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Determination of cellular RNA concentrations by electron microscopy of R loop-containing DNA

(R loop electron microscopy/recombinant DNA/mRNA abundance)

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ABSTRACT **R loop hybridizations and electron microscopy have been used to determine cellular RNA concentrations for cloned genes. In plasmid DNA sequence excess, all the complementary RNA is driven into R loop structures that can be assayed by electron microscopy. To determine the concentration of a particular poly(A)$^+$ RNA, plasmid DNA crosslinked once every 2000–5000 base pairs with trioxsalen and UV light is hybridized in DNA sequence excess to various known amounts of total poly(A)$^+$ RNA, and the R loops are stabilized by treatment with glyoxal. If necessary, excess nonhybridized RNA is removed by Sepharose 2B chromatography, which enables the visualization of less abundant transcripts. Reconstruction experiments demonstrated that electron microscopic determination of the fraction of plasmid DNA molecules containing specific RNA loops gives accurate values of specific RNA weight fractions or concentrations in the total poly(A)$^+$ RNA populations. These methods were also used to determine the concentrations of five RNA species complementary to sequences on TRT3, a recombinant DNA plasmid containing yeast histone 2A and 2B genes and three other nonhistone genes. The methods described allow one to visualize the R loop structures for both abundant and nonabundant transcripts and to estimate concentrations of these RNA species simply by determining the fraction of DNA containing R loops.**

Eukaryotic cells contain several hundred (1) to many thousands of different species of mRNA molecules (2–6). Most mRNA species are in concentrations of one or fewer copies to several copies per cell (the low prevalence or complex class). Smaller numbers of mRNA species are present at hundreds to thousands of copies per cell (the moderately abundant and abundant classes).

Relative amounts of both abundant and nonabundant transcripts can be measured by several methods. These include comparing autoradiographs of RNA blots (unpublished observations) and hybridization of labeled cDNA from an mRNA population to bacterial colonies from a cDNA library (7). More demanding hybridization experiments are necessary to quantitate the amount of a single RNA species in an RNA population (4, 8).

R loop analysis of DNA·RNA hybrids in the electron microscope provides a fast and accurate method for identifying and mapping transcribed regions in DNA molecules (9–11). Under appropriate conditions RNA will hybridize with double-stranded DNA, displacing the noncomplementary DNA strand. The structure that forms is easily recognized in the electron microscope as a bubble in an otherwise duplex DNA structure. Kaback *et al.* (12) have described improved methods for R loop formation and stabilization. Prior to hybridization, the DNA is crosslinked with trioxsalen (4,5′,8-trimethylpsoralen) once per 2–5 kilobases (kb). DNA·RNA hybridization is followed by treatment of R loop-containing DNA with glyoxal, which stabilizes the R loops. These modifications overcome difficulties due to slow rates of formation of some R loops and due to their instability and thus usually increase the efficiency of R loop formation. These improved techniques have been applied to mapping transcripts on recombinant DNA plasmids (13–16). Hybrid formation does not require prior enrichment of a particular RNA species and can be done with large amounts of total poly(A)$^+$ RNA. If the concentration of unhybridized RNA is high, the DNA and stabilized R loops can be separated from free RNA by gel filtration (12, 17). Therefore, large amounts of total or poly(A)$^+$ RNA can be used to form sufficient numbers of R loops so even nonabundant transcripts are visualized in R loop structures.

In the present communication we report that hybridizations done in sufficient plasmid DNA sequence excess will drive all the RNA complementary to sequences on the plasmid into stable R loop hybrid structures. It is then possible to measure the abundance of a given RNA by determining the fraction of R loop-containing DNA molecules on an electron microscope grid. This technique can be applied to measure the fraction of RNA for both abundant and rare mRNA species and has advantages in simplicity and convenience over other published methods. The technique is illustrated here by measuring the steady-state levels of yeast histone 2A and 2B mRNA species as well as of several transcripts present in much lower abundance.

MATERIALS AND METHODS

DNA Preparations. The DNA for recombinant plasmid pJHC11 containing yeast ribosomal DNA (18), TRT3 containing yeast histone 2A and 2B genes, and pOV230, a chicken ovalbumin cDNA plasmid (19), was isolated by published procedures (20). DNA was digested with restriction endonucleases according to the manufacturers' instructions and extracted with phenol. DNA concentrations were determined by measuring the absorbance at 260 nm and multiplying by 50 μg/ml. Recombinant DNA isolation and manipulations were carried out under appropriate biohazard containment conditions.

