Cloned Complementary Deoxyribonucleic Acid of *Drosophila* Cells. Relationship of Genome Copy Number to Messenger Ribonucleic Acid Abundance†

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ABSTRACT: Recombinant DNA plasmids containing DNA sequence complementary to poly(adenylic acid) [(poly(A)] containing RNA from the cytoplasm of *Drosophila* Kc tissue culture cells were constructed. The reiteration frequency in the genome of the RNA homologous to the 20 randomly selected clones was determined by two rapid methods. Of the 20, 17 were determined to be single copy, 2 were repeated several (2–4) times, and 1 was repeated approximately 10 times. The steady-state level of mRNAs homologous to the 20 cDNAs was quantitated and varied more than 160-fold. The RNAs ranged from 0.16% to less than 0.001% of the poly(A)-containing RNA.

The DNA of higher eucaryotes is composed of repetitive and nonrepetitive DNA sequences. The *Drosophila* genome contains 12% rapidly reassociating, highly reiterated DNA and 12% middle repetitive DNA with an average reiteration frequency of 70 and 70% single copy DNA (Manning et al., 1975). The analysis of cytoplasmic poly(adenylic acid) [poly(A)] containing RNA of tissue culture cells showed that some 40% of the most abundant poly(A) RNAs (R_0t = 0.2) are encoded by repeated genes; the remaining frequent cytoplasmic RNAs are derived from single copy DNA (Levy W., & McCarthy, 1975). Such information concerning the relationship between reiteration frequency of genes and the level of mRNA in the cytoplasm has resulted from experiments using fractionated cDNA derived from very complex RNA populations. Since recombinant DNA technology has allowed isolation and amplification of DNA sequences complementary to individual mRNAs, the abundance of particular RNAs and the reiteration frequency of the gene from which it is transcribed can now be analyzed directly. We have used this approach to examine 20 randomly selected cDNAs representing different RNAs expressed in tissue culture cells of *Drosophila melanogaster*.

Materials and Methods

RNA Isolation. Cytoplasmic RNA was isolated from the *Drosophila melanogaster* Kc cell line (Echalier & Ohanessian, 1970). Cells were grown in spinner culture in D22 medium at 25 °C and harvested in midlog phase [(3–5) × 10^6 cells/mL]. RNA to be used as substrate for cDNA synthesis was isolated from cells washed 2 times in 0.03 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.3, 0.1 M NaCl, and 0.01 M $MgCl_2$. After resuspension in the same buffer, NP40 was added to a 0.2% final concentration. The cells were disrupted on ice by 15 strokes of a Dounce homogenizer, and the nuclei were pelleted at 3000 rpm for 10 min in a Sorvall SS34 rotor. After a 10 000 rpm centrifugation for 10 min, the supernatant was made 10 mM in ethylenediaminetetraacetic acid (EDTA) and 0.2% in sodium dodecyl sulfate ($NaDodSO_4$) and extracted 3 times with phenol/chloroform/isoamyl alcohol (25:24:1). NaCl was added to 0.2 M, and the RNA was precipitated with 2 volumes of ethanol. The poly(A)-containing fraction was prepared by chromatography of oligo(dT)–cellulose (Collaborative Research).

RNA to be used for hybridization was isolated in a very similar manner (Biessmann et al., 1979). Two successive passages through the oligo(dT) column (Boehringer) were made and the RNA was heated at 100 °C for 3 min before the second passage. The yield of poly(A) RNA from Kc cells after two successive columns varied between 0.5% and 1.5% of the total cytoplasmic RNA.

Complementary DNA Synthesis. Poly(A)-containing RNA from Kc cells was transcribed into cDNA by using avian myeloblastosis virus reverse transcriptase (Efstratiadis et al., 1976). Two micrograms of RNA was added to a 100-μL reaction containing 1 mM dATP, dGTP, and TTP, 200 μM dCTP, 20 mM Tris-HCl, pH 8.2, 10 mM dithiothreitol (DTT), 8 mM $MgCl_2$, 20 μg/mL oligo(dT) (12–18), and 100 μCi of [α-^{32}P]dCTP (250 Ci/mmol). Prior to addition of 4 units of reverse transcriptase, the mixture was heated to 65 °C for 2 min and chilled to 0 °C. After incubation at 45 °C for 15 min, the reaction was terminated by the addition to EDTA to 10 mM and extracted with an equal volume of phenol. After chromatography over Sephadex G-75 in 100 mM, 10 mM Tris-HCl, pH 9.0, and 1 mM EDTA, the nucleic acid was precipitated with ethanol and dissolved in 20 μL of 0.1 N NaOH. A 20-min incubation at 70 °C was carried out to ensure degradation of the RNA template. The mixture was neutralized with HCl, 5 μg of *Escherichia coli* tRNA (Miles) was added as carrier, and the nucleic acid was precipitated again with ethanol.

