Volume 14 Number 19 1986 Nucleic Acids Research

In situ dot blots: quantitation of mRNA in intact cells

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Received 30 June 1986; Revised and Accepted 28 August 1986

ABSTRACT

A rapid, simple and reproducible dot blot method is described for quantitating the amounts of specific messages in small numbers of intact cells. The method has been used to accurately determine the number of histone H4 mRNA molecules in growing (~40,000) and in starved (~1600) Tetrahymena thermophila, and to measure the amount of message contributed by an E. coli plasmid containing part of the S10 ribosomal operon. Use of the method is illustrated to optimize in situ hybridization protocols and to measure mRNA amounts in cell lysates. Preliminary studies also indicate that the method can be used to detect mRNA in intact yeast cells.

INTRODUCTION

Recent studies have demonstrated that in situ hybridization is a powerful tool for localization and quantitation of mRNA in individual cells and in tissue sections (1-7). However, the critical variables (fixation and pretreatment of cells, hybridization parameters) vary markedly among different investigators, making it necessary to optimize conditions for any new cell type being examined. To facilitate our studies on regulation of histone gene expression in the ciliated protozoan Tetrahymena thermophila, we sought to develop a method whereby optimum conditions for in situ hybridization could be easily determined. In so doing, we have developed a simple, rapid and sensitive dot blot method that can be used to quantitate levels of specific mRNAs in small numbers of intact cells and in cell homogenates. The method is also applicable to yeast and to E. coli.

MATERIALS AND METHODS

Preparation of Tetrahymena

Tetrahymena thermophila (strain 399) were grown axenically in enriched proteose peptone (8). Log phase cells were grown to a density of 2 x 10^5 cells/ml at 28°C. Starved cells were obtained by washing once in 10 mM Tris-HCl, pH 7.4 and starving in the same buffer at 28° C for 20 hrs.

Prior to fixation, cells were collected by centrifugation at 1500 x g for 1 min at room temperature and washed once in 10 mM Tris-HCl, pH 8.0. The cell pellet was gently but thoroughly resuspended in water. Glutaraldehyde (Sigma, Grade I, 25% aqueous solution) was added to a final concentration of 1%. The cell suspension was mixed for 30 sec at room temperature and placed on ice immediately. Except where noted, cells were fixed for one hour on ice, shaking occasionally during the first 30 min. Fixed cells were then washed twice in ice-cold water for 10 min each, twice in 50% ethanol at room temperature for 15 min each and once in 70% ethanol at room temperature for 15 min. Cell concentration was adjusted to 10^6 cells/ml with 70% ethanol and the cell suspension stored at -20° C for up to seven months with no obvious reduction in signal.

Preparation of Glass Fiber Filters

Glass fiber filters (Whatman Glass Microfiber Filters 934-AH) cut to fit a 96-well BRL filter manifold were soaked in 50 µg/ml poly-L-lysine (150-300,000; Sigma) in 10 mM Tris, pH 8.0, at room temperature for 10 min, air-dried in a folded piece of Blot Block paper (Schleicher and Schuell) and stored with dessicant at room temperature until use.

Loading and Pretreatment of Cells

Filters were placed on the manifold and moistened with 10 mM Tris-HCl, pH 8.0. The manifold was tightened and connected to an aspirator. The desired number of cells in 100 µl of 70% ethanol was loaded into each well under strong suction. The filter was removed and air-dried on a piece of blot block paper.

Filters were incubated with proteinase K (2-5) in proteinase K buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA) at 37° C for 1 hr. The filters were rinsed once with proteinase K buffer, immersed in 0.1 M triethanolamine (J. T. Baker, Trolamine) pH 8.0 at room temperature for 10 min, and then in 0.1 M triethanolamine plus 0.25% acetic anhydride (added immediately before use) at room temperature for 10 min. The filter was then rinsed with 2X SSC (20X SSC is 3.0 M NaCl and 0.3 M sodium citrate, adjusted to pH 7.0 with HCl) and air-dried on a piece of Blot Block paper.

Preparation of Cell Lysate

Growing cells were collected from culture medium by centrifugation at 1500 x g for 1 min at room temperature and washed once in 10 mM Tris-HCl , pH 8.0. The cell pellet was gently loosened in a minimum volume of water and cells were lysed on ice in a medium previously shown (9) to preserve polysomes (30 mM Tris, 20 mM KAc, 50 mM $MgCl_2$, 2% spermidine tri-HCl, 1 mg

heparin-sulfate/ml, 50 µM aurin tricarboxilic acid, 2 mM dithiothreitol, 10 µg cycloheximide/ml, 0.5% v/v NP-40, adjusted to pH 7.1 with acetic acid). The lysate was kept on ice for 10 min and then fixed in glutaraldehyde (final concentration of 1%) for 1 hour on ice. At the end of fixation, 1/15 volume of 3 M NaAc, pH 5.2, and 2 volumes of 100% ethanol were added and the lysate was kept at -70° C for 30 min. The precipitate was pelleted by centrifugation at 9,000 x g for 15 min at 4°C. The pellet was washed twice with 70% ethanol and re-suspended by sonication in a volume of 70% ethanol to give a concentration equivalent to 10^5 cells/ml. Sonication was carried out in an ice water bath with a sonifier (Branson, Cell Disruptor 200) at the lowest power using two 15 sec pulses. The sonicated lysate was spotted onto a coated filter and pretreated as described above for whole cells.

Preparation of Probes

To prepare a template containing the histone H4 - I gene of Tetrahymena, the 1.3 kb Sau 3A to Hae III fragment containing the histone mRNA coding and 3' flanking region was excised from plasmid p508.8 (10). The 1.3 kb fragment was ligated into Bam HI and SmaI digested transcription vectors pSP 64 and pSP 65 (Promega Biotec, Inc.) and the recombinants were used to transform E. coli SK 1592. Recombinants containing the H4 genes in both orientations relative to the Sp6 phage promoter were isolated and were designated pSP 64/H4IHS and pSP 65/H4IHS, respectively (Fig. 1).

