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Rapid quantitation of individual RNA species in a complex population

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ABSTRACT

Many investigations require quantitation of one or more individual RNA species in complex populations. Existing methods are tedious when multiple samples are to be assayed. A method is presented which allows rapid and accurate quantitation of many species of RNA simultaneously. Recombinant plasmids containing cDNA inserts are electrophoresed in agarose and blotted to nitrocellulose. After hybridization with labeled RNA and autoradiography, bands are quantitated by scanning. The results were calibrated by solution hybridization. The approach has been validated through the use of plasmids containing inserts of Drosophila cDNA and RNA of cultured cells.

INTRODUCTION

Quantitation of individual species of RNA present in a heterogeneous population is usually accomplished by following the kinetics of RNA excess/cDNA hybridization reactions in solution (1,2) or by titration of cDNA with RNA (3). Such assays are precise but laborious especially if multiple samples are involved. With the advent of recombinant DNA methodology, it has become possible to conduct more rapid assays using colony hybridization (4) where each colony harbors a plasmid with a different DNA insert or plaque hybridization (5) where each plaque contains phage with a different insert to detect the presence of different RNAs. A variant on this theme employs an array of spots on a filter containing purified DNA from recombinant plasmids (6). While these techniques are valuable for comparing the extent to which transcripts of the various DNA segments are represented in the population and for identifying clones containing DNA sequences which are developmentally regulated (4), they do not yield accurate estimates of the absolute concentration of given RNA sequences in the population. In principle, quantitation is also possible using labeled RNA with cDNA in excess either in solution or linked to a solid phase (7,8). In

this case it is also necessary to determine the efficiency of hybridization, preferably with the aid of differentially labeled RNA as internal standard. In the succeeding pages we present a method which is also convenient for screening multiple species of RNA and comparing their abundance in different preparations and yields estimates of their absolute concentration.

MATERIALS AND METHODS

Enzymes

Restriction endonucleases (EcoRI, BamHI, PstI and HindIII) and DNA polymerase I and polynucleotide kinase were purchased from Boehringer, Mannheim. S1 nuclease was obtained from Miles. Bacterial alkaline phosphatase (BAPF) and DNAse I were purchased from Worthington.

Recombinant Plasmids

Double stranded cDNAs were synthesized from Kc cell polyA RNA and inserted with synthetic HindIII linkers into the HindIII site of pBR322. This procedure will be described in detail elsewhere. From this population of recombinant plasmids containing a short Drosophila melanogaster cDNA insert several were selected which gave a sufficient hybridization signal with polyA containing cytoplasmic RNA from Kc cells in a colony hybridization test (9). Plasmids used in these experiments were pKc1 (250 bp insert), pKC43 (190 bp insert) and pKC47 (260 bp insert), all cytoplasmic polyA RNA derived from the nuclear genome and pKc9 (250 bp insert) which includes a sequence complementary to the large mitochondrial ribosomal RNA. The plasmids were grown and amplified in E. coli RRI. Purification was accomplished using two successive CsCl-ethidium bromide equilibrium gradients. Plasmids were linearized with restriction enzymes which did not cut within the Drosophila insert (pKc1 and pKc9 with PstI, pKc43 and pKc47 with BamHI). To prepare the insert for nick-translation, the plasmids were cleaved with HindIII, and the cDNA insert purified by preparative acrylamide gel electrophoresis. DNA was recovered from the gel by electrophoresis and precipitated with ethanol.

Gel Electrophoresis and Transfer to Nitrocellulose Filters

Linearized plasmids were loaded onto 2mm thick 1% agarose gels and electrophoresed in Tris-acetate buffer (40mM Tris, pH 8.2, 20mM sodium acetate, 20mM NaCl, 2mM EDTA) at 40 mA for 2 hr. The gels were stained

with ethidium bromide and photographed under UV light with a Polaroid camera. Transfer of DNA from the gels to nitrocellulose filters (Schleicher & Schuell, BA85, Millipore SM 11306 or Sartorius Type SM 11306) was accomplished according to the method described by Southern (10).