The second strand was synthesized by using either reverse transcriptase (Kc clones) or DNA polymerase I (R1 and *Bam*HI clones). The reverse transcriptase reaction was performed essentially as described above except that 300 μCi of [^{3}H]dCTP (25 Ci/mmol) was added, and the reaction was carried out for 2 h at 37 °C. The polymerase I reaction was carried out in 0.12 M potassium phosphate, pH 6.9, 10 mM $MgCl_2$, 10 mM DTT, and 500 μM dXTP and included 100 μCi of [^{3}H]dCTP. In this case, 15–30 units of enzyme was added; incubation was carried out for 6 h at 15 °C. In either case, the reaction was terminated by addition of EDTA to 10

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0006-2960/81/0420-2651$01.25/0

mM and was made 50 mM in Tris-HCl, pH 9.0, extracted with phenol, and chromatographed over Sephadex G-75. The average size of the double-stranded (ds) cDNAs after purification was 500–600 base pairs (bp).

Blunt ends of cDNA were created in either of two ways. The cDNA was treated with S1 nuclease (Sigma) (pKc clones) or *Hae*III restriction endonuclease (pRI and p*Bam*HI clones). A total of 200 units of S1 was used to digest 10 μg of carrier tRNA and cDNA for 1 h at 25 °C in 0.3 M NaCl, 0.05 M NaOAc, pH 4.5, and 1 mM $ZnCl_2$. After digestion, the pH was raised to 8.5 with Tris base, 5 μg of carrier RNA was added, and the mixture was extracted with phenol and chromatographed over Sephadex G-75.

Either *Hin*dIII or *Bam*HI decamers or RI octamers (Collaborative Research) were blunt end ligated to the double-stranded cDNA. The linkers were end labeled by using [γ-^{32}P]ATP and T4 nucleotide kinase prior to ligation. The ligation reaction which included 66 mM Tris-HCl, pH 7.6, 10 mM DTT, 6.6 mM $MgCl_2$, 1 mM ATP, 100 pmol of linkers, and approximately 250 ng of cDNA was incubated for 2 h at 15 °C in the presence of 2 units of T4 DNA ligase. The ligation mixture was then treated with the appropriate restriction enzyme to cleave the linkers and produce staggered ends. The cDNA was separated from linkers either by chromatography on Sephadex G-100 or by electrophoresis on a 6% polyacrylamide gel. The purified cDNA was then ligated to the vector pBR322 and used to transform *E. coli* as previously described (Craig et al., 1979).

In Vitro Labeling of RNA and DNA. Poly(A) RNA was labeled by using polynucleotide kinase after partial alkaline hydrolysis as previously described (Biessmann et al., 1979). DNA was labeled in vitro by the nick translation reaction as described by Maniatis et al. (1975), except that 1 ng of DNase I was added 2 min prior to the addition of polymerase I. Insert DNA which was to be radiolabeled was separated from the parent plasmid by electrophoresis in a 5% polyacrylamide gel, electroeluted from the gel, and concentrated by ethanol precipitation.

Filter Hybridization. Kc cell or embryonic Oregon R DNA cleaved with restriction endonucleases was loaded onto 2 mm thick 1% agarose gels and electrophoresed in Tris–acetate buffer (40 mM Tris-HCl, pH 8.2, 20 mM sodium acetate, 20 mM NaCl, and 2 mM EDTA) at 40 mA. The DNA was stained with ethidium bromide (0.5 μg/mL) and photographed with Polaroid film by using ultraviolet illumination. Transfer of DNA from the gels to nitrocellulose was performed according to the method of Southern (1975). After being heated to 80 °C for 2 h, the filters were preincubated for 4 h according to Denhardt (1966). Hybridization was carried out in 50% formamide, 5 × SSC containing 0.1% $NaDodSO_4$, 1 mM EDTA, and 10 mM Hepes, pH 6.9, and 1 × Denhardt's solution at 37 °C for 20 h. After incubation, the filters were washed 3 times in 5 × SSC containing 0.2% $NaDodSO_4$ at 65 °C for 45 min, followed by 2 h at room temperature in 2 × SSC. Kodak Royal X-Omat film and Lightning Plus Screens amplifying screens (Du Pont) were used. Signals were quantitated by scanning and integrating band intensity as described previously (Biessmann et al., 1979).