Prior to transcription, templates (pSP 64/H4IHS and pSP 65/H4IHS) were truncated with either Sst I or Xba I, respectively, extracted with phenol-chloroform and precipitated with 1 volume of isopropanol. RNA was transcribed from truncated templates using an in vitro transcription kit (Amersham) according to the manufacturer's instructions. ^{32}P-UTP (Amersham, SP-6 tested) was used for all experiments except the in situ hybridization described in Fig. 9.

Following RNA synthesis, the DNA template was removed by digestion with RNase-free DNase I (50 µg/ml, Worthington DPRF) for 10 min at 37°C in the presence of 1200 units/ml RNasin (Promega). RNA was extracted with phenol-chloroform and precipitated with isopropanol after addition of tRNA carrier (BRL) and ammonium acetate to 0.3 M. The precipitates were dissolved in sterile, diethylpyrocarbonate (DEPC) treated water. Alternatively RNA was purified from unincorporated triphosphates by Sephadex G-50-150 spun-column chromatography. The specific activity of the probes ranged from 1-7 x 10^7 cpm/µg.

The lengths of denatured transcripts were determined by agarose gel

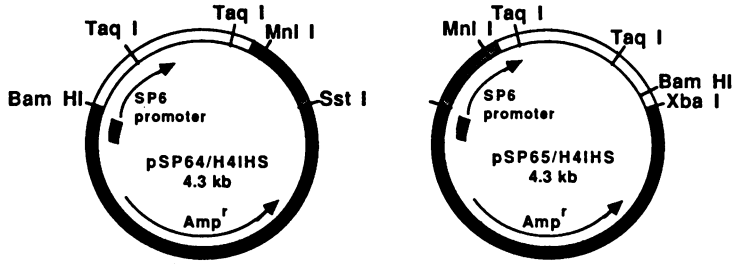

Figure 1 - Structures of pSP64/H4IHS and pSP65/H4IHS. The black areas represent pSP64 and pSP65 sequences. The cross-hatched areas represent the coding sequences of histone H4-I of Tetrahymena and the white areas represent the 3' I flanking region of H4-I.

electrophoresis (11-13). RNA was incubated in 1 M glyoxal, 10 mM $NaPO_4$, pH 6.5, at 50°C for 1 hour. Samples were electrophoresed on 0.8% agarose gels in 10 mM phosphate buffer, pH 6.5. Only preparations consisting mostly of full-length transcripts were used. Unless otherwise stated, the fragment sizes of RNA probes were adjusted to a mass average of approximately 150 bases by limited alkaline hydrolysis (6) prior to hybridization.

Hybridization

Unless otherwise indicated probe concentration was 6 ng/ml and 0.08 ml of hybridization solution was used per cm^2 of filter. The hybridization solution contained 50% formamide, 0.75 M NaCl, 0.15 M Tris-HCl, pH 8.0, 10 mM EDTA, 0.2 M phosphate buffer, pH 6.8, 1X Denhardt's and 10% dextran sulfate (14). Glass fiber filters were placed in Seal-A-Meal bags, the probe and hybridization solutions were mixed thoroughly and added. Hybridization was at 45°C for 16-20 hrs unless otherwise indicated.

After hybridization, glass fiber filters were washed with gentle shaking in three changes of 2X SSC for 5 min each at room temperature, two changes of 0.1X SSC for 20 min each at 55° C, and finally with 0.1 X SSC for 5 min at room temperature. Filters were placed on Block Blot paper and air-dried or dried under a heat lamp followed by exposure to Kodak XAR-5 X-ray film at -70°C with or without intensifying screens (DuPont Cronex Quanta III) for various times. After suitable (often multiple) exposures, the autoradiograms were quantitated by densitometer (LKB soft laser densitometer) tracing, or by cutting spots out of the filters and counting in a liquid scintillation counter.

Data Analysis

Densitometer tracings were quantitated by planimetry using a Tektronx

4956 digitizer and 4052 computer. All quantitative data are presented in terms of either counts per minute or relative areas of the densitometer tracings.

In situ hybridization

Glutaraldehyde-fixed cells were spotted on poly-L-lysine coated glass slides (6) and air-dried. Slides were then pretreated with the procedures described above for glass fiber filters except that after rinsing with 2X SSC, they were dehydrated by passing through 30%, 50%, 70%, 85%, 95%, 99% (twice) ethanol and air-dried.

Tritium-labeled probes were prepared and hybridization procedures performed as described by Cox et al. (6). The specific activity of the probe was 4 x 10^7 cpm/µg. Cells were stained with 1% Giemsa (Sigma) in 10 mM phosphate buffer, pH 6.5, for 15 min. Light microscopic autoradiography was performed as described by Angerer and Angerer (2).

Preparation of Yeast Cells

Yeast cells were fixed by a modification of the method of Novick and Botstein (15). Saccharomyces cerevisiae were grown in YPD medium (1% yeast extract, 2% Bacto-peptone, 2% dextrose) at 30°C to early log phase (~10^7 cells/ml), harvested by centrifugation (1500 x g for 3 min) at room temperature, washed once and then resuspended thoroughly in 0.1 M potassium phosphate (pH 7.5). Glutaraldehyde was added to a final concentration of 1% and the cell suspension was immediately placed on ice for 1 hr with occasional shaking for the first 30 min. The fixed cells were pelleted, washed twice with phosphate buffer and divided into aliquots. One aliquot was resuspended in 1.2 M sorbitol, 0.1 M potassium phosphate (pH 7.5), 24 mM 2-mercaptoethanol and 0.05 mg Zymolyase 60,000 (Seikagaku Kogyo LTD) and placed at 30°C for 2 hrs. These cells were pelleted, resuspended in 1.2 M sorbitol in 0.1 M potassium phosphate (pH 7.5), loaded onto coated filters and air-dried. Other cells were left untreated. Filters were pre-treated and hybridized as described above for Tetrahymena. The remainder of the fixed cells were resuspended directly in 1.2 M sorbitol - 0.1 M phosphate, loaded onto filters, and air dried. These filters were incubated in the zymolyase solution described above for 2 hr at 30°C. The filters were washed twice in sorbitol-phosphate, air-dried, pretreated and hybridized.

