nuclei as described elsewhere (Levy W. and McCarthy, 1975).

cDNA Fractionation. Nuclear cDNA was fractionated into probes representing frequent and infrequent RNA as described previously (Levy W. and McCarthy, 1975).

Results

Hybridization between Nuclear cDNA and Various RNAs. A complementary DNA probe was synthesized using polyadenylated nuclear RNA as a template and hybridized with various RNAs. As was the case for cytoplasmic polyadenylated RNA, the synthesis of cDNA was totally dependent upon the addition of oligo(dT) as primer. However we cannot eliminate

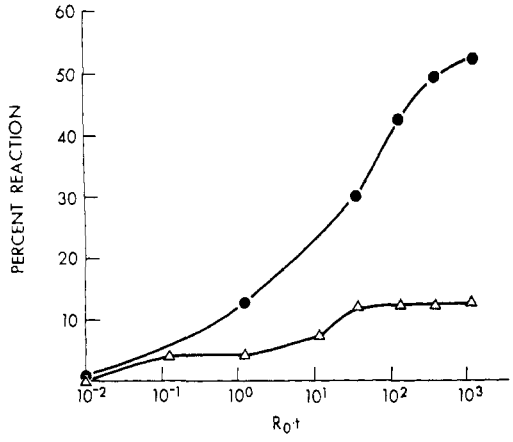

FIGURE 3: Kinetics of hybridization of fractionated nuclear cDNA. Nuclear cDNA was fractionated after partial reaction with cytoplasmic polyadenylated RNA as described in the legend to Figure 2. The unreacted cDNA was recovered and hybridized with nuclear polyadenylated RNA (●) and cytoplasmic polyadenylated RNA (△).

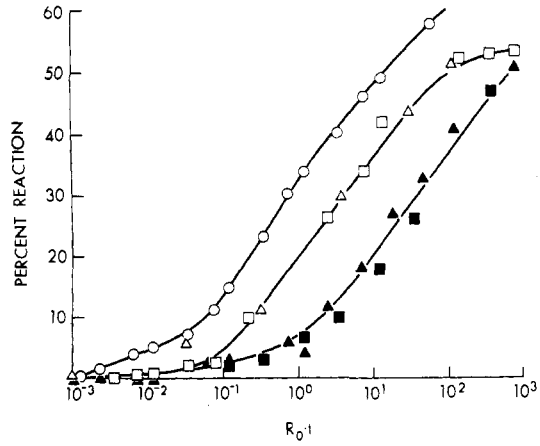

FIGURE 4: Kinetics of hybridization of cDNA complementary to cytoplasmic polyadenylated RNA with various RNAs. Five-microliter capillaries containing 500 cpm of cDNA and various RNAs at concentrations ranging between 100 μg/ml and 2 mg/ml were annealed as described in Materials and Methods. One hybridization experiment is shown between cytoplasmic cDNA and its template RNA (○). Two hybridization experiments are shown for polyadenylated nuclear RNA (△; □). Two hybridization experiments are shown for total nuclear RNA (■; ▲). For each duplicate experiment, a different batch of cDNA and RNA was used.

the possibility that the initiation of cDNA synthesis occurs on internal oligo(A) sequences as well as 3′-terminal polyadenylate. The hybridization of the cDNA with its template gives information concerning the complexity of the polyadenylated nuclear RNA. Data in Figure 1 show the hybridization of the cDNA with an excess of polyadenylated nuclear and cytoplasmic RNA and with total nuclear and cytoplasmic RNA. It is evident that neither polyadenylated nor total cytoplasmic RNA are capable of driving the nuclear cDNA reaction to completion. Both RNA preparations are complementary to only about two-thirds of the nuclear cDNA, suggesting that there exist nuclear RNA sequences represented in cDNA, which are undetectable in the cytoplasm in either polyadenylated or nonpolyadenylated form. Therefore we conclude that some RNA sequences are polyadenylated in the nucleus but fail to be transferred to the cytoplasm. It is also notable that the $R_0t_{1/2}$ value for the reaction with total nuclear RNA is some ten times higher than for polyadenylated RNA, implying that about 10% of nuclear RNA is complementary to the probe. Likewise the reaction with total cytoplasmic RNA is displaced by two orders of magnitude from that with polyadenylated cytoplasmic RNA.