Liquid Hybridizations. Denatured nick-translated *Drosophila* cDNA inserts were reassociated in the presence of calf thymus DNA or *Drosophila* DNA. Hybridizations were carried out at 70 °C in 0.3 M NaCl, 0.01 M Tris-HCl, pH 7.5, and 0.001 M EDTA in 200-μL reactions. Reassociation was measured by using S1 nuclease as described previously (Craig et al., 1975). The method of calculation was essentially as described by Sharp et al. (1974). The specific activity of the insert was determined from the rate of self-reassociation of the labeled fragment.

Isolation of Mitochondrial DNA. Mitochondrial DNA was isolated from Kc cells as follows. Cells were collected by centrifugation and washed once in phosphate-buffered saline. The pellet was resuspended in 4 volumes of 10 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 0.15 mM $CaCl_2$, and the cells were disrupted in a Dounce homogenizer. Sucrose was added to 0.25 M, and the nuclei were removed by several centrifugations for 10 min each at 3000 rpm. Mitochondria were collected from the supernatant by centrifugation at 12 000 rpm for 30 min and then further purified by centrifugation in a sucrose step gradient (1.1–1.5 M) as described by Bultmann & Laird (1973). Mitochondria were removed from the interphase, collected by centrifugation as above, and lysed by adding an equal volume of 2% $NaDodSO_4$, 10 mM Tris, pH 7.4, and 1 mM EDTA for 5 min at 37 °C. The lysate was made 1 M in CsCl and chilled to 0 °C, and the precipitated $NaDodSO_4$ was removed with 12 000 rpm for 10 min. CsCl was added to 1.57 g/mL and ethidium bromide to 0.6 mg/mL, and the solution was centrifuged at 40 000 rpm for 36 h in a 50Ti rotor at 18 °C. Mitochondrial DNA was removed from the gradient with a syringe and further purified by velocity gradient centrifugation. Samples were diluted to 1.36 g/mL CsCl and spun through 3 mL of 1.4 g/mL CsCl into a 0.8-mL cushion of 1.7 g/mL CsCl in a SW50.1 rotor at 4.5 h at 38 000 rpm. Finally, mitochondrial DNA was again collected by CsCl equilibrium centrifugation as described above, and the ethidium bromide was removed by extractions with 2-propranol.

Results

Construction and Identification of Recombinant Plasmids. Cytoplasmic poly(A)-containing RNA, isolated from the cytoplasm of Kc cells as described under Materials and Methods, was used as a template to construct recombinant plasmids. The initial set of cDNA-containing plasmids (designated pKc plasmids) was constructed by using S1 nuclease to create flush ends of the cDNAs which were inserted into the vector pBR322 by using *Hin*dIII linkers. Those clones containing inserts larger than 160 bp as judged by analysis of restriction enzyme digests were selected for further screening.

Since our goal was to study the relationship between nuclear DNA repetition and mRNA concentration, we designed experiments to allow elimination of clones of mitochondrial origin. Mitochondrial DNA was isolated from the cytoplasm of Kc cells. DNAs isolated from whole cells and mitochondria were cleaved with restriction endonucleases *Eco*RI, *Hin*dIII, and *HaeIII*, electrophorsed on 1% agarose gels, and transferred to nitrocellulose filters. Hybridization with ^{32}P nick translated probes synthesized from plasmids was used to determine the molecular weight distribution of homologous nuclear or mitochondrial fragments.

In an initial colony screen (Grunstein & Hogness, 1975), approximately 25% (10 out of 42) of the clones tested hybridized very strongly to cytoplasmic poly(A+) RNA. When seven of these recombinant plasmids representing very frequent cytoplasmic RNA were hybridized to fragmented nuclear and mitochondrial DNA, they showed identical patterns of hybridization (Figure 1). All seven clones hybridized to a 0.45 × 10^6 dalton *Eco*RI fragment of mtDNA which is internal to the coding region of the large mt ribosomal RNA as determined by Klukas & Dawid (1976).

When 17 clones picked at random were analyzed by Southern blot hybridization, 4 were identified as of mitochondrial ribosomal origin; 3 were of mitochondrial origin but