Preparation of E. coli Cells and RNA Probes

Plasmid pLL36 contains the E. coli ribosomal protein S10 promoter, leader, S10 structural gene and part of the L3 gene inserted into plasmid pSC101 (16). E. coli strain LL308 (17) harboring pLL36 or pSC101 were grown

to log phase (2 x 10^8 cells/ml) at 37°C in AB minimal medium (18) supplemented with 2.5 µg thiamine/ml, 0.2% (w/v) glucose, and casamino acids at 20 µg/ml. Cells were harvested by centrifugation (12,000 x g for 5 min) at 4°C, washed once and then resuspended in 10 mM $MgSO_4$. Glutaraldehyde was added to a final concentration of 1% and the cell suspension was immediately placed on ice for 30 min with occassional shaking. Following fixation, cells were pelleted, washed twice with ice cold 10 mM MgSO4, and once with 50% ethanol at room temperature for 10 min. Cells were then resuspended in 50% ethanol at a concentration of 10^8 cells/ml and stored at -20°C and the desired numbers were loaded onto poly-L-lysine coated glass fiber filters (see above) and air-dried. Filters were incubated with 2 µg/ml proteinase K at 37°C for 1 hr, pretreated, hybridized and analyzed as described above for Tetrahymena.

Plasmid pT712 carrying part of S10 and L3 genes inserted into plasmid pT7-1 (United States Biochemical Co.) and plasmid pT724 carrying the leader, S10 and part of L3 genes inserted to plasmid pT7-2 (United States Biochemical Co.) provided by Drs. L. Lindahl and J. Zengel were used to transcribe ^{32}P-labeled RNA probes. pT712 and pT724 were truncated with Hind III or Eco RI, respectively, and transcribed with T7 RNA polymerase (Pharmacia, Inc.) to yield antimessage (500 b) and message (750 b) strand transcripts. Transcription reactions were carried out according to procedures described above.

RESULTS AND DISCUSSION

Analysis of fixation and proteinase treatment

Extensive quantitative in situ analyses of mRNA have been done by the Angerers and their colleagues (6) using glutaraldehyde fixed materials. We reasoned that their methods could be rapidly adapted and optimized for Tetrahymena simply by using poly-L-lysine-coated glass fiber filters instead of coated slides and by substituting ^{32}P-labeled probes for ^{3}H-labeled probes and (7) analyzing the results by densitometry or scintillation counting (7) rather than by counting silver grains. We chose a cloned H4 gene as our probe since the relative amounts of this message have been measured previously in growing and starved Tetrahymena (9,10), and an additional independent estimate of the relative abundance of the message can be obtained (see below).

Figure 2 demonstrates that when cells were fixed for various times in 1% glutaraldehyde and digested with 8 µg/ml proteinase K for 1 hr at 37°C, the intensity of hybridization with an H4 anti-message probe progressively decreased with increased fixation. However, digestion with 32 µg proteinase

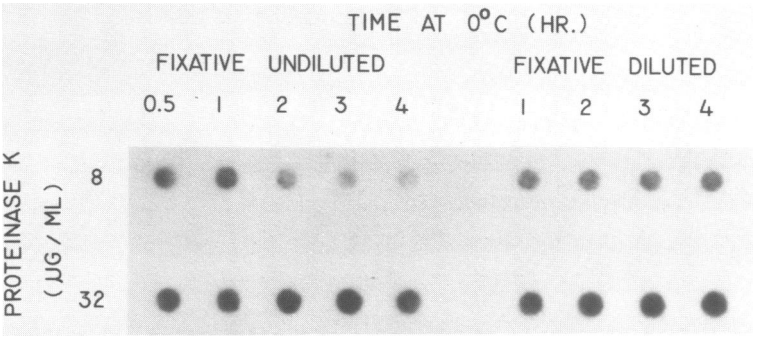

Figure 2 - Effects of Fixation Time on Hybridization. Growing cells were brought to 1% glutaraldehyde, then placed on ice for the indicated times. After 30 min, some samples were diluted with 10 volumes of ice-cold water and retained on ice for the indicated times. Fixatives were washed out immediately after the indicated incubation time. Prior to hybridization, cells were incubated with 8 or 32 µg/ml of proteinase K at 37°C for one hour. 2×10^3 cells were loaded into each well of a filter manifold and aspirated onto glass fiber filters. Anti-message transcripts of pSP64/H4IHS were hybridized to the cells for 16 hrs. Autoradiography was done with an intensifying screen for 16 hrs at -70°C.

K/ml resulted in a strong signal that was independent of fixation time. If cells were fixed for 0.5 hr, diluted 10-fold with ice-cold water and kept on ice for various times, the signal was relatively independent of fixation time at both proteinase concentrations, but more extensive proteinase K digestion resulted in a stronger signal. We conclude that there is a relationship between extent of fixation and subsequent treatment with proteinase K; more extensive fixation requires more intensive proteinase treatment.

For dot blot analyses, it seems clear that increased proteinase K digestion significantly increases the signal for glutaraldehyde-fixed cells. It should also be noted that fixation either in 1% glutaraldehyde for more than 0.5 hr followed by digestion at 32 µg/ml or fixation for 0.5 hr followed by dilution (1:10) and proteinase digestion at either concentration can be used to analyze multiple, serially collected samples from rapidly changing populations of cells over a period of 4 hrs.