In order to validate the absence of certain nuclear cDNA sequences from the cytoplasm, a recycling experiment was performed. The nuclear cDNA was annealed with polyadenylated cytoplasmic RNA to R_0t of 12 and the reacted, double-stranded cDNA was separated from the unreacted single-stranded cDNA by hydroxylapatite.

In principle, the previously reacted cDNA should hybridize completely with both poly(A)(+) nuclear RNA and cytoplasmic RNA. On the other hand, the unreacted cDNA should hybridize appreciably with poly(A)(+) nuclear RNA but not with cytoplasmic RNA. The data in Figure 2 show that the previously hybridized cDNA reacted with both polyadenylated RNAs with similar kinetics. The unreacted cDNA hybridized with polyadenylated nuclear RNA but not with cytoplasmic RNA (Figure 3).

Hybridization of Cytoplasmic cDNA with Various RNAs. If a precursor–product relationship exists between nuclear and cytoplasmic RNA, all of the sequences that are found in the cytoplasmic polyadenylated RNA should be represented in the population of nuclear RNA molecules. To test this prediction, a cDNA was synthesized on a template of cytoplasmic polyadenylated RNA. The cDNA was annealed with its template and also with an excess of unlabeled polyadenylated and total nuclear RNA (Figure 4). The hybridization experiments between the cDNA and the nuclear RNAs were performed in duplicate, using different batches of cDNA and different RNA preparations.

The results show that total and polyadenylated nuclear RNAs react well with the cytoplasmic cDNA. The reaction with the polyadenylated HnRNA is about ten times slower than the reaction of the cDNA with its template RNA, reflecting a dilution of polyadenylated RNA sequences complementary to the cyptoplasmic fraction by other nuclear polyadenylated sequences. However, it should also be kept in mind that a contamination of the nuclear RNA by 10% of the cytoplasmic RNA would yield a similar result. The value of $R_0t_{1/2}$ for the reaction with total nuclear RNA is ten times higher than the corresponding $R_0t_{1/2}$ for the reaction with the polyadenylated nuclear RNA, consistent with previous data showing that 10% of the nuclear RNA is polyadenylated.

Isolation of Fractionated Nuclear cDNA Probes. The kinetics of hybridization between nuclear cDNA and HnRNA are complex, extending over 5 log units (Figure 1). This kinetic behavior is indicative of the existence of molecules present at very different frequencies within the nuclear RNA population. In order to obtain a cDNA probe corresponding to either abundant or rare nuclear RNA molecules, the nuclear cDNA was partially hybridized with an excess of nuclear RNA. Hybridization of the cDNA remaining single stranded or the previously hybridized cDNA with nuclear RNA validated the successful fractionation of cDNA (Figure 5).

In order to determine the extent to which abundance of nuclear sequences reflected gene dosage, the fractionated nuclear cDNA probe was annealed with cellular DNA. The kinetics of reaction suggested that the abundance of nuclear polyadenylated RNA sequences could be partially accounted for by gene dosage. Abundant cDNA reacted with kinetics diagnostic of repetitive DNA while the cDNA corresponding to rare sequences reacted almost exclusively with unique sequence DNA (Figure 6). As in the case of some abundant cytoplasmic DNA (Levy W. and McCarthy, 1975), it appears that this apparent dosage effect is real since the thermal sta-