RNA Preparations. Total yeast RNA was isolated from cells growing logarithmically at 30°C in 1.0% yeast extract/2.0% peptone/2.0% glucose. Harvested cells were broken by rapid Vortex mixing for 1 min in 0.3 M NaCl/0.03 M sodium citrate/0.5% sodium dodecyl sulfate/0.1% diethylpyrocarbonate in the presence of glass beads. The extract was centrifuged at 1000 × *g* and the supernatant was extracted with phenol three times. The aqueous phase was precipitated with ethanol and passed over oligo(dT)-cellulose two times to isolate poly(A)$^+$ RNA (5). Yeast poly(A)$^+$ RNA preparations were estimated to contain 40% rRNA contamination by observing 18S and 25S rRNA bands on CH_3HgOH agarose gels (21). RNA concentrations were determined by multiplying the absorbance at 260 nm by 40 μg/ml.

Abbreviations: kb, kilobase(s); Pipes, 1,4-piperazinediethanesulfonic acid.

R Loop Hybridizations and Hybrid Purification. DNA at a concentration of 50 μg/ml in 10 mM Tris·HCl/1.0 mM EDTA/10 mM NaCl, pH 7.4, was crosslinked once every 2–5 kb by treating with trioxsalen at 1.0 μg/ml and long-wavelength ultraviolet light as described (12). DNA and RNA were hybridized in 70% (vol/vol) formamide/0.1 M 1,4-piperazinediethanesulfonic acid (Pipes), pH 7.2/0.01 M EDTA/0.4 M NaCl at various temperatures and times as referred to in the text. To ensure kinetic completion with complex poly(A)$^+$ RNA populations, the reactions were usually allowed to proceed for 18–24 hr. All reactions that were allowed to continue for longer than 18 hr gave equivalent results. The hybrids were chilled to 0°C and, where noted, were treated with 1.0 M glyoxal for 2 hr at 12°C (12). Extreme care was taken to ensure that the temperature of DNA solutions never exceeded 12°C in the presence of glyoxal. If the concentration of RNA in the hybridization reaction was greater than 100 μg/ml, it obscured the DNA in the electron microscope field. To separate the unhybridized RNA from the R loop-containing DNA, free glyoxal was removed by dialysis against 0.5 M NaCl/0.1 M Pipes/0.01 M EDTA, pH 7.2. The R loop mixture was then passed over a Sepharose 2B column equilibrated with the same buffer at 4°C. DNA was excluded whereas unhybridized RNA was an included fraction. The column fractions were dialyzed and prepared as described below. When the RNA concentration was lower than 100 μg/ml, the R loop mixture was dialyzed directly against 10 mM Tris·HCl/1.0 mM EDTA, pH 7.4. Storage for several days in this buffer at 4°C showed no changes in the length or quantities of the observed R loops.

Electron Microscopy. R loop samples were spread from 50% (vol/vol) formamide onto a 15% (vol/vol) formamide hypophase as described (12, 22). Sample grids were viewed in the electron microscope and the fraction of DNA containing R loops was determined by counting 200–300 DNA molecules. Only clear R loops approximately 50% of full length or greater, present on approximately full-length linearized DNA molecules, were counted. Double-stranded replicative form DNA of phage φX174 (5378 base pairs) (23) was included in all spreads for length calibration. In all the plasmids studied here, the positions of R loops in the DNA were sufficiently unambiguous to permit identification by visual inspection in the electron microscope alone. R loop maps and representative molecules of each plasmid utilized are shown in Fig. 1.