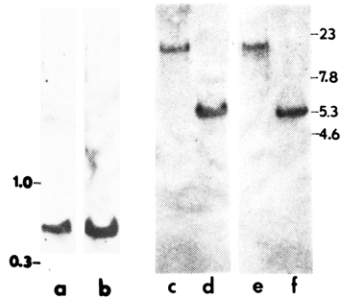

FIGURE 1: Hybridization of a cDNA clone (pKc9) to total cellular DNA and purified mitochondrial DNA. 5 μg of total cellular *Drosophila* or 5 ng of mitochondrial DNA was cleaved with *Eco*RI, *Pst*I, or *Hae*III, electrophoresed on a 1% agarose gel, and transferred to nitrocellulose filters. The recombinant plasmid pKc9 was nick translated and hybridized (3×10^6 cpm) to the DNA bound to the filters. (a) *Eco*RI-cleaved total DNA; (b) *Eco*RI-cleaved mitochondrial DNA; (c) *Pst*I-cleaved total DNA; (d) *Hae*III-cleaved total DNA; (e) *Pst*I-cleaved mitochondrial DNA; (f) *Hae*III-cleaved mitochondrial DNA. The numbers indicate the size in kilobases and position of selected *Hin*dIII-cleaved Ad2 and *Eco*RI-cleaved λ DNA fragments.

were not homologous to the large ribosomal gene, and 10 were of nuclear origin. The high content of mitochondrial ribosomal RNA in our cytoplasmic poly(A) RNA preparation was also demonstrated by electrophoresis on glyoxal–agarose gels (data not shown) and has also been recently discussed by Izquierdo & Bishop (1979).

Drosophila mtDNA is cleaved by *Hae*III into three fragments of 8.5, 5.5, and 3.5 kb (Klukas & Dawid, 1976) whereas the average size of chromosome DNA cleaved with *Hae*III is approximately 300 bp. Since the average size of our ds cDNA molecules was 500–600 bp, cleavage with *Hae*III would strongly select for complements of nuclear coded RNA molecules, and only very few of the resulting clones should be of mitochondrial origin. Using this procedure, we constructed two other sets of cDNA clones (designated pRI or p*Bam*HI) and reduced the proportion of mt clones to about 1 in 12.

Analysis of Repetition Frequency. Twenty clones, 7 of pKc, 2 of p*Bam*HI, and 11 of pRI type of nuclear origin, were chosen for determination of repetition frequency in the genome. *Drosophila* DNA isolated from Kc cells or from Oregon R embryos was cleaved with different restriction endonucleases, *Eco*RI, *Hin*dIII, *Pst*I, *Bam*HI, or *Hae*III, electrophoresed on a 1% agarose gel, and transferred to a nitrocellulose filter. Hybridization with ^{32}P nick translated probes synthesized from the 20 plasmids was used to determine the molecular weight distribution of homologous genome fragments (Table I). No two plasmids gave the same pattern of hybridization, indicating that representatives of 20 different genes had been selected for study. Seventeen of the 20 clones produced a simple hybridization pattern; one band of hybridization was observed in each enzyme digest. The hybridization pattern of one, pKc24, was quite complicated, giving 8–9 bands with each restriction enzyme. The relative intensities and size of the bands were identical with those of Kc or embryonic DNA, indicating no detectable DNA rearrangement between tissue culture cells and embryos. Two, pKc16 and pKc43, gave a somewhat less complicated pattern. pKc16 showed a single band with *Eco*RI and *Hae*III and two bands with *Hin*dIII and *Pst*I. pKc43 showed from two to four bands of differing intensities with several enzymes. This may be due to restriction site polymorphism or cross-reacting nonidentical sequences in the genome.

The number of bands observable in each case gave a suggestion of the copy number of the genes in the haploid genome.

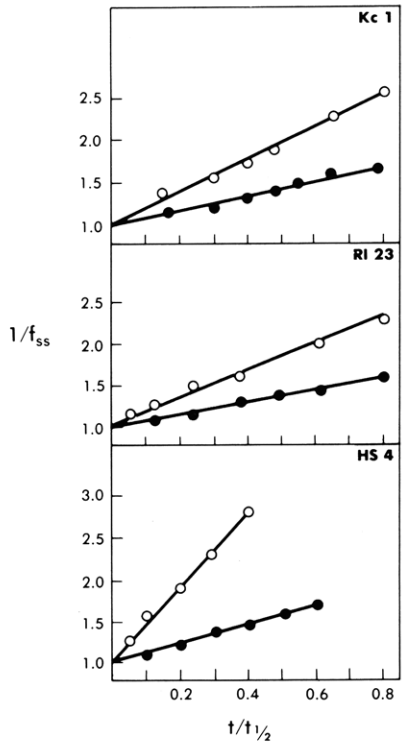

FIGURE 2: Kinetics of reassociation of ^3H-labeled Kc1, RI23, and HS4 inserts in the presence of unlabeled DNAs from calf thymus and *Drosophila* Kc cells. Each reaction contained 2 mg of DNA/mL of either calf thymus (●) or *Drosophila* DNA (○). The hybridizations were carried out as described under Materials and Methods for times ranging from 0.5 to 5 h. The specific activity of the cDNA inserts was 5×10^6 cpm/μg (Kc1), 4.5×10^6 cpm/μg (RI23), and 2.4×10^6 cpm/μg (HS4) as determined by the rate of self-reaction. The hybridization reactions contained 1200 (HS4), 1300 (RI23), and 1150 cpm (Kc1). Samples were removed from the incubation and diluted in S1 buffer, and the fraction of single-stranded DNA was determined by digestion with S1 nuclease.