Preparation of RNA

Cytoplasmic RNA was isolated from the Drosophila melanogaster Kc cell line (11). Cells were grown in spinner culture in D22 medium at a constant temperature of 25°C and harvested in mid-log phase (3-5x10^6 cells/ml). For a typical preparation 2-3 liters of cells were collected by centrifugation and washed once in 10mM Tris, pH 8.0, 10mM NaCl, 3mM $MgCl_2$. After suspending in 50 ml of the same buffer, dithiothreitol was added to 5mM and NP40 to 0.2% final concentration. The cells were disrupted on ice by 15 strokes in 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 10mM in EDTA and 0.5% in SDS and extracted once with phenol/chloroform (1:1) and twice with chloroform/isoamylalcohol (24:1). Potassium-acetate was added to 0.2M and the RNA precipitated with two volumes of ethanol at -20°C overnight. The polyA containing fraction was prepared by chromatography on oligodT-cellulose T-1 (Boehringer, Mannheim). Two successive passes through the column were made, the RNA was heated to 100°C for 3 min before the second passage. The yield of polyA RNA from Kc cells after two successive oligodT-cellulose columns was 0.5-1.5% of the total cytoplasmic RNA.

In Vitro Labeling of RNA and DNA

PolyA RNA was labeled using polynucleotide kinase; 10 µg of RNA were partially hydrolyzed by incubation in 80 ml of 50mM Tris, pH 9.5 at 90°C for 70 min (12,13). The RNA was then incubated in a final volume of 150 µl containing 10mM $MgCl_2$, 5mM DTT (50 µCi of ^{32}P-γ-ATP >3000 Ci/mmol, Amersham or NEN) and 3 units of T4 kinase for 50 min at 37°C (13). The reaction was terminated by the addition of 100 µl of 4M ammonium acetate, E. coli carrier RNA was added and the mixture was extracted once with phenol and twice with chloroform/isoamyl alcohol. The RNA fragments were precipitated with 2 volumes ethanol, after resuspension in H_2O, 1/10 volume of 2.5M LiCl and 0.5M $MgCl_2$ was added and the RNA precipitated by the addition of 3 volumes of ethanol for 1 hr at -70°C.

Linearized plasmid DNA was end-labeled using polynucleotide kinase

by the procedure described by Maxam and Gilbert (14) with minor modifications. The DNA was not denatured prior to labeling and spermidine was not included in the reaction mixture.

For preparation of probes in excess RNA hybridization experiments in solution, purified Drosophila cDNA inserts were nick-translated (15) using 50 µCi each of ^{3}H-dCTP (22 Ci/mmol) and ^{3}H-dTTP (105 Ci/mmol) (NEN). After the reaction the solution was extracted with phenol/chloroform and chromatographed on a Sephadex G-50 medium column (Econo-column 15 cm, BioRad). Radioactivity in the excluded volume was pooled and ethanol precipitated. The S1 resistant background of these probes averaged 22%.

Hybridization Reactions and Autoradiography

The polynucleotide kinase labeled polyA RNA was hybridized in 5 ml solutions of 50% formamide (deionized), 5xSSC, 0.1% SDS, 1mM EDTA, 10mM HEPES, pH 7.5, 0.2 mg/ml E. coli RNA (Boehringer, Mannheim) at 37°C in plastic tubes with immobilized plasmid DNA on nitrocellulose filters no larger than 20 cm^{2}. After the desired period of time the filters were washed with 4 changes of 500 ml 5xSSC-0.5% SDS at 70°C and dried. Autoradiography was conducted either with Kodak No-Screen (Kodirex) without amplifying screen at room temperature or on Kodak X-Omat R film with amplifying screens, Quanta 2 (DuPont) at -70°C. The films were developed in Kodak DX-80 developer for 4 min at room temperature, and fixed in Ilford Hypam fixer. For quantitative analysis of the autoradiograph, the films were scanned in a Vitatron densitometer, the tracings copied and the peaks cut out and weighed.

Hybridizations in solution were done in 0.3M NaCl, 20mM Tris, pH 7.5, 1mM EDTA. Samples containing 500-1000 cpm of nick-translated probe and an excess of polyadenylated RNA were sealed in 5 µl capillaries, boiled for 5 min and incubated at 70°C for the desired period of time. The amount of DNA in hybrid was determined by digestion with S1 endonuclease (16).

