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Polyadenylated RNA Complementary to Repetitive DNA in Mouse L-Cells†

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ABSTRACT: Complementary DNA, synthesized with L-cell polyadenylated RNA as template, renatured with total L-cell DNA to about 70%. About 30% complementary to unique sequence DNA and another 10 and 30% corresponded to sequences about 20- and 500-fold repetitive. Complementary DNA was fractionated after partial hybridization with total polyadenylated RNA to obtain preparations enriched or impoverished in complements of the most frequent polyadenylated RNA. Renaturation of these complementary DNA fractions with L-cell DNA revealed that most frequent RNAs are transcribed from repetitive DNA sequences. Complementary DNA, density labeled with bromodeoxyuridine, was fractionated by renaturation with L-cell DNA to yield fractions enriched in repetitive and unique sequence DNA. The density labeled complementary DNA was purified by equilibrium centrifugation in an alkaline Cs_2SO_4 gradient. The complementary DNA representing mainly repetitive DNA sequences hybridized preferentially to frequent polyadenylated RNA.

The genome of eucaryotic cells is made up of interspersed repetitive and unique sequences (Davidson et al., 1973). Although the primary transcript HnRNA[1] certainly contains both unique and repetitive sequences (Jelinek et al., 1973), conflicting results have been obtained concerning the existence of repetitive sequences in the messenger fraction. In the case of poly(A) containing messengers such as those for hemoglobin (Bishop and Rosbash, 1973; Harrison et al., 1972), ovalbumin (Harris et al., 1973; Sullivan et al., 1973), silk fibroin (Suzuki et al., 1972), and immunoglobulin κ chain (Faust et al., 1974; Stavnezer et al., 1974; Honjo et al., 1974), convincing evidence exists that they originate from unique sequence DNA. On the other hand, histone mRNA, which is devoid of 3′-terminal poly(A), is encoded by multiple genes (Kedes and Birnstiel, 1971; Farquhar and McCarthy, 1973). However, other messenger RNAs may be transcribed from repetitive sequences and they may be polyadenylated.

The results of hybridization of total cytoplasmic polyadenylated RNA under conditions of DNA excess led several investigators to conclude that all of this RNA was derived from unique sequences (Goldberg et al., 1973). On the other hand, several reports have appeared in which from 6 to 40% of the total polyadenylated RNA hybridizes with repetitive sequences (Greenberg and Perry, 1971; Firtel and Lodish,1973; Dina et al., 1974; Klein et al., 1974; Rabbitts et al., 1974; Spradling et al., 1974). The tendency for polyadenylated RNA to hybridize with repetitive sequences is greater for mammalian than for insect DNA (Spradling et al., 1974). Similar experiments, in which cDNA[1] complementary to HeLa mRNA was used, demonstrated that some 10% renatured with repetitive DNA (Bishop et al., 1974).

In an attempt to resolve these issues cDNA was prepared from mouse L-cell polyadenylated RNA. This probe was used to determine the fraction of mRNA derived from re-

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[1] Abbreviations used are: cDNA, complementary DNA; SDS, sodium dodecyl sulfate; HnRNA, heterogeneous nuclear RNA; R_0t, the product of the total RNA concentration (R_0) in molarity of nucleotides and the time (t) in seconds; C_0, concentration of dCTP; BrdUTP, bromodeoxyuridine triphosphate.