Those producing multiple bands of hybridization were candidates for repeated genes. A single band indicated either a single copy gene or tandemly repeated genes. In an attempt to distinguish between these two possibilities, DNA reassociation experiments were performed to estimate the number of gene copies present in the haploid genome. The cDNA inserts of plasmids pRI23 and pKc1 were isolated and labeled with ^3H by nick translation. Denatured ^3H-labeled fragments were then reassociated in the presence and absence of *Drosophila* DNA, and the initial rate of reassociation was measured (Figure 2); data were corrected to account for the specific activity as well as the size of the DNA fragment (see legend to Figure 2 and Materials and Methods). The *Drosophila* DNA increased the rate of reassociation by the factor expected if the genes were repeated approximately once per haploid genome.

Since performing DNA/DNA reassociation kinetics on each cDNA is very time consuming, we used two simpler methods to determine reiteration frequencies. The reconstruction method (Lis et al., 1978) involves hybridization analysis with fragmented DNA electrophoresed on an agarose gel as described above but in which successive lanes contained increasing concentrations of plasmid DNA. The plasmid was cleaved with a restriction endonuclease to produce a single linear fragment; included in four adjacent lanes was sufficient DNA to be equivalent to 1, 5, 10, and 20 copies of the sequence per haploid genome. Isolated cDNA inserts were ^{32}P labeled by nick translation and used as probes. Figure 3 shows the increasing hybridization signal with increasing amounts of

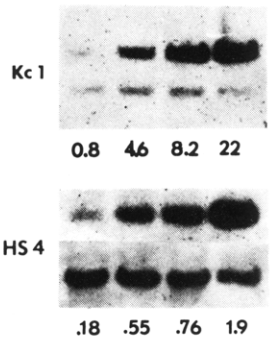

FIGURE 3: Hybridization of pKc1 DNA to mixtures of *Drosophila* DNA and increasing amounts of pKc1 DNA. *Drosophila* DNA, 3 μg, was digested with *Pst*I and mixed with 0.07, 0.35, 0.7, or 1.4 ng of *Pst*I-digested pKc1 DNA, equivalent to 1, 5, 10, and 20 copies of the cDNA segment per haploid genome. The mixtures were electrophoresed in a 1% agarose gel, transferred to nitrocellulose, and hybridized with nick-translated pKc1 frament (1.5×10^6 cpm). The autoradiographs of the hybridized filters were scanned. The numbers below the autoradiographs are the ratio of the intensity of the bands. In each case, the upper bands result from hybridization to added plasmid DNA and the lower to *Drosophila* genome DNA. A similar experiment was carried out by using HS4 DNA using 0.07, 0.21, 0.35, or 0.7 ng of cDNA segment, equivalent to 1, 3, 5, and 10 copies per haploid genome.

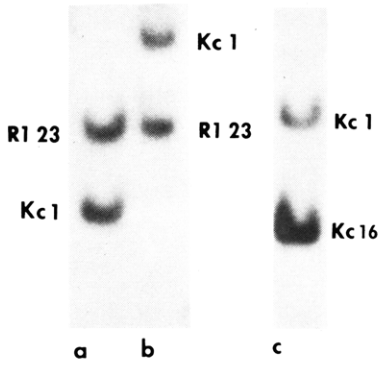

FIGURE 4: Simultaneous hybridization of pKc1 and pRI23 to *Drosophila* DNA. *Drosophila* DNA, 3 μg, was digested with *Bam*HI (a) or *Eco*RI (b), electrophoresed in agarose, and transferred to nitrocellulose. pKc1 and pRI23, 100 ng each, were mixed and labeled by nick translation; 4×10^6 cpm of the mixed probe was hybridized to the filter. In a similar experiment (c), a mixed pKc1 and pKc16 DNA probe was hybridized to *Pst*I-digested DNA. After exposure with an intensifying screen, the autoradiographs were scanned, and the areas under the peaks were determined. The ratio of intensity of bands was (a) RI23/Kc1, 1:1.07; (b) Kc1/RI23, 1:1.1; (c) Kc1/Kc16, 1:3.1.