RESULTS

Our intention was to develop a rapid and convenient method for quantitating several species of RNA present at different concentrations within a population. The method involves electrophoresis in adjacent lanes of an agarose gel of several linearized plasmid DNAs each con-

taining a different insert. Upon hybridization with ^{32}P labeled RNA and autoradiography signals present in sharp bands at the position of the linear plasmid appear proportionate to the relative concentration of the RNA species in question. The bands on the autoradiograph are then scanned in a densitometer. The absolute concentration of RNA was calculated from a calibration curve constructed by plotting peak intensity against $R_o t_{1/2}$ values obtained from conventional solution hybridization reactions. In order to validate this approach it was necessary to demonstrate reproducibility of transfer to the nitrocellulose paper, that the signal is proportional to the amount of input RNA, i.e. that DNA excess can be achieved, that the autoradiographic image is proportional to the amount of radioactivity present in the hybrid and that the extent of reaction accurately reflects the concentration of that species of RNA. We have established the range throughout which each of these conditions prevails as well as the kinetics of the hybridization reaction.

Efficiency of transfer to Nitrocellulose Paper

In order to quantitatively analyze hybridization of ^{32}P-RNA to linearized recombinant plasmids, it was necessary to minimize differences in the efficiency of transfer of DNA from the agarose gels to nitrocellulose filters. Since the efficiency depends on the molecular weight of the DNA fragment (10) it was convenient to use recombinant plasmids which differ little in size. For this reason we used double stranded cDNA fragments synthesized from Drosophila melanogaster Kc polyA cytoplasmic RNA which were inserted in the vector pBR322 (4600 bp) as described elsewhere (12). The inserts varied in size between 170 and 600 bp. These small size differences in the size of the insert have little effect on the size of the overall plasmid. To investigate reproducibility of the transfer we performed two experiments: The plasmid pKc1 was linearized with BamHI, end-labeled and purified by preparative electrophoresis on an agarose gel. By adding unlabeled pKc1 the specific activity was adjusted to 3500 cpm/µg and several samples containing 300 ng of plasmid were electrophoresed in a 1% agarose gel. After transfer, pieces of the nitrocellulose filter containing the DNA band were cut out and counted. The results of such an experiment are shown in Table 1. Some 69% of the TCA precipitable radioactivity was reproducibly transferred to the nitrocellulose filter.

In a second experiment different quantities of DNA were electrophoresed on an agarose gel and blotted as above. Figure 1 shows that

TABLE 1: Transfer Efficiency of Linear DNA from an Agarose Gel to Nitrocellulose Filter

^{32}P-Kcl Loaded per gel slot (cpm)	^{32}P-Kcl Bound to nitrocellulose filter	Average % recovered (S.D.)	Weight of peak after autoradio-	Average peak weight (mg S.D.)
	73.1%		116	
	77.5%		121	
	69.8%		114	
	74.6%	731±65 cpm	112	
1065	62.4%	68±6%	95	105±14
	65.1%		103	
	62.3%		98	
	60.8%		78	

Transfer efficiency of linear DNA from an agarose gel to nitrocellulose filter. The recombinant plasmid Kcl was linearized with BamHI, end-labeled after phosphatase treatment with nucleotide kinase and γ-^{32}P-ATP. The labeled product was purified by agarose gel electrophoresis, recovered by freezing at 70°C and thawing three times and squeezing through a needle and subsequent ethanol precipitation. Unlabeled linear Kc DNA was added to adjust the specific activity to 3500 cpm/µg. Samples of 300 ng PKcl were loaded on a 1% agarose gel and electrophoresed in Tris-acetate buffer for 3 hr. DNA in the gel was denatured and transferred to a nitrocellulose filter. Individual bands were cut out of the filter and counted. A background of 30 cpm was subtracted. The filter was placed on Kokak X-Omat R film and autoradiographed with DuPont Quanta 2 amplifying screen overnight at -70°C. The bands were quantitated by scanning the autoradiogram cutting out the peaks and weighing them.

the efficiency of transfer was independent of the amount of DNA loaded up to 600 µg per band. Thus we conclude that under our conditions 60 to 70% of the material loaded on an agarose gel is transferred to nitrocellulose. This DNA remained firmly bound to the filter after baking for 2 hr at 80°C. When the filters were incubated overnight at 37°C in hybridization buffer used in the later experiments, dried and recounted, no loss of radioactive material from the filters was detected.