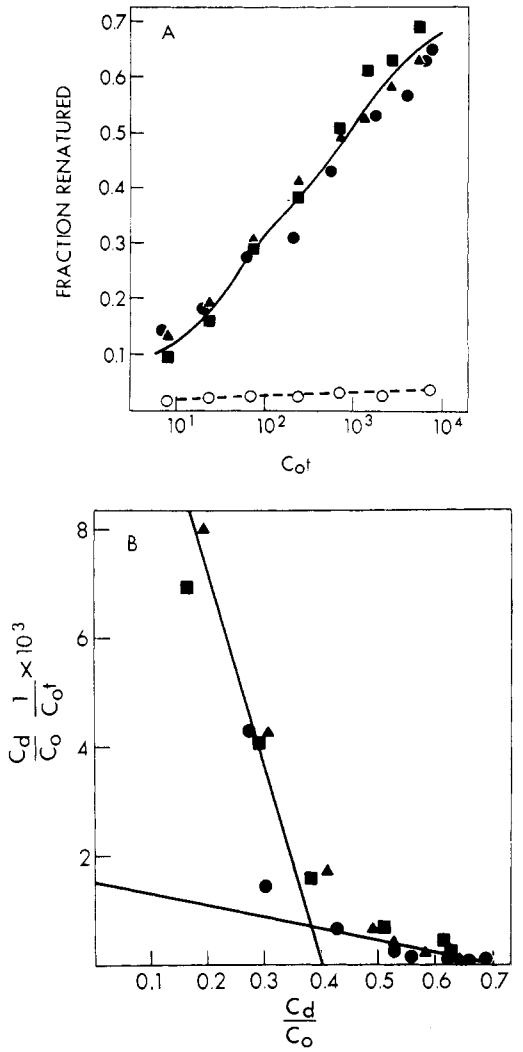

FIGURE 1: Renaturation of cDNA with an excess of L-cell DNA. Three different preparations of cDNA, synthesized on polyadenylated L-cell RNA as a template, were renatured with a large excess of L-cell DNA; experiments 1 (●), 2 (■), and 3 (▲). The cDNA from experiment 3 was also renatured with calf-thymus DNA (O- - -O). No background was subtracted. (A) Fraction renatured (C_d/C_0) was plotted against the log of C_0t. (B) Fraction renatured (C_d/C_0) over C_0t was plotted against the fraction renatured (C_d/C_0).

petitive DNA. In addition, the cDNA was fractionated by limited hybridization with polyadenylated mRNA. In this way, it was possible to determine whether frequent mRNA is preferentially encoded by repetitive DNA. As an alternative approach to the same question cDNA density labeled with bromodeoxyuridine was separated into repetitive and unique sequence classes by renaturation with DNA. The results reveal that about half of the cDNA renatures with repetitive DNA and that it represents mainly polyadenylated RNA in the high or middle frequency class (Bishop et al., 1974; Ryffel and McCarthy, 1975).

Materials and Methods

Preparation of RNA. Polyadenylated RNA was extracted from L-cells (LA-9) as described elsewhere (Ryffel and McCarthy, 1975).

Preparation of DNA. DNA was extracted with phenol–chloroform (1:1) from a crude nuclear pellet obtained in 0.5% NP40. The DNA was sheared by depurination for 30 min at 70° in 0.1 *M* sodium acetate (pH 4.2) and treated with 0.2 *M* NaOH at 50° for 10 min to hydrolyze any contaminating RNA (Grouse et al., 1972).

Synthesis of cDNA. The reverse transcriptase from avian myeloblastosis virus was donated by Drs. Julian Wells and William Rutter. The cDNA was synthesized using [^{3}H]dCTP (Schwarz/Mann, 20 Ci/mmol) as the labeled triphosphate (Ryffel and McCarthy, 1975).

To synthesize density labeled cDNA, dTTP was replaced by the same amount of bromodeoxyuridine 5′-triphosphate (Boeringer, No. 15218). This triphosphate was equally well incorporated into the cDNA.

Isolation of cDNA Representing Frequent RNAs. A large batch of cDNA was hybridized with polyadenylated RNA to a R_0t^1 of 0.3 *M* sec and the single-stranded and hybridized cDNA were separated on hydroxylapatite as described (Ryffel and McCarthy, 1975).