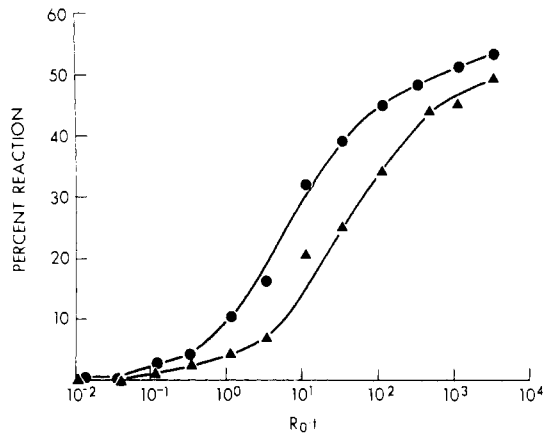

FIGURE 5: Kinetics of hybridization of fractionated nuclear cDNA with total nuclear RNA. Nuclear cDNA (3.5×10^5 cpm) was annealed with total nuclear RNA to a R_0t of 12 and fractionated on hydroxylapatite. Aliquots with 1000 cpm of previously reacted cDNA (●) or unreacted cDNA (▲) were annealed with total nuclear RNA. Two reaction mixtures were used containing 500 μg/ml and 2 mg/ml RNA.

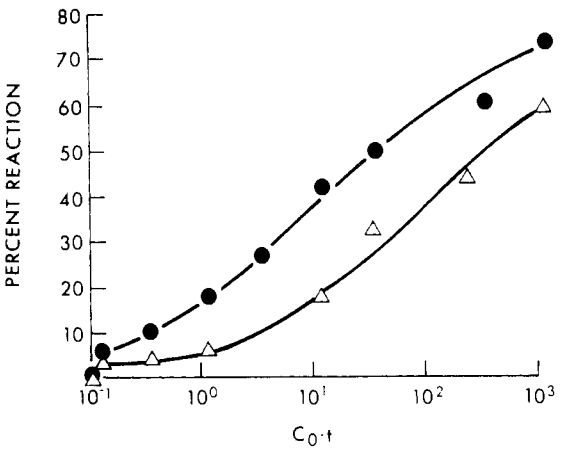

FIGURE 6: Kinetics of reaction of nuclear cDNA fractionated on hydroxylapatite with an excess of unlabeled *Drosophila* DNA. cDNA synthesized with polyadenylated nuclear RNA as template was annealed to total nuclear RNA to a R_0t value of 12 and fractionated on hydroxylapatite as described in Materials and Methods. Aliquots containing 1000 cpm of previously hybridized cDNA (●) or unreacted cDNA (△) were annealed with unlabeled *Drosophila* DNA. The DNA was present at a concentration of 1 mg/ml.

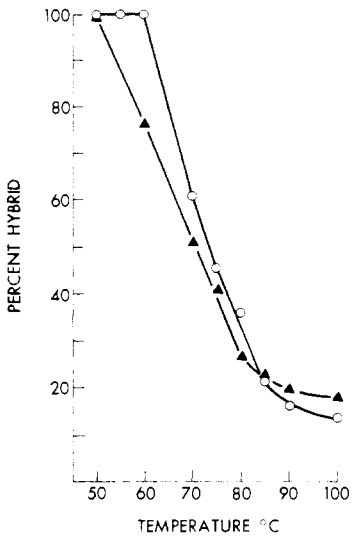

FIGURE 7: Thermal stability of nuclear cDNA and cytoplasmic cDNA duplexes formed with repetitive cellular DNA. cDNA/DNA duplexes were formed by renaturation of either cytoplasmic or nuclear cDNA with *Drosophila* DNA to $C_0t = 6$. After reaction 5-μl samples were diluted and the thermal dissociation profiles determined. (○) nuclear cDNA/DNA duplexes; (▲) cytoplasmic cDNA/DNA duplexes.