Proportionality Between The Filter Bound Radioactivity and the Autoradiograph Scans

Having established conditions resulting in reproducible transfer in the blotting procedure it was also necessary to demonstrate that a linear relationship existed between the amount of radioactivity on the nitrocellulose filter and the size of the peak obtained from a scan of the autoradiograph. The filters used in the two experiments described above were placed on Kodak X-Omat R film and exposed with amplifying screens overnight at -70°C. After development, the films were scanned and the peaks of the densitometer tracing cut out and weighed. Those data, also included in Table 1, establish a linear relation between radioactivity on the filter and intensity of the bands on the X-ray

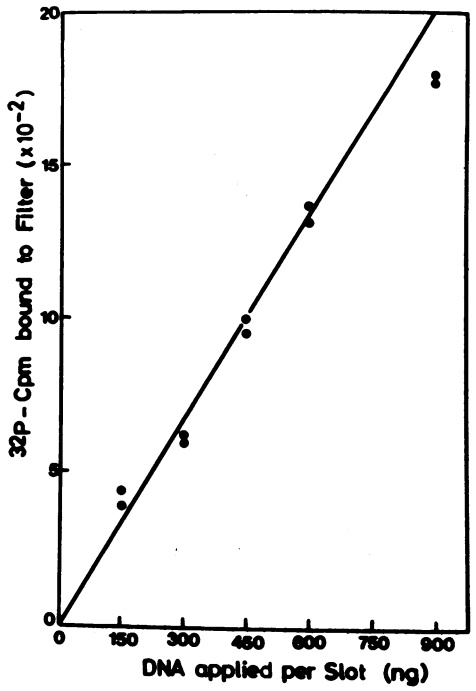

Fig. 1: Quantitation of transfer efficiency of different amounts of linear DNA from an agarose gel to nitrocellulose filter. Up to 900 ng of linearized and end-labelled pKcl plasmid (specific activity 3500 cpm/ug) was loaded in duplicate on a 1% agarose gel, electrophoresed and blotted to a nitrocellulose filter. After autoradiography, individual ^{32}P labeled bands were excised and counted in a liquid scintillation counter.

film. To establish the range of linearity, the nitrocellulose filter containing increasing amounts of ^{32}P-DNA was also autoradiographed. As shown in Figure 2 the correlation between radioactivity and intensity is linear up to 1000 cpm of ^{32}P per band for an overnight exposure.

To investigate the hybridization kinetics it was important to obtain a high excess of immobilized DNA to the labeled RNA probe. If the excess of DNA is sufficient, the amount of RNA hybridizing after a certain period of time should only depend on the concentration of the complementary RNA in the population. The use of cloned double stranded cDNA fragments from Drosophila Kc cell polyA RNA made it feasible to electrophorese and transfer large amounts of a single sequence. For the experiment shown in Figures 3 and 4 we used 0.3 µg of the PstI linear-

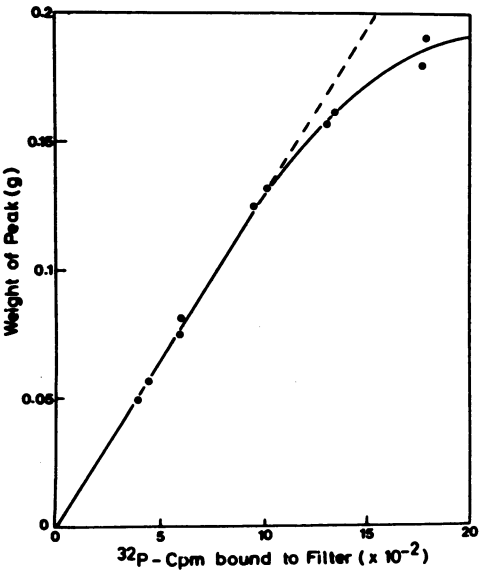

Fig. 2: Quantitation of transfer efficiency of different amounts of linear DNA by autoradiography. After transfer of different amounts of end-labeled pKc1 the nitrocellulose filter was exposed on Kodak X-Omat R film with Quanta 2 amplifying screen at -70°C overnight. Intensities of individual bands were monitored by densitometer scanning. The weight of each peak is plotted against the amount of ^{32}P-radioactivity in each band (from Fig. 1).