Isolation of cDNA Representing Repeated DNA Sequences. [^{3}H]dCTP- and BrdUTP-labeled cDNA (2×10^5 cpm) was renatured with 500 μg of sheared L-cell DNA to a C_0t of 70 *M* sec under standard conditions. The reaction mixture was diluted 50-fold into 0.01 *M* sodium phosphate. One milliliter of a hydroxylapatite suspension (Bio-Rad, DNA grade) was added for each 100 μg of DNA and shaken for 5 min at room temperature. Single-stranded and double-stranded DNA were separated by elution with 0.14 and 0.4 *M* sodium phosphate as for the separation of single-stranded cDNA from cDNA–RNA hybrids. Since bromodeoxyuridine-substituted DNA has a higher T_m the fractionation was performed at 75°. The single-stranded and double-stranded DNAs were ethanol precipitated and put over a Sephadex G-50 column developed in 0.1 *M* NaCl, 0.5 m*M* EDTA, 0.1% SDS, and 10 m*M* Tris (pH 7.4). The DNA containing fractions were pooled and the DNA precipitated with ethanol. The pellet was dissolved in 0.05 *M* $NaHCO_3$ (pH 11.5) and mixed with a concentrated Cs_2SO_4 solution (2 g/cm^3 in 0.05 *M* $NaHCO_3$ (pH 11.5)) to obtain a final volume of 7 ml and an initial density of 1.47 g/cm^3 (Inman and Baldwin, 1964). Centrifugation to equilibrium was performed in polyallomer tubes in the Beckman No. 40 rotor at 33,000 rpm for 60 hr at 15°. Samples (200 μl) were collected from the bottom of the tube. Fractions containing the cDNA were pooled and dialyzed against H_2O. The cDNA was ethanol precipitated in 0.2 *M* NaCl and dialyzed against H_2O.

Renaturation of cDNA. Small amounts of cDNA (300 cpm) were sealed in 5-μl capillaries with the appropriate amount of sheared DNA. DNA concentration was between 20 μg and 4 mg per ml in 0.24 *M* sodium phosphate, 1 m*M* EDTA, and 0.05% SDS. The capillaries were boiled for 10 min and then incubated at 70° for the time required to reach the desired C_0t value. The renatured cDNA was quantitated by S1 nuclease digestion (Leong et al., 1972).

Hybridization with polyadenylated RNA was performed as described earlier (Ryffel and McCarthy, 1975).

Results

Renaturation of cDNA with DNA. In order to determine if the polyadenylated RNA sequences from L-cells are transcribed from unique or repetitive DNA sequences, the cDNA synthesized on polyadenylated RNA as template was renatured with L-cell DNA. From Figure 1A it can be seen that renaturation occurs between 10 and 10^4 *M* sec. Since renaturation occurs over more than 2 log units of C_0t, more than one component must be present. After plotting the fraction reassociated C_d/C_0 over C_0t against the frac-