To study the effects of proteinase K digestion more systematically, cells fixed for 1 hr were digested with varying concentrations of proteinase K and hybridized with either message or anti-message transcripts (Figure 3). The hybridization signal with the anti-message transcript increased progressively with increasing digestion, although the differences between 8 and 48 µg/ml are less than 1.7 fold. No background hybridization (assayed using the message transcript) was detected even though our protocol does not use the RNAse

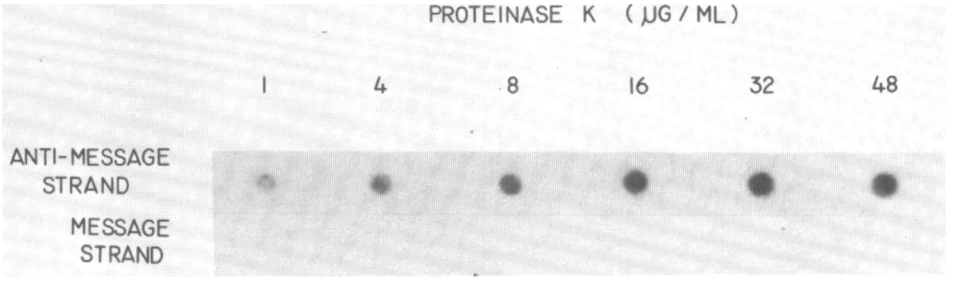

Figure 3 - Effect of Proteinase Digestion on Hybridization. 2 x 10^3 growing cells were spotted on glass fiber filters. Prior to hybridization, samples were incubated with the indicated concentrations of proteinase K at 37°C for one hour. Transcripts from pSP 64/H4IHS (anti-message) and pSP 65/H4IHS (message) were hybridized to cells for 15½ hrs. Autoradiography was done with an intensifying screen for 16 hrs at -70°C.

digestion common to *in situ* protocols using RNA probes (2,6). Similar studies using starved cells (data not shown) indicated that they fell off the filter upon treatment with 32 µg/ml proteinase K. Since, as described below, accurate quantitation can be achieved at either high or low proteinase K concentrations, once an adequate signal has been obtained the particular choice of digestion conditions may be of little importance or may (as for *in situ* hybridization) be dictated by other considerations. In practice, useful fixation and proteinase K treatments either for dot blots or for *in situ* hybridization (see below) can be established quickly by experiments such as those in Figs. 2 and 3 and simple morphological criteria. It should also be noted that the levels of proteinase K digestion found to be optimal for *Tetrahymena* are considerably higher than those used in other systems (6).

Quantitation and Kinetic Parameters

Our initial studies on the quantitative aspects of this method were performed at low concentrations of probe to save time and expense. Figure 4A illustrates that, when small numbers (2-4000) of cells and low concentrations (6-24 ng per ml) of probe are used, signal strength is proportional to time over the entire range in which the film response was linear. In Fig. 4B, the slopes of the lines in Fig 4A have been plotted against the product of cell number and probe concentration. These results demonstrate that, under these conditions, hybridization signal is directly proportional to cell number, time of incubation and probe concentration, providing the essential prerequisites for quantitative analysis. It should noted that 2-fold differences in amounts of RNA hybridizing per dot are easily and accurately measured. In other

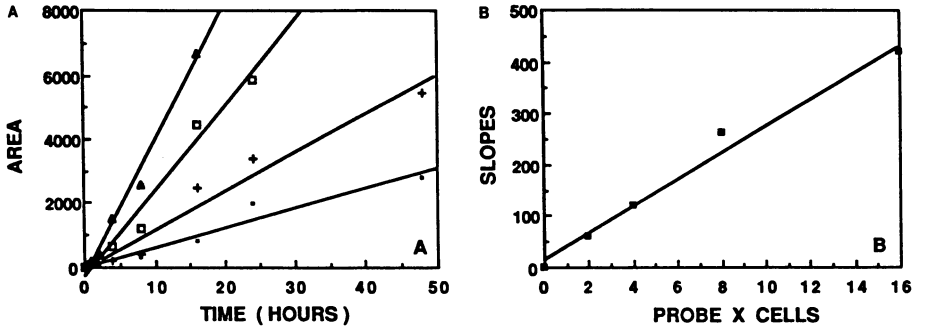

Figure 4 - Dependence of Hybridization on Time, Cell Number and Probe Concentration. Transcripts of pSP 64/H4IHS were hybridized to growing cells for various times. (A) Linear regression lines were plotted of area vs. hybridization time. Symbols denote combinations of cell number and probe concentration: ( )-2 x 10^3 cells, 6 ng/ml of probe; (+)-2 x 10^3 cells, 12 ng/ml of probe; ( )-4 x 10^3 cells, 12 ng/ml of probe; (Δ)-4 x 10^3 cells, 24 ng/ml of probe. Note that while we have approximated these curves with straight lines, more extensive kinetic analyses (Figs. 5 and 6) suggest they are actually the early portions of first order curves and that signals approximately proportional to time can only be expected at probe concentrations well below saturation. (B) A regression line was plotted with the slopes of lines in (A) vs. the products of cell number and probe concentration.

studies (data not shown) we found that hybridization is proportional to cell numbers from 1000-10,000 log phase cells (or 4000-32,000 starved cells) per dot. When larger numbers of cells were used, the signal was non linear, probably because the capacity of the filter to retain cells was exceeded.