ized plasmid pKc9 per gel slot. Since the Drosophila double stranded cDNA insert in this recombinant plasmid is 250 bp this was equivalent to 18 ng of Drosophila DNA; assuming a 65% transfer efficiency from the gel, 12 ng of insert was present in each band on the nitrocellulose filter. The plasmid pKc9 contains a double stranded cDNA insert synthesized from the large mitochondrial ribosomal RNA which comprises by far the most abundant RNA species in the cytoplasmic polyA containing RNA preparation. Different amounts of kinased cytoplasmic polyA RNA from Kc cells were hybridized to the cut nitrocellulose filter and the amount of radioactivity bound to each pKc9 band was determined by counting the strips in a liquid scintillation counter (Fig. 3) or by autoradiography overnight on Kodak No-Screen film (Fig. 4). At least up to an input of 7×10^4 cpm of ^{32}P-RNA/cm^2 the DNA on the filter was sufficient to bind all complementary RNA molecules. The mitochondrial ribosomal RNA accounts

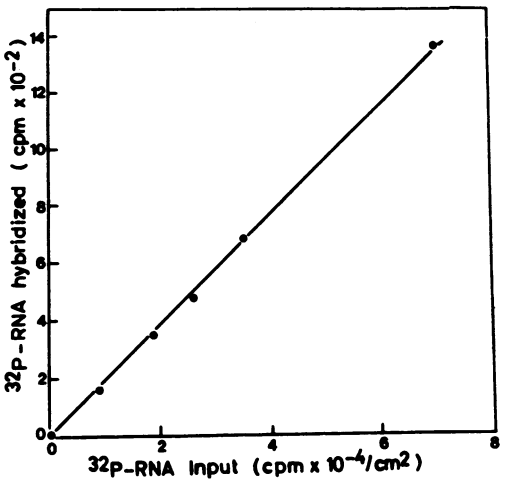

Fig. 3: Relation between RNA input and RNA hybridized. 300 ng of linearized plasmid pKc9 were loaded on each slot of an agarose gel, electrophoresed and blotted to nitrocellulose. The filter was cut into pieces of identical size, each containing one band, and hybridized for 4 hr at 32°C. In all cases the incubation volume was 5 ml. The filters were washed extensively and counted. From each value a background obtained with a blank filter and the same amount of radioactivity was subtracted.

for as much as 40% of the mass of the total polyA RNA preparation. With the obtained specific activity of $2x10^6$ cpm/µg RNA we calculate that 12 ng of a single DNA would be in reasonable excess over a RNA species comprising 1% of the population if up to $2.8x10^6$ cpm of polyA RNA/cm^2 were applied. This value was never exceeded in the following experiments. Quantitation of the scanned autoradiograph showed that using a Kodak No-Screen film the intensity of the signal was again strictly correlated with the amount of radioactivity on the filter up to about 700 ^{32}P-cpm in a band in an overnight exposure (Fig. 4). Since the experiments described subsequently employed cDNA clones derived from nuclear genes, autoradiography was conducted with intensifying screens at -70°C on Kodak X-Omat R film. The corresponding transcript in the polyA cytoplasmic RNA population was never found to be more than 1% of the total mass.

Kinetics of the Reaction

The kinetics of the hybridization of ^{32}P-labeled RNA to immobilized plasmids was studied using the recombinant plasmid pKc1. Different

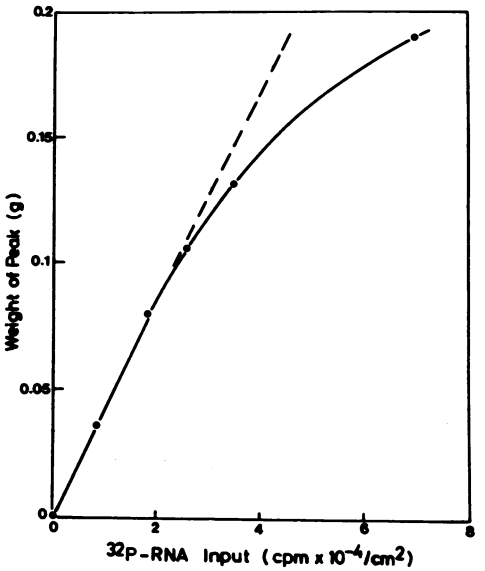

Fig. 4: Quantitation of hybridization of different amounts of ^{32}P-RNA to pKc9 DNA on nitrocellulose filters. The filters after hybridization and washing as described in the legend to Fig. 3 were exposed on Kodak No-Screen film at room temperature overnight. The extent of reaction was quantitated by scanning, followed by weighing of the resulting peaks. The weight of each peak is plotted against the amount of ^{32}P-RNA hybridized to each band as determined by scintillation counting (from Fig. 3). Incubation volume was 5 ml.