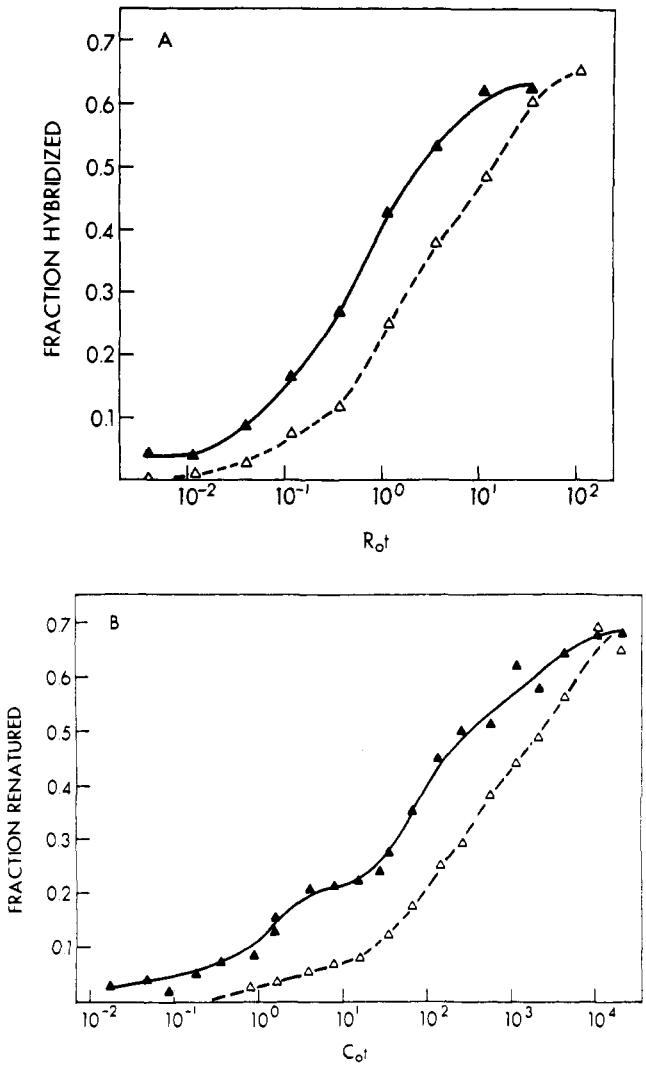

FIGURE 2: Isolation of cDNA representing frequent RNA. cDNA (4 × 10^5 cpm) was hybridized under standard conditions with 50 μg of polyadenylated RNA to an R_0t value of 0.3 *M* sec under standard conditions. The single-stranded and hybridized cDNA were separated by hydroxylapatite chromatography. (A) Small amounts of single-stranded cDNA (△- - -△) and hybridized cDNA (▲–▲) were renatured with polyadenylated RNA. (B) Small amounts of single-stranded cDNA (△- - -△) and hybridized cDNA (▲–▲) were renatured with L-cell DNA. Neither cDNA fractions exhibited self-annealing as assayed by renaturation with bacterial DNA. The S1 nuclease resistant material of the renaturation with bacterial DNA (3%) was subtracted as background in the renaturation curves with the homologous DNA.

tion reassociated according to Marsh and McCarthy (1974), the points can be fitted to two straight lines (Figure 1B). From the reciprocal value of the slope the $C_0t_{1/2}$ of the components can be calculated to be 500 and 30 *M* sec, respectively. The component with the $C_0t_{1/2}$ of 500 *M* sec corresponds to the renaturation of unique sequence DNA (Faust et al., 1974; Stavnezer et al., 1974), whereas that with a $C_0t_{1/2}$ of 30 *M* sec corresponds to sequences 15–20 times repeated. Figure 1A also shows that about 10% of the cDNA became S1 resistant at a time when only 2% was resistant after renaturation with calf thymus DNA. Therefore, a very highly repetitive component of cDNA must exist. Since the same proportion of S1 resistant cDNA was found after renaturation with bacterial DNA, this 2% was assumed to represent the background value. The lack of renaturation with calf thymus DNA demonstrated that all of the cDNA sequences are species specific.

Isolation of cDNA of Frequent Polyadenylated RNA. The population of polyadenylated RNAs consists of several frequency classes (Bishop et al., 1974; Ryffel and McCarthy, 1975). If the most frequent mRNAs are derived from repetitive sequences, then cDNA representing the most prevalent mRNA would be complementary to rapidly renaturing DNA. To investigate this possibility a large batch of cDNA was hybridized with an excess of polyadenylated RNA to a R_0t value of 0.3 *M* sec. Under these conditions 25% of the cDNA hybridizes and represents mainly the most frequent mRNA together with some of intermediate frequency (Ryffel and McCarthy, 1975). The single-stranded and hybridized cDNA were separated by hydroxylapatite chromatography and treated with alkali to destroy the RNA. Hybridization of the single-stranded and previously hybridized cDNA to polyadenylated RNA validated the successful separation of cDNA enriched and impoverished in terms of reaction with frequent RNA sequences (Figure 2A). When the cDNA enriched for frequent polyadenylated RNAs was incubated with L-cell DNA, a large proportion renatured rapidly (Figure 2B). The data suggest a component of about 20% with a $C_0t_{1/2}$ of 1–2 *M* sec, another 30% with a C_0t of 50, and some unique sequence cDNA. On the other hand, the cDNA fraction enriched for complementarity to infrequent RNAs renatured mainly with unique sequence DNA.