In an attempt to establish the concentration of probe required to saturate hybridization to cellular RNA in a given hybridization time, a more extensive analysis of the effects of probe concentration was performed (Fig. 5). An unusual result was obtained. Although saturation was consistently reached at about ~2 µg/ml, the curve had a distinctly biphasic nature when proteinase K concentrations of 8 µg/ml were used. Pretreatment with different batches of proteinase K led to saturation at the same RNA concentration and reached similar saturation values (Fig 5), but showed varying percentages of the two components of the curve. We reasoned that the simplest explanation for these results was that, for unknown reasons, there were 2 different states of accessibility of the probe to the messages in the cell and that the extent of proteinase K digestion determined either the fraction of mRNA per cell or the fraction of cells in each state. To test this hypothesis, cells were digested more extensively (32 µg/ml) with proteinase K. As can be seen in Fig. 5, more extensively digested cells saturated at approximately the same

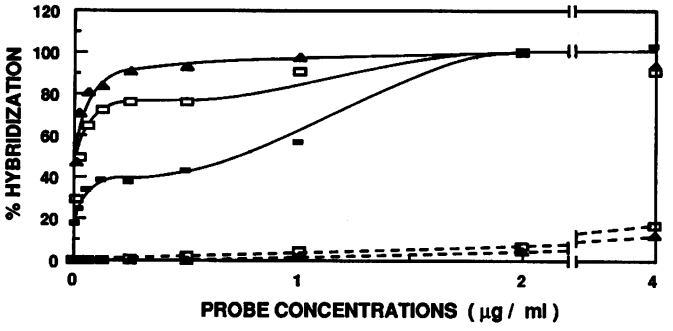

Figure 5 - Dependence of Hybridization on Concentration of Probe. Solid lines represent the pSP 64/H4IHS anti-message probe, dashed lines represent the pSP 65/H4IHS message probe. Probes were hybridized to 8 x 10^5 growing cells per dot for 16 hrs. Symbols denote two concentrations of proteinase K: ( )-32 µg/ml, (Δ) and (•)-8 µg/ml from two different preparations of proteinase K. Values in each curve are normalized to the saturation value to facilitate comparison of kinetics. Actual saturation values varied by two-fold or less (Table I).

probe concentration and showed a similar saturation value, but appeared to contain only a single, fast component. This result is consistent with our interpretation and suggests that extensive deproteinization converts all of the H4 mRNA to a more accessible form.

Because our interest was in developing a simple method for quantitating RNA in intact cells (see below), we have not performed extensive kinetic studies or attempted a more detailed analysis of the biphasic curves in Fig. 5. We have, however, determined the kinetics of hybridization using

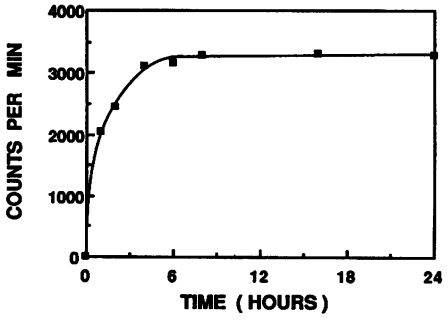

Figure 6 - Time Dependence of Hybridization. Transcripts of pSP 64/H4IHS were hybridized to 8 x 10^5 growing cells per dot at a probe concentration of 1.5 µg/ml for the indicated times.

Table I. Number of Histone H4 Messages in Growing Tetrahymena

Experiment	Proteinase K	Message Number
1.	8 $\mu g/ml$	56,000
2.	8 $\mu g/ml$	39,000
3.	8 $\mu g/ml$	28,000
4.	32 $\mu g/ml$	38,000
Average (±S.E.)		40,000 ± 6000

saturating amounts of probe, since that is a condition likely to be used in many studies. Saturation was reached in about 6 hrs (Fig. 6), a rate similar to that (4-10 hrs) found in *in situ* studies (6,7).

Figure 5 also provides an estimate of the background hybridization at the highest required probe concentrations (i.e., at saturation). Even without RNAse treatment, backgrounds are not a problem. Interestingly, background hybridization is slightly lower with more extensive protease digestion.

The H4 Message Content of Growing Tetrahymena

The number of H4 messages per cell was calculated (7) using the saturation values for H4 mRNA obtained in 4 dot blot experiments (Table 1). The measured value, 40,000 messages per cell, can be compared with calculated estimates of the H4 mRNA content of Tetrahymena. Tetrahymena macronuclei in G_1 contain about 10 pg of DNA; G_1 micronuclei contain about 0.46 pg (19). Since eukaryotic chromatin contains a 1:1 complex (by weight) of histone and DNA, Tetrahymena must synthesize about 10.5 pg of histone per generation. Approximately one-fifth of the histone, or 2 pg, is H4. Estimates of the rate of protein synthesis (20) indicate that Tetrahymena synthesize approximately 1000 pg of protein per cell generation. Since the translational efficiency of histone message is similar to that of other messages (10,20), it is reasonable to assume that H4 messages represent approximately (2.1/1000) 0.0021 of the total cell messages. The number of messages in growing Tetrahymena has been estimated by a variety of methods to be about 3×10^7/cell (21). Thus, we calculate there are approximately ($3 \times 10^7 \times 2 \times 10^{-3}$ =) 60,000 H4 messages per cell. An alternative method for estimating the histone message amount takes advantage of the fact that Hallberg *et al.* (22) have shown that ribosomal protein synthesis accounts for about 20% of the total synthesis in growing Tetrahymena. Since ribosomes are a 1:1 complex by mass of RNA and protein and there are 320 pg of rRNA per cell in growing Tetrahymena (21), the number of total H4 messages per cell can be estimated as follows: Number of

H4 messages = 2 pg histone protein/320 pg ribosomal protein x 20% x 3 x 10^7 = 37,500. The values we have obtained by saturation hybridization are in good agreement with these estimates.

Perhaps the most important point to emerge from these studies is that quantitative analyses are best done using the highest possible level of proteinase K digestion consistent with good retention of cells and RNA, and with saturating amounts of probe. However, as described below, highly reliable, quantitative results also can be obtained at very low probe levels using less extensive protease digestion.