amounts of the linearized plasmid were loaded on the gels amounting to 6, 12, 18 and 36 ng of the Drosophila cDNA insert and blotted to nitrocellulose filters. All filters were trimmed to the same size and hybridized with ^{32}P-labeled polyA RNA from Kc cells for various lengths of time. Autoradiographs with Kodak X-Omat R and amplifying screen were scanned and quantified by weighing the peaks. Figure 5 shows the kinetics of hybridization. These data establish that DNA excess conditions were operative throughout this range and that the reactions reached a plateau at about 24 hr. A parallel kinetic study with pKc43 DNA gave similar results.

Calibration of Filter Reactions with Solution Hybridization Kinetics

Under DNA excess conditions, the plateau reached at maximum hybridization with a complex RNA mixture should reflect the relative frequency of a RNA species in the population. To verify this expectation,

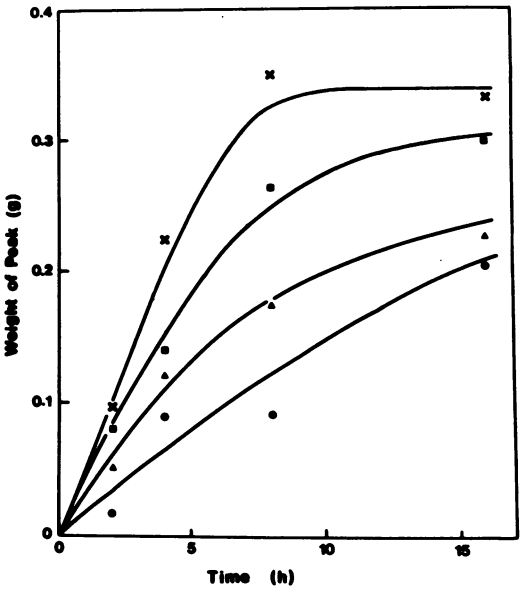

Fig. 5: Kinetics of hybridization of ^{32}P RNA to DNA bound to nitrocellulose filters. Filters with linearized pKc1 DNA containing 6 ng (O), 12 ng (Δ), 18 ng (■) and 36 ng (X) of Drosophila DNA insert were hybridized with 10^6 cpm/cm^2 ^{32}P labeled RNA for 2-16 hr at 37°C. Exposure was with Kodak X-Omat R film with an amplifying screen for 7 days.

recombinant plasmids were selected which represented RNA of different frequencies in the cytoplasm of Kc cells. Amounts equivalent to 18 ng of Drosophila cDNA insert were loaded and after hybridization with polyA RNA the autoradiographic signals were quantitated as before. Figure 6 shows that RNA reacted to different extents with each plasmid DNA. The relative amounts of RNA hybridized was calculated from the weight of the peaks obtained by scanning the autoradiograph.

In order to obtain absolute numbers for the abundance of each RNA, inserts were prepared from the same three plasmids, nick-translated to introduce ^{3}H and used in conventional RNA excess solution reactions (1). The excess of RNA was sufficient to prevent any self-reassociation of the ^{3}H-nick-translated DNA fragments over the time scale employed. The hybridization kinetics of these RNA driven reactions are shown in Fig. 7a and 7b. All three reactions approached the theoretical maximum of

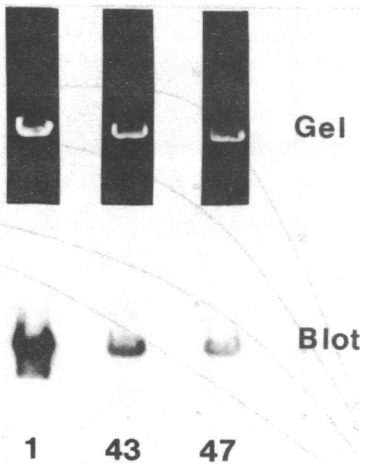

Fig. 6: Hybridization of ^{32}P-RNA (1.5×10^6 cpm/cm^2) to 0.3 µg of recombinant plasmids bound to nitrocellulose filters. (A) is a representation of the ethidium bromide stained agarose gel with the linearized plasmids before blotting. After blotting, the filter was incubated in 5 ml hybridization buffer overnight at 37°C and washed. (B) Autoradiography was conducted with Kodak X-Omat R film with amplifying screen for 6 days. The bands were quantitated by scanning as recorded in Table 2.