Isolation of cDNA Representing Repeated DNA Sequences. The experiment described demonstrates that cDNA molecules which hybridize most rapidly are preferentially representative of repetitive DNA. To verify this conclusion in an independent manner we sought to isolate the cDNA representing repeated DNA by annealing the cDNA to a C_0t value at which most repetitive DNA would have renatured. In order to be able to remove the cellular DNA used to drive the renaturation, it was necessary to add a density label to the cDNA. This was achieved by replacing thymidine 5′-triphosphate (dTTP) with its analog bromodeoxyuridine 5′-triphosphate (BrdUTP). As is the case for other DNA polymerases, reverse transcriptase incorporates BrdUTP equally well as it does dTTP. A large batch of cDNA radiolabeled with [^{3}H]dCTP and density labeled with BrdUTP was renatured in the presence of an excess of sheared L-cell DNA to a C_0t of 70 *M* sec under standard conditions. The single- and double-stranded DNA was isolated by hydroxylapatite chromatography. To separate the cDNA from the cellular DNA the single-stranded and double-stranded DNA were placed on an alkaline Cs_2SO_4 gradient. Figure 3 shows that the density labeled cDNA has a mean density of 1.52 g/cm^3 whereas the cellular DNA bands at 1.41 g/cm^3. The bands are relatively broad due to the low molecular weight of the DNA. Furthermore, it is expected that the poly(A) ends of the RNAs are copied to different extents resulting in a variability in the bromodeoxyuridine content of different cDNA molecules. Nevertheless the density difference was sufficient to achieve satisfactory separation. To validate the fractionation both subfractions of density labeled cDNA were annealed with DNA. Figure 4A shows that previously renatured cDNA anneals at a lower C_0t value than does the single-stranded cDNA and is therefore enriched for repeated DNA sequences. When these same cDNA fractions were hybridized with polyadenylated RNA the renatured cDNA reacted more rapidly indicating an enrichment for cDNA molecules representative of the most abundant polyadenylated RNA (Figure 4B). Conversely, the single-stranded

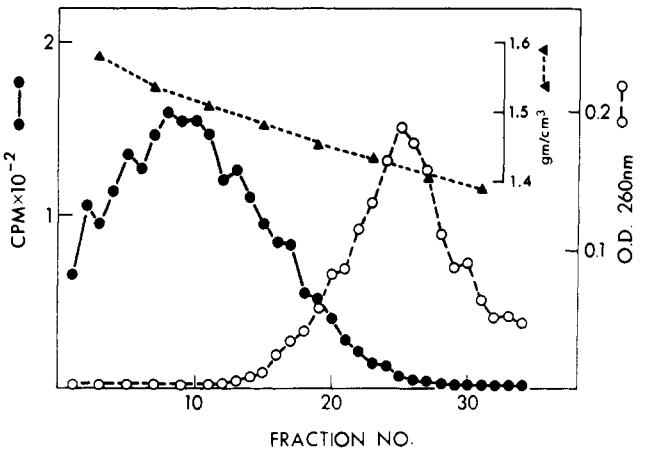

FIGURE 3: Separation of cDNA labeled with BrdUTP from L-cell DNA on an alkaline Cs_2SO_4 equilibrium gradient. cDNA (4×10^5 cpm) labeled with [^{3}H]CTP and bromodeoxyuridine triphosphate (BrdUTP) was renatured with an excess of sheared L-cell DNA to a C_0t of 70 M sec and the double-stranded and single-stranded DNA separated on hydroxylapatite. The DNA fractions were placed on alkaline Cs_2SO_4 gradient. Only the separation with the double-stranded DNA is shown; the gradient for the single-stranded fraction was essentially the same. The cDNA was assayed by counting small aliquots and the DNA by optical density at 260 nm after a three-fold dilution.