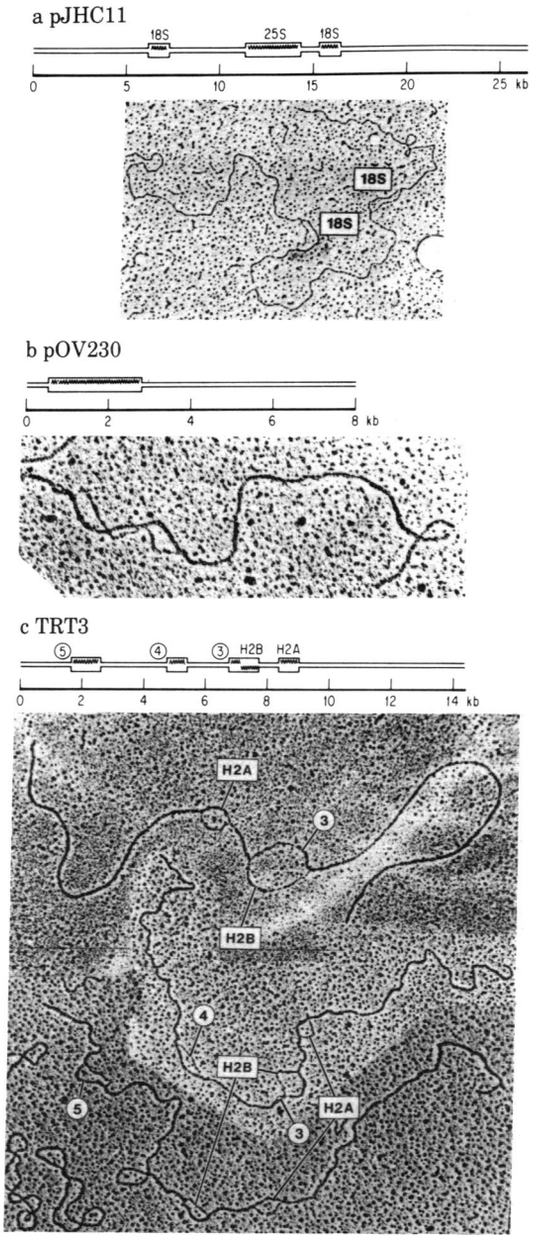

FIG. 1. Maps and representative electron micrographs of molecules containing R loops.

RESULTS

Quantitative Recovery of RNA in R Loops. To determine if RNA could be quantitatively recovered in R loop structures, known amounts of purified 18S yeast rRNA or chicken ovalbumin mRNA were hybridized to known excess amounts of either yeast ribosomal DNA (pJHC11) or chicken ovalbumin cDNA (pOV230) recombinant DNA plasmids, respectively. For ovalbumin, the hybrids were first treated with glyoxal and then fixed for the electron microscope. For rRNA, the hybrids were prepared directly for electron microscopy, because these R loops are known to be stable except for a small amount of branch migration at the 5′ end (10, 12). In both cases, the duplex DNA was relatively intact. Representative electron micrographs are shown in Fig. 1. The fraction of R loop-containing DNA molecules was determined by counting three independent 100-molecule samples from two or three different grids. As shown in Table 1, each DNA contained 88% or greater of the fraction of R loops expected if all of the added complementary RNA was present as R loops. Doubling or halving either the DNA or RNA concentration had no effect on the yield of RNA in R loops. In addition, a reconstruction experiment with pOV230 and ovalbumin mRNA was done to mimic hybridization of a low-abundance specific sequence in a total poly(A)$^+$ RNA population. Heterologous yeast poly(A)$^+$ RNA in a 10,000-fold excess over the ovalbumin mRNA was added to the hybridization reaction. The yield of ovalbumin mRNA sequences present in R loops was again quantitative (±10%).

Rate of R Loop Formation. Table 2 presents the results of a preliminary study of the rate of R loop formation as a function of DNA concentration. If the reaction between RNA and DNA is a conventional second-order reaction, the rate equation should be $-dR/dt = kRD$, in which R and D are the concentrations of RNA and DNA, respectively.

For experiment A in Table 2, the DNA was preincubated for 15 min at 52°C, a temperature that is estimated to be several degrees above the strand separation temperature of the DNA in the solvent used (12). Under these circumstances, the effects of DNA concentration and time on the extent of reaction are at