50%. The observed $R_o t_{1/2}$ values were calculated and used to compute the concentration of each hybridized RNA species. As an absolute standard the $R_o t_{1/2}$ value of the reaction between globin cDNA and its mRNA was taken to be 3×10^{-4} (17). The concentrations of RNAs complementary to Kc1, Kc43 and Kc47 were thus determined to be 0.11%, 0.05% and 0.017% of cytoplasmic polyA RNA (Table 2). Estimates of the relative concentration of the three species of RNA obtained by the two methods are in reasonable agreement. Therefore, it is possible to calculate absolute concentrations of many RNA species from such autoradiographic data provided that at least one of the species has been assayed by the solution hybridization method.

DISCUSSION

It is frequently necessary to quantitate one or more RNA molecules within a complex population. Such assays are often performed to follow gene induction or to compare the levels of gene expression in different cell types. Currently, the most common approach relies upon the kinetics

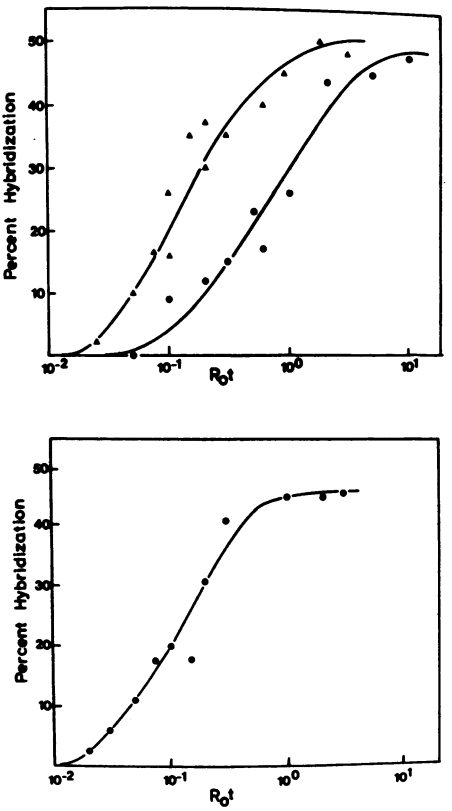

Fig. 7: RNA excess/^{3}H cDNA hybridization kinetics in solution with the inserts from: A. pKc1 (Δ) and pKc47 (0); and B. pKc43.

of hybridization of a labeled cDNA probe under conditions of RNA excess. Although such assays are precise they suffer from two major disadvantages. In the first place, calculation of the absolute rate constant requires establishing a kinetic curve with multiple points, a procedure which can be tedious if multiple samples are involved. Furthermore even with very high specific activity probes, considerable amounts of RNA must be used particularly if the species in question is not abundant in the population. The second difficulty can be obviated if conditions of cDNA excess are used although an internal standard of differentially labeled RNA must be used to enable correction for the intrinsic efficiency of hybridization.

Table 2: Quantitation of ^{32}P-RNA Hybridization to Excess Recombinant Plasmids on Nitrocellulose Filters

Plasmid	Size of Drosophila Insert (bp)	Weight of Peak after Autoradiography (mg)	Relative Maximum Hybridization	$Rot_{1/2}$ of RNA-Excess Hybridization	$Rot_{1/2}$ if Pure	% PolyA Cytoplasmic RNA	Relative Amounts of RNA
pKc1	250	180	1	$1.0x10^{-1}$	$1.1x10^{-4}$	0.11	1
pKc43	190	58	0.32	$1.5x10^{-1}$	$0.84x10^{-4}$	0.05	0.51
pKc47	260	21	0.12	$7x10^{-1}$	$1.2x10^{-4}$	0.017	0.15

Summary of data on the frequencies of three RNA species in the cytoplasmic polyA RNA from Kc cells obtained by hybridization to plasmids on nitrocellulose filters and by RNA excess hybridization in solution. The weight of the cut out peaks of the densitometer tracings were taken from Fig. 6B. The $Rot_{1/2}$ values of the RNA excess reactions were taken from Figs. 7A and 7B. Calculation of the theoretical $Rot_{1/2}$ value if the sequence in question were pure was made using a $Rot_{1/2}$ value of $3x10^{-4}$ for the reaction between Hb cDNA and mRNA and taking into account the size of the insert.