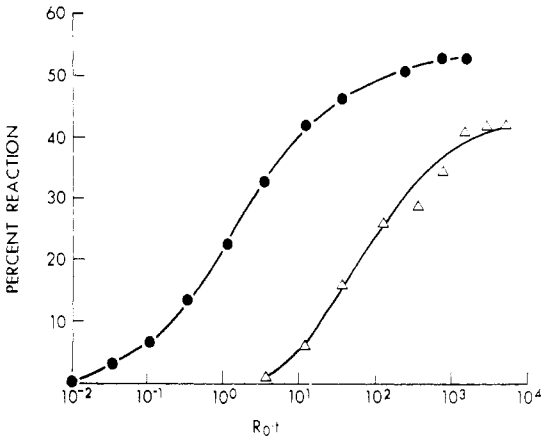

FIGURE 8: Kinetics of hybridization of fractionated nuclear cDNA with polyadenylated cytoplasmic RNA. cDNA representing polyadenylated nuclear RNA was partially annealed with total nuclear RNA and fractionated on hydroxylapatite as described in the legend to Figure 6. Aliquots containing 1000 cpm of previously hybridized (●) or unreacted cDNA (△) were annealed with polyadenylated cytoplasmic RNA.

bility of rapidly reacting cDNA/DNA duplexes is essentially the same whether cytoplasmic or nuclear cDNA is used (Figure 7). Previously published data showed that frequent and infrequent cytoplasmic cDNA/DNA duplexes are equally stable. If the rapid reaction of nuclear cDNA with cellular DNA were due to cross-reaction among intermediate repetitive DNA, a lower thermal stability would be predicted.

cDNA probes enriched to represent frequent or rare nuclear RNA sequences were also annealed with cytoplasmic RNA. The kinetics of reaction between cytoplasmic RNA and frequent nuclear cDNA are much more rapid than that for the probe representative of infrequent nuclear RNA (Figure 8). From this we conclude that molecules which are abundant in the nuclear RNA population become also preferentially over-represented in cytoplasmic RNA.

Discussion

In several kinds of eucaryotic cells which have been studied, nuclear RNA represents much more of the total complexity of the genome than does cytoplasmic RNA. For example, in sea urchin embryos and mouse Friend cells, the complexity of nuclear RNA is some five to ten times greater than that of cytoplasmic RNA (Smith et al., 1974; Hough et al., 1975; Getz et al., 1975). Essentially the same conclusion has recently been reached for cultured *Drosophila* cells (Levy W., Johnson, and McCarthy, unpublished results). In the case of *Drosophila* cells and Friend cells, a large difference in complexity also exists between polyadenylated nuclear and cytoplasmic RNA. It would seem, therefore, that not all polyadenylated nuclear RNA can be precursor to cytoplasmic messenger and that polyadenylation is not causally related to transport to the cytoplasm or to polysome association. The existence of a substantial proportion of messenger RNA which is devoid of poly(A) (Milcarek et al., 1974) is consistent with this view. Furthermore, some polyadenylated cytoplasmic RNA exists in nonpolyribosome-associated ribonucleoprotein particles (McLeod, 1975).

In the present study we have attempted to examine directly the relationship between nuclear and cytoplasmic RNA through hybridization of cDNA synthesized as a complement to each population. As in all studies of this type, the interpretation is complicated by one major difficulty: that of cross-contamination between nuclear and cytoplasmic fractions. For example, the data of Figure 4 demonstrate that nuclear RNA hybridizes efficiently with cDNA complementary to cytoplasmic RNA. This result appears to validate the precursor–product relationship between nuclear and cytoplasmic RNA. Alternatively the result could be attributable to contamination of the nuclear RNA by cytoplasmic RNA. This possibility is especially difficult to eliminate if, in *Drosophila* as in mammalian cells, most of the polyadenylated RNA molecules reside in the cytoplasm (Johnson et al., 1975; Sheiness and Darnell, 1973).