Table 1. Quantitative recovery of RNA in R loop structures

DNA·RNA hybrid	cDNA, μg/ml	RNA, μg/ml	Fraction of DNA sites with R loops	RNA in R loops, %
*Hin*dIII	1.83	0.45	0.27 ± 0.01	109
pOV230·oval-	1.83	0.22	0.14 ± 0.03	115
bumin mRNA	1.83	0.22*	0.12 ± 0.03	100
	1.83	0.14	0.10 ± 0.02	125
*Eco*RI	0.80	0.08	0.12 ± 0.02	116
pOV230·oval-	0.40	0.08	0.19 ± 0.03	96
bumin mRNA				
*Bam*HI	0.50	0.20	0.35 ± 0.03	88
pJHC11·18S	1.00	0.20	0.18 ± 0.03	92
rRNA	0.50	0.10	0.18 ± 0.02	90

Restriction enzyme-linearized, crosslinked recombinant DNA plasmids were hybridized for 1–3 hr to RNA under conditions favoring R loop formation. For ovalbumin the R loop preparations were treated with glyoxal for 2 hr and spread from 50% formamide. For 18S rRNA the preparations were spread directly from 50% formamide. The fraction of the complementary sites containing R loops was scored in three different 100-molecule samples for each hybridization. The error shown is the SD observed for the three samples. The conversion factors between plasmid DNA concentration and mRNA complementary sequence concentration are (2 × 8.0/1.8) and (2 × 26/3.5) for pOV230 and pJHC11, respectively.

* In addition to ovalbumin RNA, yeast poly(A)$^+$ RNA was added to the reaction mixture at 2 mg/ml. In this case the preparation was passed over Sepharose 2B.

least roughly consistent with the rate equation $dR/dt = -kRD$. However, the ranges of concentrations and times studied are limited, and these preliminary results are not a critical test of this plausible rate equation.

The predicted value of k in the same solvent for a DNA·DNA reaction with single strands of the length and complexity of 18S rRNA (1800 nucleotides) is about 10^3 M^{-1} sec^{-1} (24). Thus the rate of R loop formation ($k \approx 300$ M^{-1} sec^{-1}) appears to be about ⅓ relative to the single-strand DNA·DNA rate. Given $k = 300$ M^{-1} sec^{-1} with DNA in a sufficient sequence excess so that the reaction is approximately pseudo-first-order in RNA, the predicted $C_0t_{1/2}$ (product of DNA concentration and incubation time when the hybridization is half complete) is 2.3×10^{-3} M

Table 2. Rate of R loop formation

Exp.	18S complementary sequence DNA, μg/ml	Fraction of 18S rRNA in R loops		k,* M^{-1} sec^{-1}	
		20 min	40 min	20 min	40 min
A	0.17	0.12	0.23	220	230
	0.50	0.50	0.64	410	310
		15 min	24 hr		
B	0.35	0.22	—		
	0.50	0.23	0.88		
	0.70	0.27	—		
	1.00	0.21	0.92		

Various amounts of crosslinked pJHC11 DNA either were preincubated in the hybridization buffer for 15 min at 52°C and then combined with 18S rRNA at 0.09 μg/ml (Exp. A) or were directly combined with 18S rRNA at 0.09 μg/ml and incubated at 52°C (Exp. B). Samples were taken at the times noted and prepared for electron microscopy, and the fraction of 18S rDNA sites with R loops was determined by counting 200–300 molecules.

* Calculated from the integrated form of the rate equation $dR/dT = -kRD$; i.e., $\ln\{[1 - (R_0 - R)/D_0](R_0/R)\} = (D_0 - R_0)kt$, in which $_0$ indicates concentration at time zero.

sec. Thus for DNA at a concentration of 1 μg/ml (for the sequences of interest, not including the flanking sequences), the calculated half-time is 12.5 min. Thus the incubations of several hours to overnight used in this study are predicted to be more than sufficient for a complete reaction. A study of the dependence of reaction rate on RNA concentration has not been made. However, the data in Tables 1, 3, and 4 show that, for several different RNA species at several different concentrations, all the RNA can be driven into R loops by sufficiently long hybridizations.

The rate experiments were carried out by preincubating the crosslinked DNA in formamide for 15 min under hybridization conditions before adding the RNA. Experiment B in Table 2 shows that when the DNA and RNA are mixed in the formamide at room temperature, the initial reaction is slower and relatively independent of DNA concentration. This surprising observation suggests that a finite time is required for the crosslinked DNA to unwind and be available for RNA·DNA hybridization. It recalls the equally unexpected observation of Thomas *et al.* (10) that, in RNA excess, the rate of R loop formation is not proportional to the RNA concentration below the DNA strand separation temperature, although it is proportional above that temperature. Our observations are insufficient to reveal the mechanism of these curious effects. However, the practical point is that for long incubations, even in the absence of preincubation of the DNA, complete hybridization of the RNA is achieved, as shown in experiment B of Table 2.