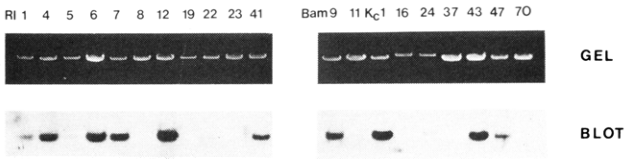

FIGURE 5: Hybridization of poly(A)-containing RNA to cDNA recombinant plasmids. The 20 plasmids were linearized with a restriction enzyne chosen because it was not used to insert the cDNA fragment and did not cut within the *Drosophila* cDNA fragment. Quantities of linearized plasmid, each equivalent to approximately 20 ng of *Drosophila* DNA insert, were electrophoresed in 1% agarose and transferred to nitrocellulose. In vitro ^{32}P-labeled poly(A) RNAs (1×10^8 cpm) were hybridized in a total volume of 5 mL. The filter was exposed for 5 h with Quanta 2 screen at −70 °C. The autoradiograph was scanned, and the areas under the peaks were integrated. For calculation of the amounts of each RNA hybridized to the cDNA clones, pKc1 = 0.11%, pKc43 = 0.05%, and pKc37 = 0.02% of cytoplasmic poly(A) RNA were used as standards (Biessmann et al., 1979).

plasmid DNA; quantitation obtained by scanning the autoradiograph established a direct correlation between the amount of DNA added and the amount of hybridization. Hybridization to the genome fragment homologous to pKc1 was the same in each lane. Scanning of the autoradiographs and comparison of the two signals obtained in each lane indicated that the gene homologous to pKc1 is repeated approximately once per haploid genome, a result in agreement with that obtained by DNA/DNA reassociation in solution.

A similar comparison was carried out by using a cDNA clone (HS4) which contains a portion of the gene for the 70K heat shock protein (Craig et al., 1979). Since a DNA/DNA reassociation experiment indicated that this gene is repeated approximately 7 times per haploid genome (see Figure 2), we asked whether this larger copy number is detectable by the method described above. Scanning of the autoradiograph indicates a repetition frequency of approximately six. The same type of quantitation experiment was performed by using eight additional cDNA plasmids all of which produced a single band of hybridization on a gel. All eight results gave a reiteration frequency of once per haploid genome.

Another approach which eliminates the necessity to isolate cDNA inserts also gave similar results. *Drosophila* genome DNA was cleaved with various restriction enzymes and transferred to nitrocellulose as described above. The probe used in the hybridization was a mixture of labeled pKcI and the plasmid of interest. Equal amounts of the two plasmids were added to the nick-translation reaction. Although the inserts are slightly different in size, the overall sizes of the two plasmids are very similar. Since the size of the restriction fragments for each cDNA is already known, the hybridization band can be assigned to the corresponding plasmid. An example of this approach is seen in Figure 4. Total DNA digested with *Eco*RI or *Bam*HI and electrophoresed on agarose gel was transferred to nitrocellulose. A mixed Kc1 and RI23 probe was hybridized to the filter. A scan of the autoradiograph indicates that Kc1 and RI23 bands are of equal intensity. We therefore conclude that their repetition frequency in the genome is the same. The repetition frequency of both of these sequences had also been determined by C_0t reduction (Figure 2) to be once per haploid genome. This approach was then used to estimate the genome reiteration frequency of the remaining recombinant plasmids (see Table I). In all cases, we assumed that the two plasmids became equally labeled. All of the plasmids which gave a single band with each of four enzymes were estimated to have a repetition frequency of one. Kc16, which gave two bands with two enzymes and one band with two other enzymes, was estimated to have a repetition frequency of three since the intensity of hybridization to the Kc16 fragment was 3 times that of the pKc1 band when cleaved with *Eco*RI (Figure 4c). Kc43 was the only plasmid which gave equivocal results. We estimate the repetition frequency to be between two and four.

RNA Frequency in the Cytoplasm of Kc Cells. The relative frequency of the RNAs represented by the 20 cDNA clones in the cytoplasm of log phase Kc cells was determined. Recently, we published a rapid method of quantitation of the steady-state concentrations of individual RNAs in a complex population (Biessmann et al., 1979). This procedure involves the electrophoresis of linearized plasmid in an agarose gel and transfer to nitrocellulose followed by hybridization with in vitro labeled poly(A)-containing RNA. The intensity of the hybridization signal detected by autoradiography is proportional to the relative abundance of the RNAs, assuming uniform RNA labeling (Figure 5). The abundance of three mRNAs,