The higher complexity of nuclear polyadenylated RNA, compared with the corresponding cytoplasmic fraction, implies that cDNA complementary to nuclear RNA would react only partially with cytoplasmic RNA. A direct comparison of hybridization kinetics between nuclear cDNA and nuclear or cytoplasmic RNA, Figure 1, demonstrated this expectation. It appeared that only the most slowly reacting cDNA corresponding to the low frequency class of nuclear RNA was absent from cytoplasmic RNA. Therefore it was necessary to perform a two-cycle experiment in which nuclear cDNA was fractionated based upon partial reaction with cytoplasmic polyadenylated RNA. A second hybridization of this fractionated probe with nuclear and cytoplasmic polyadenylated RNA provided clear evidence that many sequences present in nuclear RNA are indeed absent from cytoplasmic RNA. Our results show that the polyadenylation of a nuclear RNA molecule is insufficient to ensure its transport to the cytoplasm. These data are also consistent with turnover of nuclear poly(A) as suggested by Perry et al. (1974). It is important to note that sequences adjacent to internal oligo(A)'s could account for the nuclear restricted fraction since internal oligo(A) sequences may also act as initiation sites for the reverse transcriptase.

Both nuclear and cytoplasmic RNA appear to be complex populations in which different sequences are present at quite different frequencies. Accordingly we asked whether any correlation exists between the relative abundance of RNA species in the nucleus with that in the cytoplasm. Using fractionated probes representing high or low frequency classes, it appears that a high degree of correlation exists. Thus those RNA species present at the highest concentration in the nucleus give rise to the most prevalent species of cytoplasmic RNA. Since kinetic experiments were not carried out, we cannot distinguish between higher rates of synthesis or longer life-time as a basis for this effect. This finding appears to conflict with the suggestion that processing of the primary transcript might act as a regulatory device to control the level of messenger RNA (Scherrer, 1975). In this event, no correlation would exist between abundance in the nucleus and abundance in the cytoplasm. However, since the present experiments were performed with the complex mixture of total RNA, we can conclude only that no distortion of the population distribution is evident in total RNA. The possibility that individual nuclear precursors are processed with quite different efficiencies cannot be discounted.

References

Adesnik, M., Salditt, M., Thomas, W., and Darnell, J. E. (1972), *J. Mol. Biol. 71,* 21.

Darnell, J. E., Jelinek, W. R., and Molloy, G. R. (1973), *Science 181,* 1215.

Derman, E., and Darnell, J. E. (1974), *Cell 3,* 255.

Getz, M. J., Birnie, G. D., Young, B. D., MacPhail, E., and Paul, J. (1975), *Cell 4,* 121.

Hough, B. R., Smith, M. J., Britten, R. J., and Davidson, E. H. (1975), *Cell 5,* 291.

Johnson, L. F., Williams, J. G., Abelson, A. T., Green, H., and Penman, S. (1975), *Cell 4,* 69.

Lengyel, J., and Penman, S. (1975), *Cell 5,* 281.

Leong, J. A., Garapin, A. C., Jackson, N., Fanisher, L., Levinson, W., and Bishop, J. M. (1972), *J. Virol. 9,* 891.

Levy W., B., and McCarthy, B. J. (1975), *Biochemistry 14,* 2440.

Lodish, H. F., Firtel, R. A., and Jacobson, A. (1973), *Cold Spring Harbor Symp. Quant. Biol. 38,* 899.

McLeod, M. C. (1975), *Biochemistry 14,* 4011.

Milcarek, C., Price, R., and Penman, S. (1974), *Cell 3,* 1.

Perry, R. P., Kelley, D. E., and La Torre, J. (1974), *J. Mol. Biol. 82,* 315.

Scherrer, K. (1975), in Control of Gene Expression, Kohn, A., and Shatkay, A., Ed., New York, N.Y., Plenum Publishing Co., p 169.

Scherrer, K., Spohr, G., Granboulan, N., Morel, C., Grosclaude, J., and Chezzi, C. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35,* 539.

Schneider, I. (1972), *J. Embryol. Exp. Morphol. 27,* 353.

Sheiness, D., and Darnell, J. E. (1973), *Nature (London) 241,* 265.

Smith, M. J., Hough, B. R., Chamberlin, M. E., and Davidson, E. H. (1974), *J. Mol. Biol. 85,* 103.