Formation of R Loops with Yeast Histone 2A and 2B Genes and Yeast Total Poly(A)$^+$ RNA. We investigated the levels of RNA abundance in total poly(A)$^+$ RNA for several genes located on a yeast recombinant DNA plasmid, TRT3. This plasmid contains one of the two histone 2A–2B (H2A–H2B) gene clusters present in the haploid genome. [Yeast histone mRNA is poly(A)$^+$ (25).] In addition, TRT3 contains DNA complementary to at least three additional poly(A)-containing RNA species identified as 3, 4, and 5 (Fig. 1*c*) (14). R loops were formed on TRT3 DNA with various concentrations of poly(A)$^+$ RNA and prepared for electron microscopy. The fraction of R loop-containing molecules was determined for each transcribed region on the plasmid DNA (Table 3). Up to the point at which approximately 80% of the DNA was saturated with R loops, an approximately linear increase in the fraction of R loops was observed with increasing RNA concentration for almost all of the R loops examined. R loop 4, corresponding to the least abundant RNA species, showed an overall increase but was not abundant enough to give reliable data for the sample size studied. Because we know the concentrations of both the added poly(A)$^+$ RNA and plasmid DNA and the approximate sizes of each of the transcripts from contour length measurements, the weight fraction of each RNA species in the total RNA population can be calculated. The data in Table 3 show that H2A and H2B RNA species are relatively abundant and are present in different concentrations accounting for 2.0×10^{-3} and 0.5×10^{-3} weight fraction of the total poly(A)$^+$ RNA, respectively. The fractions of R loops observed for the three other transcripts were much lower, indicating that these RNA species are present in much lower amounts than the histone messages in poly(A)$^+$ RNA.

In experiments with TRT3 DNA and a different RNA preparation, the weight fractions of H2A and H2B transcripts were measured as $1.35 \pm 0.10 \times 10^{-3}$ and $0.65 \pm 0.14 \times 10^{-3}$, respectively. These results are quantitatively different than those in Table 3, although a significant difference between H2A and H2B RNA is still observed. Single experiments with the same RNA preparation were in agreement to ±10% or the expected statistical variation, whichever was larger.

When a mixture of TRT3 and TRT1 plasmid DNAs [the latter contains the second set of H2A and H2B genes (14)] was used,

Table 3. Quantitation of R loops of TRT3

Gene		Poly(A)$^+$ RNA, μg/ml 22	44	110	220	330	Mean wt fraction of RNA × 10^3	RNA molecules per cell
H2A	1	0.40	0.70	1.00	0.95	0.99		
	2	0.0021	0.0019	Sat'd	Sat'd	Sat'd	2.0 ± 0.05	170
H2B	1	0.09	0.22	0.51	0.79	0.97		
	2	0.0043	0.00058	0.00054	0.00042	Sat'd	0.5 ± 0.05	40
3	1	0.01	0.03	0.16	0.33	0.31		
	2	NS	NS	0.00015	0.00016	0.00010	0.14 ± 0.03	13
4	1	0.01	0	0.03	0.03	0.02		
	2	NS	NS	3.5×10^{-5}	1.8×10^{-5}	8.0×10^{-6}	0.02 ± 0.015	2
5	1	0.02	0.02	0.06	0.13	0.16		
	2	NS	NS	0.00012	0.00012	0.00011	0.12 ± 0.01	6

Total yeast poly(A)$^+$ RNA was hybridized to crosslinked TRT3 at 6 μg/ml in 70% formamide. The R loops were treated with 1.0 M glyoxal and unhybridized RNA was removed where necessary. The preparations were spread from 50% formamide and observed; 100 or 200 molecules were scored. Lines 1 give fraction of plasmids with the given R loop; lines 2 give calculated weight fraction of the RNA, assuming molecular lengths of 450 nucleotides (H2A, H2B, and gene 4), 400 nucleotides (gene 3), and 800 nucleotides (gene 5). Mean weights are given ± SD. NS signifies not statistically significant; Sat'd signifies that the RNA is in excess over the DNA. RNA molecules per cell for any given mRNA is calculated by assuming 22,000 mRNA molecules of average length 1000 nucleotides from the length measurements of Kaback *et al.* (12) and amount of poly(A)$^+$ RNA per cell from Hereford and Rosbash (5). The observed weight fraction of any RNA refers to the fraction of the RNA sample that we estimate contains 40% rRNA. The calculated RNA molecules per cell is corrected for this factor by multiplying by 1.67.