Quantitative Analyses with Sub-saturating Amounts of Probe

To measure the absolute number of messages per cell it is necessary to hybridize to saturation which can be reached in conveniently short times if large amounts of probe are used (Fig. 6). The results in Fig. 4 suggest that saturation may also be approachable by hybridizing low concentrations of probe for longer times. In situ hybridizations usually use high resolution but low efficiency ^{3}H-labeled probes that frequently require saturating levels of probe to give detectable signals in reasonable exposure times. The extremely small volumes needed for in situ hybridization makes the use of high concentrations of probe practicable. Dot blots require larger volumes of hybridization solution, making the use of saturating probe concentrations expensive. However, the high sensitivity of detection afforded by the use of ^{32}P-labeled probes and large numbers (1000-8,000) of cells per dot also makes it possible to detect messages using extremely low probe concentrations (Figs. 2-4). Since most experiments require relative rather than absolute measurements of message amounts (i.e. comparison of the message amounts in different cell types or in the same cell type in different physiological states), it seemed important to demonstrate that the method described here could accurately estimate the relative amounts of messages using small amounts of probe.

As a test system we measured the relative amounts of histone H4 message in growing and starved cells. Previous studies using isolated RNA (cytoplasmic or poly A^+) estimated the relative amounts of H4 mRNA in growing cells to be 30 to 40 times that in starved cells (9,10). Because of the possibility that starved cells and growing cells have different susceptibilities to proteinase K, we measured relative amounts of H4 mRNA at three different proteinase K concentrations. Hybridization values were estimated by calculating the slope of signal intensity versus cell number for each treatment. As seen in Fig. 7, identical results were obtained in each

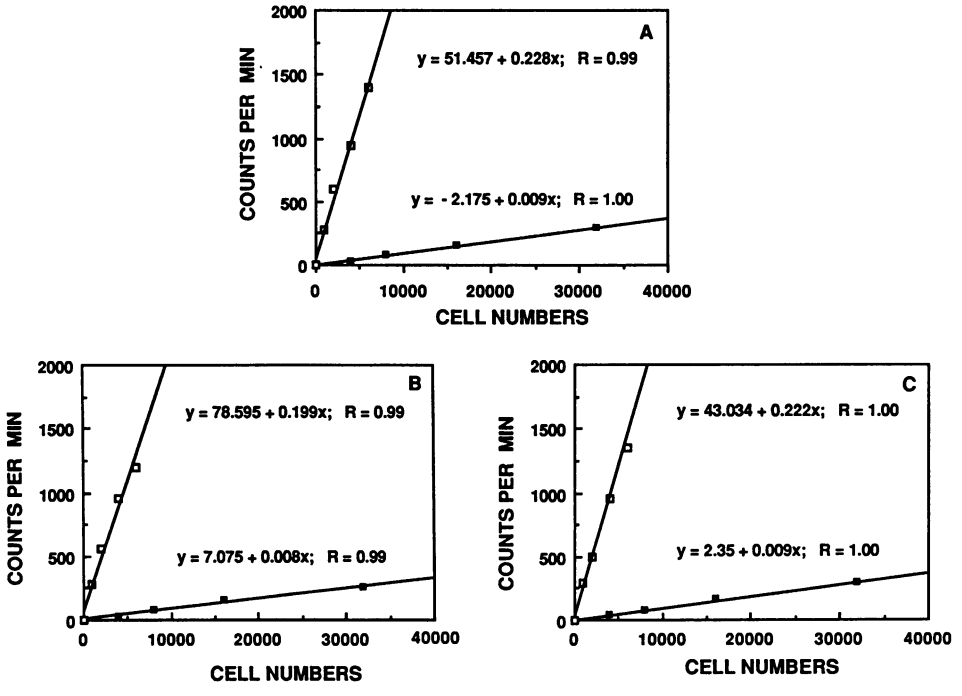

Figure 7 - Hybridization to Growing and Starved Cells Treated with Different Concentrations of Proteinase K - A, B, C: Growing ( ) or starved ( ) cells digested with 4, 8, and 16 µg/ml of proteinase K, respectively.

case: the ratio of H4 mRNA in growing and starved cells measured by the dot blot method was 25, regardless of which proteinase K concentration was used. This result is in good agreement with the much more laborious and variable measurements on isolated RNA (9,10). These results indicate that, as for *in situ* hybridization (2), accurate quantitation is independent of probe concentration. Accurate quantitation also appears to be unaffected by protease treatment, although we suggest that all comparisons be done initially at two different extents of proteinase K digestion.

Hybridization to Cell Lysates

Many tissues cannot be easily dispersed into suspensions of single cells. Also, it is frequently desirable to measure mRNA amounts in cell fractions. For these reasons, we tested the feasibility of analyzing cell lysates by the methods described above. Previous studies have demonstrated that most if not all of the mRNA in *Tetrahymena* can be found in the post-nuclear supernatant (9). Figure 8 demonstrates that signals of similar intensities are obtained

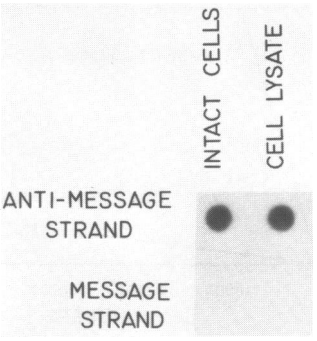

Figure 8 - Comparison of Hybridization to Intact Cells and Cell Lysate. Intact cells and cell lysate were prepared as described under methods. 10^4 intact cells or cell lysate derived from 10^4 cells were spotted onto glass fiber filters. Samples were treated with 8 µg/ml proteinase K at 37°C for 1 hr. Message or anti-message transcripts were hybridized to the samples for 16 hrs. Autoradiography was done with an intensifying screen for 3 hrs at -70°C.

from equivalent amounts of cells or of cell lysate. Background hybridization assayed using the message transcript was undetectable in both cases. These results indicate that it should be possible to use this method on homogenized tissues and on cell fractions.

Miscellaneous Other Conditions

(a) Poly-L-lysine. Coating the glass filter (or slides) with poly-L-lysine is absolutely required for retention of cells. Filters (or slides) can be stored for at least 6 months without loss of binding capacity for cells. Poly-L-lysine coated nitrocellulose can also be used but gives variable results and is significantly more expensive than glass filter.