The approach summarized here exploits the well-known Southern (10) gel transfer technique to construct filters containing many parallel bands of plasmid DNA harboring different cDNA inserts. Following hybridization with randomly labeled RNA the extent of reaction with each DNA can be assessed visually by examining the film or quantitatively by densitometric scanning. This approach is conceptually similar to that of Kafatos et al. (6) wherein aliquots of each plasmid are simply spotted to form a matrix on the filter. Both allow a rapid visual estimate of the relative concentration of RNA complementary to the array of plasmids present on the filter. However the filters constructed by the gel transfer technique lend themselves to more convenient quantitation. Data presented here establish that scans of the autoradiographs give accurate estimates of the radioactivity on the filter. Furthermore the assay can be made more sensitive by using intensifying screens during autoradiography. Even with Kodak No-Screen film a band containing 100 cpm was visible after an overnight exposure. The use of amplifying screens with Kodak X-Omat R improved the sensitivity by about a factor of five. In both cases the intensity is linear with the amount of ^{32}P over a considerable range.

The rate of hybridization of RNA was dependent on the concentration of DNA. Under our conditions maximum hybridization was obtained with about 18 ng of hybridizable DNA loaded on the gel in an overnight reaction. During this period of incubation no degradation of probe or bound DNA was detected which could have an influence on the hybridization.

For example if more than break per labeled RNA molecule had occurred, the middle part of the molecule would be without ^{32}P-label and therefore not detected. Also, RNA molecules which are larger than the DNA fragment on the filter may lose their terminal ^{32}P during the washing procedure after the hybridization. It was for these reasons that the RNA was degraded by mild alkaline treatment to fragments of some 50 nucleotides in length before labeling. This procedure obviates such end effects as well as creating more 5' ends for labeling.

The limit of detection of a RNA molecule in a population does not depend only on the specific activity of the probe. Because of the high sensitivity of amplifying screen enhanced autoradiography the absolute amount of ^{32}P radioactivity per band is usually not the limiting parameter. More important is the signal to noise ratio. Treatment of the filter after the hybridization with RNAse did not improve this ratio because of loss of radioactivity from the bands. Extensive washing under hybridization conditions gave the best, most reproducible results. We do not know how much of the total ^{32}P-RNA in the hybridization solution is available for hybridization to each band of DNA on the filter. A precise calculation of the lowest amount of RNA detectable is therefore not possible. However, comparison of the solution kinetics for Kc47 with those of total cDNA/mRNA reactions (18) shows that Kc47 complementary RNA is present at less than average abundance in the total populations. Nevertheless the method presented here has adequate sensitivity to quantitate them. We estimate that with an input of 1.5×10^6 cpm RNA/cm^2 (i.e. 2.7×10^7 cpm/filter), extensive washing and exposure with amplifying screen for one week, an RNA which is 1 part in 10^5 of the total is detectable.

In summary, quantitative analysis of the hybridization of RNA to Southern blots has many advantages: a) several recombinant plasmids can be hybridized on the same filter to the same RNA probe under identical conditions; b) little RNA is necessary, which makes it possible to analyze RNA populations not available in large enough amounts for RNA excess hybridization in solution; and c) different RNA populations can also be compared by hybridization to the same set of DNA molecules. Although experiments discussed here were designed to measure the steady state level of RNA molecules the same methodology is in principle adpatable to measurements of rates of synthesis provided that pulse-labeled RNA of sufficient specific radioactivity can be obtained. An apparent limitation of this method is that coding DNA fragments which do not differ substantially in size must be used to ensure similar efficiency of transfer from the gel.

Short double stranded cDNA fragments cloned in bacterial plasmids are ideal for this purpose. However the procedure could be used with recombinant plasmids containing larger genome DNA inserts, a modification which would also increase the sensitivity of the method. If more than one transcript is synthesized from a given DNA, each could be quantitated simultaneously by using restriction endonucleases which separate the transcription units. If length dependent elution from the gel and transfer to the filter do reduce precision, the procedure of Alwine et al. (19), wherein the DNA is transferred to a covalent linkage to paper, could be substituted.

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