the observed H2A and H2B RNA weight fractions were 1.37 ± 0.2 and 0.87 ± 0.05 × 10^{-3}, respectively, in close agreement with the results for the same RNA preparation with TRT3 alone. Furthermore, carrying out the hybridization first at 52°C for 12 hr and shifting to 46°C for an additional 6 hr gave no significant differences in the levels of H2A and H2B RNA from TRT1/TRT3 mixtures incubated continuously at 52°C for 18 hr (data not shown). These results eliminate a possible interpretation that the measured difference in H2A and H2B RNA levels was due to the failure of H2B RNA coded by TRT1 to form R loops with TRT3 DNA.

Table 4. Removal of complementary poly(A)$^+$ RNA sequences by R loop hybridization with TRT3 DNA

Gene	Fraction of DNA with R loops: First hybridization, Glyoxal before Sepharose (n = 59)	First hybridization, Glyoxal after Sepharose (n = 50)	Second hybridization (n = 437)	% of total RNA in R loops in second hybridization
H2A	0.93	0.72	0.30	64
H2B	0.80	0.72	0.02	12
2	0.17	0.14	0.007	18
3	0.02	0.04	0	3
4	0.17	0.10	0.002	6

Crosslinked TRT3 DNA (5 μg/ml) was hybridized to poly(A)-containing RNA at 165 μg/ml. Part of the reaction mixture was treated with glyoxal, freed of unhybridized RNA by Sepharose 2B chromatography, and fixed for electron microscopy, and the fraction of DNA containing each R loop species was determined (column 2 of the table). Numbers in parentheses are the numbers of molecules scored. The remaining hybridization mixture was not fixed with glyoxal but was directly passed over a Sepharose 2B column to separate the R loop-containing DNA from the unhybridized RNA. The excluded DNA fractions were treated with glyoxal and observed in the electron microscope to assess any loss of R loops due to running the column prior to glyoxal treatment (column 3 of the table). The included RNA was eluted, precipitated with ethanol, and resuspended in hybridization buffer to a final concentration of 30 μg/ml with crosslinked TRT3 DNA at 5 μg/ml, and the second hybridization reaction was allowed to proceed. The preparation was treated with glyoxal and the fraction of DNA containing each R loop was determined (2) (column 4 of the table). Appropriate correction was made for the fact that the RNA sample was diluted from 165 to 30 μg/ml by the gel filtration step.

Removal of Complementary RNA Sequences from Total Poly(A)$^+$ RNA by R Loop Hybridizations. A test was performed to determine if all the hybridizable RNA is removed by the DNA-excess R loop hybridization procedure. TRT3 DNA hybrids were formed with poly(A)$^+$ RNA as described in the legend to Table 4. An aliquot was removed and treated with glyoxal, and the R loop-containing DNA was purified and the fraction of DNA containing R loops was determined (Table 4, column 2). The remainder of the hybridization mixture was not treated with glyoxal because this treatment would modify any nonhybridized RNA. The mixture was passed over Sepharose 2B to separate the duplex and R loop-containing DNA from the unhybridized RNA. The DNA fraction was then treated with glyoxal and the fraction of R loops was determined by electron microscopy to assess if there was any loss of R loops due to the absence of glyoxal fixation and chromatography (Table 4, column 3). Finally, the eluted RNA was rehybridized to additional TRT3 DNA to determine the fraction of TRT3 complementary RNA sequences remaining (Table 4, column 4). The results confirm that, after the first hybridization with excess TRT3 DNA, little RNA complementary to H2B, gene 2, gene 3, and gene 4 remained in the unhybridized RNA fraction. There is some release of RNA by gel filtration without glyoxal fixation. This may be responsible for some of the RNA found during the second hybridization.