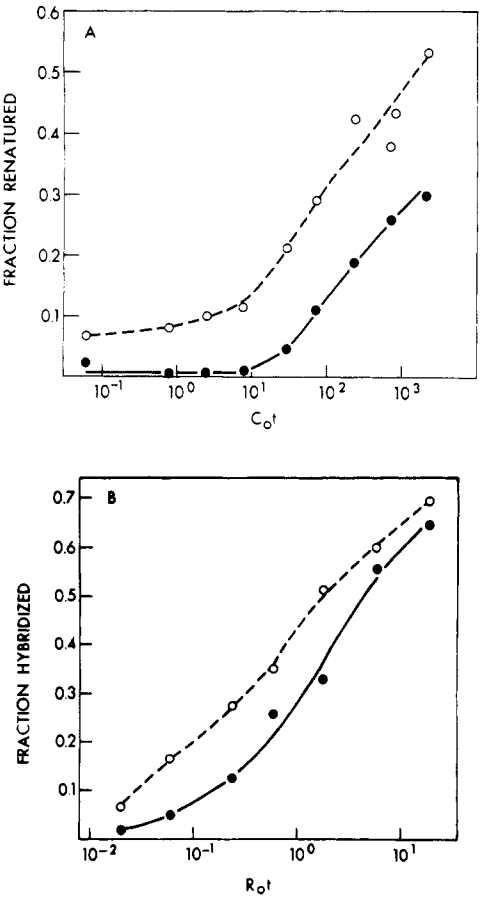

FIGURE 4: Annealing of cDNA representing repetitive DNA sequences. (A) The renatured (O- - -O) and single-stranded (●-●) cDNA recovered from the alkaline Cs_2SO_4 gradient (Figure 3, fractions 1–15) was incubated with L-cell DNA. The S1 nuclease resistant material after zero time renaturation with bacterial DNA was subtracted as background. Self-annealing of the previously renatured cDNA was 6% after 16 hr. Previously unreacted cDNA self-annealed to less than 1%. (B) Previously renatured (O- - -O) and single-stranded (●—●), cDNA were hybridized with polyadenylated RNA from L-cells.

cDNA reacted at a rate consistent with complementarity to middle and low frequency polyadenylated RNA. When cDNA enriched for repetitive sequences is used the ratio of RNA to cDNA is decreased from the usual 3000 to about 200 since these sequences are about 15-fold repetitive. This may not provide sufficient RNA excess to derive all of the repeated cDNA.

Renaturation of both density labeled cDNA fractions in the presence of bacterial DNA resulted in 6% reaction for the previously reacted cDNA after 16 hr and less than 1% for the other fraction. This is probably attributable to the presence of residual unlabeled cellular DNA not removed in the Cs_2SO_4 equilibrium gradient. The presence of this contaminating DNA made it impossible to measure the renaturation at low C_0t values.

Discussion

Our results show that about half of the cDNA synthesized with polyadenylated RNA as template renatures with repetitive DNA sequences. A number of possible interpretations may be offered. It could be argued that the cDNA renaturing to repeated DNA sequences is a transcript of the contaminating ribosomal and transfer RNA in the polyadenylated RNA preparation. This can be excluded since the cDNA hybridized with essentially the same kinetics to total RNA as to polyadenylated RNA except for a displacement by a factor of 30 to higher R_0t values. No additional early transition appeared as predicted if ribosomal or transfer RNA were copied. Any poly(A) sequences present in ribosomal and transfer RNA are probably of insufficient length to allow the oligo(dT) primer to initiate transcription. Since oligo(dT) stimulates the synthesis of cDNA by about 70-fold, less than 2% of the cDNA can represent RNA containing no poly(A).