(b) Probe size. It has long been thought that small probe fragments (<200-300 nucleotides) penetrate fixed cells more effectively than larger fragments (1,2). However, some studies indicate that higher signals are obtained with larger sized probe fragments (7). Using sub-saturating probe concentrations we have found that, without RNAse treatment, the effect of probe size varies with the extent of proteolytic digestion. At low proteinase K concentrations (< 4 µg/ml) hydrolyzed probe (~150 nucleotides) gives a 2-fold higher signal than unhydrolyzed probe (~1300 nucleotides). However, at higher proteinase K concentrations (> 8 µg/ml), the larger probe shows a 2-8 fold greater signal. Thus, it is possible to obtain stronger signals with larger probe fragments. However, it should be noted that all of our analyses have been performed with sheared probes and the validity of using larger probes for quantitative studies remains to be established.

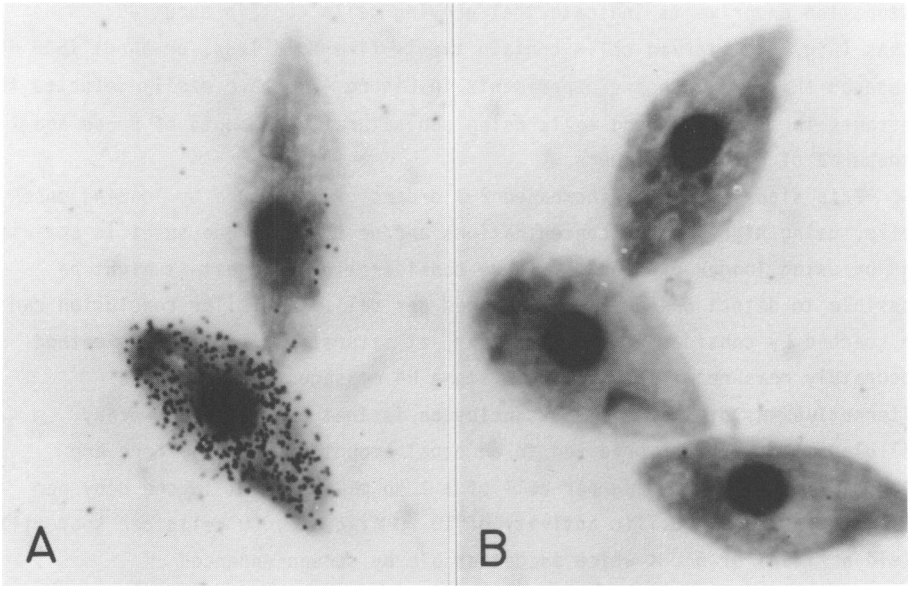

Figure 9 - Autoradiography of Growing Cells with ^{3}H-labeled Probes. (A) -Anti-message transcripts of pSP 64/H4IHS; (B) -Message transcripts of pSP 65/H4IHS. Specific activities for both probes were 4 x 10^{7} cpm/ug. The heavily labeled cell is presumed to be in macronuclear S-phase while the unlabeled cell is assumed to be a non-S-phase (G_1 or G_2) cell.

Application to In Situ Hybridization

For in situ hybridization, morphological criteria often dictate minimum fixation times and maximum proteinase treatments. For example, in Tetrahymena fixed for 1 hr, nuclei frequently fell out of cells digested with more than 8 µg proteinase K/ml. Using the maximum proteinase K concentration consistent with reasonable morphology (8 µg/ml), tritiated probes were hybridized to cells on poly-L-lysine coated glass slides. Fig. 9 shows log phase cells labeled with H4-I anti-message or message transcripts. Intense signals were obtained after 2 weeks exposure and little or no background hybridization was observed. It should be noted that this result was achieved the first time in situ hybridization was attempted, using conditions otherwise determined from the dot-blot experiments described above, coupled with RNAse treatment as described by Cox et al. (6).

Sensitivity

The dot blot method described here is extremely sensitive. The histone H4 messages in Tetrahymena are about 800-1000 nucleotides long (10). Our

saturation experiments indicate that growing cells contain about 40,000 H4 mRNAs (Fig. 5); starved cells contain twenty-five-fold less, or about 1600 H4 messages (Fig. 7). In the experiments in Figure 4 we have easily detected the messages in a few thousand cells using sub-saturating amounts of probe and exposures of only a few hours.

This signal could be increased 2-3 orders of magnitude by loading more cells, using higher probe concentrations and/or higher probe specific activity and by using longer exposures. These considerations suggest it might be possible to detect one or a few messages per cell. A similar conclusion can be reached by considering the fact that, at saturation (Fig 5), the method accurately measures the number of histone H4 messages per cell. An alternative way of stating this conclusion is that at saturation every cellular message has hybridized to an equal amount of probe. There are approximately 5×10^{-13} µg per cell of a 1 kb mRNA present in one copy per cell. At a probe specific activity of 10^9 CPM/µg, 10,000 cells per spot will yield a signal of 5 CPM which is detectable by screen-enhanced autoradiography. It should be noted that these calculations apply to Tetrahymena which is a very large cell. For more typical, smaller cells, it should be possible to increase the signal by applying more cells per spot.

The above considerations indicate that detection of extremely low levels of mRNA per cell is feasible. It is likely that noise levels rather than signal detection will ultimately limit the sensitivity of this method. It should be noted that few, if any, biological situations require accurate measurment of as few as one mRNA molecule per cell and, in the results described here, we have not yet found it necessary to employ RNAse treatment to reduce backgrounds.

Hybridization to Intact Yeast Cells

Fig. 10 shows that a strong signal is obtained using the Tetrahymena H4 gene to probe yeast cells. Similar intensities are obtained if cell walls are removed either before or after spotting on the filter; little or no signal is obtained if the cells are not treated with zymolyase to remove the cell wall. Although we have not attempted to optimize the hybridization conditions or to estimate quantitative aspects of hybridization to yeast, these results suggest that this method should be applicable to yeast. Cell types lacking cell walls (e.g. mammalian tissue culture cells) have been analyzed by similar methods using gelatin coated glass coverslips (7). We think it likely that the methods described here (or slight modifications of them) should be applicable to most eukaryotic cell types and tissues.