DISCUSSION

We have shown that with the preparation conditions described and with a modest sequence excess of a cloned DNA, all of the complementary RNA in a complex RNA preparation can be driven into R loops. R loops can be formed with large amounts of total cellular RNA and the unhybridized RNA can be separated from the R loop-containing DNA by gel filtration. Thus the method can be used to measure the fraction of a specific RNA in a complex population, even for rare transcripts.

Unlike other techniques, it does not require radioactive labeling of any of the reaction components or prior DNA strand separation (7). A problem might arise in using eukaryotic genes

containing multiple intervening sequences because R loops formed by these genes can be quite complex and difficult to score. In this case the use of cDNA recombinant clones would facilitate quantitation.

Factors Affecting Quantitation of RNA Levels. Accurate estimation of RNA levels is dependent on the fact that the DNA is almost completely intact before and after the hybridization reaction. Without crosslinking, denatured DNA is found in most R loop mixtures (unpublished observation). Hybrids of RNA with single-stranded DNA might form in preference to R loops. These structures are difficult to recognize by electron microscopy and might lead to an underestimate in the measurements of the concentration of a specific RNA. Most of the DNA renatures into unit-length linear duplexes when previously crosslinked and most of the hybridized RNA is found in R loop structures that are readily recognized and counted on electron microscope grids when the methods described here are used.

Another factor affecting accurate quantitation is the assumption that the R loops that form are all stable. This assumption appears to be valid when the R loops are stabilized by glyoxal treatment and may not be true otherwise. This stability is directly demonstrated by the reconstruction experiments in Table 1. Evidence for the instability of many yeast poly(A)$^+$ RNA·genomic DNA R loops in the absence of glyoxal fixation has been observed (12).

The R loop method for RNA quantitation is applicable only to RNA preparations that remain relatively undegraded, because it requires hybridization of single intact RNA molecules to single plasmid DNA sites. RNA that is broken in half might give an overestimate because two RNA fragments from a single RNA molecule would almost certainly hybridize to two different DNA molecules under conditions of DNA excess and would be counted as two hybrids. Alternatively, more degraded RNA would lead to underestimates of the RNA concentration because the hybrids might be too small to be counted. Slightly degraded mRNA may be the cause of the 5–25% higher than expected yield with ovalbumin mRNA (Table 1). If necessary, these problems can be partially overcome by measuring the contour lengths of the R loops in question.

In our hands, independent experiments with a given RNA sample give measurements of the RNA concentration that agree within ±10% or the expected statistical variability, whichever is larger (usually the latter). Counting many molecules on electron microscope grids is tedious; thus from a practical point of view, the method is most useful when an uncertainty of 10% or greater is acceptable.

Yeast H2A and H2B Transcripts. We find that the average concentration of H2A RNA in a rapidly growing logarithmic phase culture is 2- to 4-fold greater than that of H2B RNA. We believe that the variability in the observed ratio is not due to experimental error but is due to real differences between different RNA preparations, because of some factor such as variations in the doubling time or harvesting time of the yeast cells. Nevertheless, in all cases observed, the average H2A mRNA concentration is greater than that of H2B, despite the probable fact that there are equal numbers of the two protein species in chromatin. It has been observed that the concentrations of histone RNAs peak transiently during the S phase of the cell cycle (L. Hereford, personal communication) at the time of histone synthesis (26). Therefore, the actual amounts of the H2A and H2B RNAs during S phase are greater than the steady-state levels observed here. Thus, the cause and significance of the observed differences in steady-state RNA levels are not clear.

Observing Rare Transcripts. The weight fractions of the other mRNA species hybridizing to TRT3 range between 1.4×10^{-4} and 2×10^{-5} (Table 3). We have estimated the level of the RNA complementary to R loop 4 as 2 (±1) copies per cell. In this case there was a large error due to the sample size and the small numbers of this specific R loop observed. For the hybridization reactions in Table 3, RNA was used at only 330 μg/ml. In principle, the experiments could be carried out with 10- to 20-fold higher RNA concentrations. We therefore suggest that one could measure the abundance of an RNA composing 2×10^{-6} of the total in a sample; this would be 0.6 cytoplasmic poly(A)$^+$ molecules for a typical mammalian cell (27).

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