Table I: Characterization of 20 Recombinant Plasmids with Respect to Size of *Drosophila* cDNA Insert, Complementary Genome Fragment Sizes, Copy Number, and Frequency of Complementary RNA in the Cytoplasmic Poly(A) Population of Kc Cells

plasmid	insert size (bp)[a]	genome fragment size (kb)[b]					no. of copies per haploid genome[c]	% poly(A) cytoplasmic RNA[d,e]
		*Eco*RI	*Hin*dIII	*Hae*III	*Pst*I	*Bam*HI		
Kc1	250	4.7	0.33	1.5	13		1	0.11
Kc16	490	2.7	1.1	0.53	5.8		3	<0.005
			0.71		0.33			
Kc24	600	15	30	5.6	18.3		10	<0.005
		9.3	13	3.3	13.6			
		7.3	10	3.1	11.0			
		5.1	8	2.0	9			
		4.7	6	1.6	6.3			
		4.2	5.3	1.3	5.6			
		1.6	4.5	0.63	4.5			
		1.0	3.5	0.46	3.3			
			2		2.7			
Kc37	160		13	1.7	6.7		1	nd
Kc43	190	6.6	13	1.5	3.7		2–4	0.05
		5.0	11.5	1.1	2.0			
		3.8						
		2.5						
Kc47	260	8.8	0.47	0.7	21		1	0.02
Kc70	220	2	0.67	0.78	7.7		1	nd
RI1	260	1.4			13	1.2	1	0.01
RI4	245	9.3	5.5		15	17	1	0.05
RI5	280	5	4		2.8	10	1	<0.005
RI6	200	3.3	2.5	1.3		5	1	0.09
RI7	255	4.5	2.5		3.2	11	1	0.07
RI8	200	13	7		17.5	3	1	nd
RI12	210	4.7	11.6		3.8	4.3	1	0.16
RI19	300			0.9	5	5.6	1	nd
RI22	245	2.5	1.3		4	10	1	<0.005
RI23	210	2	1.8		1.7	2.2	1	<0.005
RI41	255	2.6		1.3	5.0	2.3	1	0.03
*Bam*9	230	5.1		0.8	4.5	6.3	1	0.03
*Bam*11	200	6			1.1	20	1	<0.005

[a] Size of the insert was determined after electrophoresis in acrylamide gels of plasmid cleaved with the restriction endonuclease specific for the linkers used to insert the cDNA into the vector. The migration of fragments were compared to that of *Hae*III-cleaved pBR322 and *Hin*cII-cleaved ϕX174. [b] Fragment sizes were determined by hybridization of nick-translated plasmid DNA to *Drosophila* DNA cleaved by restriction endonucleases and fractionated on agarose gels. The migration of hybridizable fragments was compared to that of *Bam*HI- and *Hin*dIII-cleaved adenovirus 2 DNA and *Eco*RI-cleaved λ DNA. [c] Reiteration frequency was determined by (1) C_0t reduction experiments, (2) hybridization relative to known amounts of added plasmid DNA, or (3) hybridization relative to a gene of known copy number, all as described in the text. [d] Three cDNAs were used as standards, pKc1, pKc43, and pKc47. Previously, the amount of these RNAs has been quantitated and determined to represent 0.11%, 0.05%, and 0.02% of the poly(A) cytoplasmic RNA in Kc cells, respectively (Biessmann et al., 1979). nd, not detectable. [e] Measurements of RNA content are not available for Kc cells. However, an approximate estimate of the number of copies of each mRNA may be made if it is assumed that the RNA content of *Drosophila* cells is about 10 times the DNA content (Church & Robertson, 1966; Turner & Laird, 1973), i.e., about 3 pg/cell for Kc cells. With the assumption that poly(adenylated) messenger RNA is 1% of the total RNA and taking 400 000 as an average molecular weight (Levy W. & McCarthy, 1975), the total number of messenger RNA molecules in each Kc cells is about 5×10^4. Thus, the most frequent species of RNA referred to in the table are present in approximately 80 copies per cell and the least abundant at about 2.5 copies per cell or less.

represented by pKc1, pKc43, and pKc47, was shown to be 0.11%, 0.05%, and 0.02%, respectively. When these were used as standards, the abundance of the other mRNAs in Kc cells was calculated (Table I). The most abundant RNA, represented by pRI12, accounts for 0.16% of the poly(A)-containing RNA. Hybridization to six clones resulted in a band which was observable but could not be accurately scanned; these cDNAs represented RNAs which accounted for between 0.001% and 0.005% of the poly(A) RNA. Hybridization of RNA to four other clones was undetectable even after long exposures; these clones must represent less than 0.001% of the poly(A) RNA, the limit of detectability of this method.