On the same basis it is very unlikely that the cDNA representing repeated DNA represents histone mRNA (Kedes and Birnstiel, 1971; Farquhar and McCarthy, 1973), since this group of messenger RNAs is devoid of terminal poly(A) (Adesnik and Darnell, 1972). A further argument against the contribution of ribosomal, tRNA, or histone mRNA is the lack of cross-reaction of the cDNA with calf-thymus DNA. In all three cases, the base sequences are conservative and cross-reaction with other mammalian DNA is to be expected (see McCarthy and Farquhar (1972) for a review).

Having eliminated these three known classes of repeated genes as the basis for repetitive cDNA, is it reasonable to conclude that other classes of repeated genes exist which are transcribed into polyadenylated RNA? Several other possibilities should be entertained. First, is the reaction attributable to viral DNA which contaminates the L-cell nuclear DNA or is integrated in multiple copies into that genome? Although this cannot be conclusively eliminated, it is unlikely since cDNA made from mouse liver RNA showed the same renaturation kinetics when incubated with excess L-cell DNA (unpublished results). If viruses are present there is no reason to expect the same infection in liver as in L-cells or that the same number of copies are integrated into the two genomes. Nevertheless, some C type virus genomes are ubiquitous in mice and integrated in multiple copies and could contribute to the repetitive cDNA (Varmus et al., 1972).

A second possible source of repetitive transcripts is the

mitochondrial genome. Since several hundred mitochondrial genomes exist in each cell, DNA isolated from crude nuclei might contain several mitochondrial genomes which would appear to be repetitive according to renaturation kinetics. Certainly mitochondrial DNA does encode polyadenylated RNA sequences (Hirsch et al., 1974; Ojala and Attardi, 1974). If each mitochondrial RNA species is present ten times per mitochondrion, each sequence would be present more than a thousand times per cell and be included in the high or intermediate frequency class. Some preliminary tests of this possibility have been made. In two separate experiments cDNA, enriched by limited hybridization in complements for frequent polyadenylated RNA (Figure 2), renatured with excess mitochondrial DNA to 3 and 14%. Therefore, some of the rapid renaturation of cDNA may be a manifestation of mitochondrial DNA contamination, although the actual extent of this effect remains unknown. Nevertheless it appears that the lack of agreement between the results of various experiments in which polyadenylated mRNA is hybridized in DNA excess (Greenberg and Perry, 1971; Firtel and Lodish, 1973; Goldberg et al., 1973; Dina et al., 1974; Klein et al., 1974; Rabbitts et al., 1974; Spradling et al., 1974) may reflect the degree of mitochondrial DNA contamination.

A third type of explanation for the repetitive sequence content of polyadenylated mRNA should be considered. Although the rapid renaturation appears to reflect multiple copies of gene sequences it could also be explained by internal repetition of sequences within genes or sequences common to several genes. Homologies of the first type do exist for Chironomous RNA derived from Balbiani ring number 2 (Lambert, 1973). The repetitive reaction of cDNA with DNA cannot be attributed to the repeated sequence at the 5′ terminus of mRNA (Dina et al., 1974) since this end is not normally copied by the reverse transcriptase (Stavnezer et al., 1974).

Thus, the meaning of repetitive sequence content in mRNA and cDNA cannot be clearly stated at present. None of the possibilities suggested are mutually exclusive and it is likely that the observation is a result of a complex situation, reflecting other as yet unknown classes of repeated genes and contaminating genomes. Clearly many more experiments are necessary. Nevertheless, it is apparent that these repetitive sequences are preferentially represented in the most frequent class of polyadenylated RNA. If these frequent mRNAs do in fact represent cellular genes, then these may code for a few common structural proteins present in all cells. For although repetitive polyadenylated RNA may constitute as much as half of the total population, it represents only a minor fraction of the total sequence complexity of the messenger population.

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