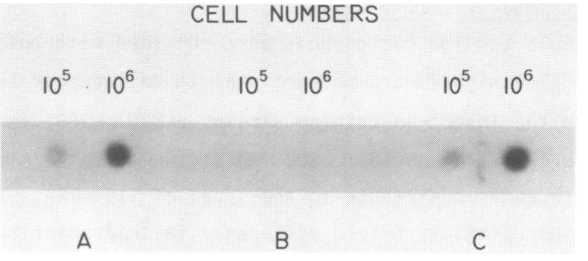

Figure 10 - Hybridization to Yeast Cells. 10^5 or 10^6 yeast cells were spotted on poly-L-lysine coated glass fiber filters (A) after (B) without (C) before removal of cell walls by Zymolyase. Proteinase K digestion was with 1 µg/ml for 1 hr at 37°C. Anti-message transcripts of pSP 64/H4IHS were hybridized to the samples for 16 hrs. Autoradiography was done with an intensifying screen for 3 hrs at -70°C.

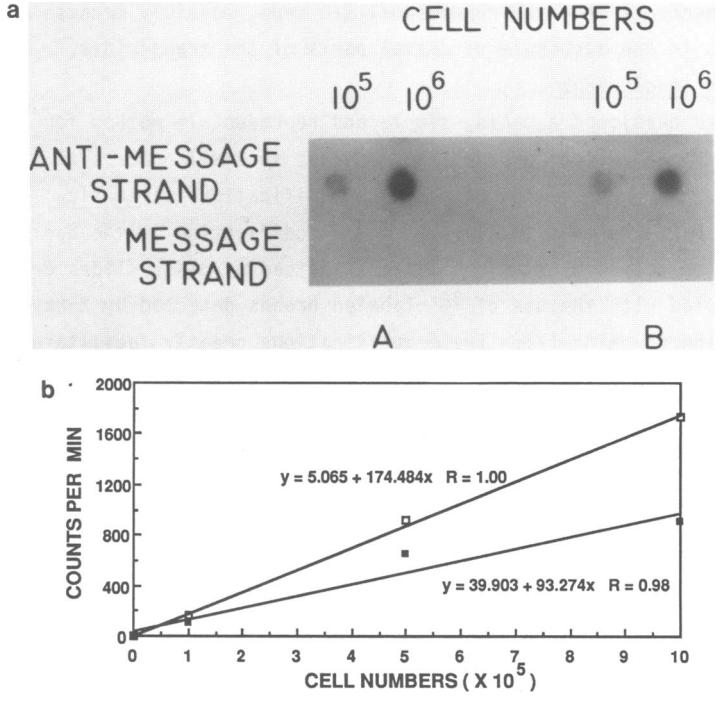

Figure 11 - Hybridization to E. coli Cells. (a) Hybridization to 10^5 or 10^6 E. coli cells from strain pLL36/LL308 (A) or pSC101/LL308 (B) on poly-L-lysine coated glass fiber filter. Proteinase K digestion was with 2 µg/ml for 1 hr at 37°C. Anti-message or message transcripts were hybridized to the samples for 16 hrs. Autoradiography was done with an intensifying screen for 4 hrs at -70°C. (b) Linear regression lines were plotted of counts per min vs. cell numbers. Counts per min at each cell concentration are the average of 5 spots. Symbols denote the two E. coli strains: ( )- pLL36/LL308, ( )- pSC101/LL308.

Hybridization to Intact E. coli Cells

Strong signals and low backgrounds were obtained with both strains of E. coli (Fig 11a). The signals are proportional to cell number (Fig. 11b) and hybridization to the insert-containing strain, pLL36/LL308, is approximately twice as high as that from pSC101/LL308 indicating that the plasmid-borne ribosomal protein genes contribute to the level of S10 mRNA. This value is lower than the approximately 6-fold difference in transcription rates of S10 message measured in these same constructs (see Table I in 16), but agrees well with the relative rates of synthesis of the S10 proteins in these cells (J. Zengel and L. Lindahl, personal communication). Taken together, these observations argue that S10 protein synthesis is regulated post-transcriptionally but not at the level of transcription or that the mRNA transcripts derived from the plasmid are translated less efficiently than are normal transcripts from the chromosomal S10 gene, possibly because of differences in the structure of distal parts of the transcripts.

Summary and Conclusions

We have developed a rapid, simple and reproducible method for quantitating the amounts of specific messages in small numbers of intact cells or cell lysates. The method is based on modifications of in situ hybridization techniques (6,7). The major modification is the use of poly-L-lysine coated glass fiber filters instead of glass slides or cover slips, coupled with the use of ^{32}P-labeled probes detected by X-ray film or scintillation counting (7). These modifications greatly facilitate the rapid examination of important methodological variables and the quantitation of mRNAs in large numbers of different samples. The method has been used to accurately measure the actual number of histone H4 mRNA molecules in growing Tetrahymena, to demonstrate a 25 fold difference in the contents of H4 messages in growing and starved cells, and to study the regulation of a ribosomal protein gene in E. coli. The method can also be used on yeast, on cell lysates and to establish and optimize the conditions for in situ hybidizations. Coupled with other methods, such as cell sorting, it should now also be possible to measure the mRNA amounts in small numbers of highly purified cell types.

ACKNOWLEDGEMENTS

This research was supported by grants from the National Institute of Health. We are indebted to Robert Angerer, Stuart Horowitz and David Pennock for stimulating discussions and advice, to Janice Zengel, Lasse Lindahl, and Alan Blowers for the wherewithal to perform studiies on E. coli and yeast.

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