Discussion

Earlier studies of poly(adenylated) messenger RNA in cultured *Drosophila* cells suggested that a few species were highly abundant (Levy & McCarthy, 1975). However, it is now apparent that mitochondrial transcripts account for many of the very abundant cytoplasmic RNAs. The larger mitochondrial ribosomal RNA contains an internal A-rich tract which causes it to contaminate 3′ poly(adenylated) mRNA isolated by oligo(dT)–cellulose chromatography (Izquierdo & Bishop 1979). This RNA is presumably derived from broken mitochondria. Since this RNA also serves as an efficient template for reverse transcriptase, cDNA with the corresponding complementary sequence is also a major component in the population. In the present study with cloned cDNA, clones containing sequences complementary to 13S mitochondrial ribosomal RNA were by far the most common species isolated. Our initial collection of cDNA clones synthesized from flush ends of cDNA ligated to *Hin*dIII linkers also contained a large number of clones complementary to mitochondrial messenger RNA. Whether all of the most abundant class of cytoplasmic poly(adenylated) RNA is attributable to the mitochondrial genome remains to be seen.

The 20 cloned cDNAs studied in detail contained sequences of nuclear origin. These cDNAs represented RNAs present in very different amounts, varying from 0.16% to less than 0.001% of the poly(adenylated) cytoplasmic RNA. The distribution of abundance does not fall into sharp frequency classes. Numerous studies of messenger RNA abundance in a variety of eucaryotic cells have been published, and, in most

cases, the distribution obtained can be approximated by a three-abundance class model (Bishop et al., 1974; Levy W., & McCarthy, 1975; Getz et al., 1976; Ryffel & McCarthy, 1975). However, several authors have questioned the biological reality of such a model, pointing out that a continuum of concentrations is the limiting case (Hereford & Rosbash, 1977; Quinlan et al., 1978). Although our sample of 20 cloned cDNAs is small, the present data add no support to the discrete abundance class model. A similar study, limited in this case to nine cloned cDNAs from Chinese hamster ovary cells, also failed to provide evidence for quantized abundance of mRNA species (Harpold et al., 1979).

Several previous studies have demonstrated that some poly(adenylated) cytoplasmic RNAs are encoded by repetitive DNA (Bishop et al., 1974; Spradling et al., 1974). Some of the most abundant RNA species appear to be encoded by repetitive DNA (Ryffel & McCarthy, 1975; Levy & McCarthy, 1975). In the light of present results, some of the data for *Drosophila* cells, at least, must be reevaluated since mitochondrial DNA contaminating nuclear DNA could act operationally as repetitive DNA in hybridizing with cDNA of mitochondrial origin. Nevertheless, in the collection of 20 cDNA clones examined here, three proved to be complementary to multicopy DNA. Two were repeated several times, i.e., 2–4 times; one was homologous to a fairly abundant poly(A) RNA, 0.05%, and the other to a rare species accounting for less than 0.001%. The third clone was moderately repeated, i.e., ten or so copies, but accounted for only 0.001% of the cytoplasmic RNA in Kc cells. Although none of these three plasmids contained sequences complementary to the most abundant RNA, there is no doubt that repetitive DNA sequences encoding abundant mRNA do exist. Among these are the transposable elements such as copia (Finnegan et al., 1977). In addition, there are six different actin genes in *Drosophila*, the DNA sequences of which are sufficiently similar to cross-react with the different actin messenger RNAs (Tobin et al., 1980; Fyrberg et al., 1980). A similar situation exists for other prevalent messengers encoding α- and β-tubulins (Sanchez et al., 1980).

Concentrations of RNA complementary to individual cDNA plasmids were estimated by a rapid gel blotting hybridization procedure (Biessmann et al., 1979). The same general principles have now been applied to measurement of the copy number of each cDNA sequence in the nuclear genome. Two variants of this approach were applied; both gave reliable estimates comparable to those made by a more laborious DNA annealing in solution method. In one, the signal obtained in a DNA blot with a ^{32}P-labeled cDNA insert is compared with that obtained with that of a second plasmid known to be single copy used as internal standard. Alternatively, since the *Drosophila* genome size and the size of the plasmid insert are known, signals obtained with a ^{32}P probe can be compared in parallel lanes containing either genome DNA or amounts of the same plasmid DNA equivalent to one or many copies per haploid genome (Lis et al., 1978). Longacre & Mach (1978) quantitated hemoglobin genes in a similar manner by mixing known amounts of plasmid with genome DNA. Again, comparison of signal intensity gives a reliable estimate of copy number. Because of their precision and convenience, these various approaches should be generally applicable to measurements of copy number.

Acknowledgments

We thank Frank Küttner for expert technical assistance.

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