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Quantitative analysis of *in situ* hybridization methods for the detection of actin gene expression

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ABSTRACT

We have implemented an efficient, quantitative approach for the optimization of in situ hybridization using double-stranded recombinant DNA probes. The model system studied was actin mRNA expression in chicken embryonic muscle cultures. Actin and control (pBR322) probes were nick-translated with P^{32} labeled nucleotides, hybridized to cells grown on coverslips, and quantitated in a scintillation counter. Cellular RNA retention was monitored via the incorporation of H^3-Uridine into RNA prior to cell fixation. Over a thousand samples were analyzed, and among the technical variables examined were the fixation protocol, proteolytic cell pretreatment, the time course of hybridization, saturation kinetics, hybridization efficiency, and effect of probe size on hybridization and network formation. Results have allowed us to develop a reproducible in situ hybridization methodology which is simpler and less destructive to cellular RNA and morphology than other protocols. Moreover, this technique is highly sensitive and efficient in detection of cellular RNAs. Lastly, the rapid quantitative approach used for this analysis is valuable in itself as a potential alternative to filter or solution hybridizations.

INTRODUCTION

We are interested in in situ hybridization as a means of studying the expression and intracellular distribution of muscle-specific mRNAs during skeletal myogenesis. The in situ hybridization technique makes possible the detection and localization of specific nucleic acid sequences within a tissue, cell or genome. Since first described in 1969 (1, 2), this method has been used primarily for the localization of DNA sequences, such as the mapping of genes to Drosophila polytene chromosomes. In more recent years cytological hybridization has begun to be applied to the investigation of cellular RNA, allowing one to obtain molecular information concerning the primary products of gene expression while preserving morphological information (3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14). The in situ hybridization approach has vast potential as a molecular tool for developmental and genetic studies. Thus far, the development and application of this approach have been largely qualitative rather than quantitative in nature. Several investigators have developed a

quantitative, methodological approach using autoradiography on cells to which probe has been hybridized (see for instance, 5, 7, 14, 15). However, because this method of assessing results is time consuming and laborious it does not facilitate extensive analyses of many technical variables. As a result critical parameters such as fixation protocol, hybridization time, cell pretreatment, probe size and probe concentration vary markedly among investigators. Therefore, the application of *in situ* hybridization would be far greater if these parameters were better characterized, so as to make the procedure more sensitive, more readily quantifiable, and less destructive to the cell.

We felt that it would contribute to the development of this technology to undertake a strictly quantitative methodological analysis of technical variables governing hybridization to cellular mRNA. To facilitate analysis of the large number of samples required, we used ^{32}P-labelled DNA probes and Cerenkov scintillation counting for rapid quantitation of hybridization to mRNA in cells grown on coverslips. We chose actin mRNA expression in chicken embryonic muscle and fibroblast cultures as our model systems, because this message has been well quantitated in these cell types (see, for instance, 16) and thus allows an evaluation of our technique. Since hybridization protocols use procedures which tend to dissociate cellular components, we also compared the effects of various experimental procedures on retention of H^3-Uridine labelled cellular RNA, quantitated by scintillation counting. The approach employed allowed us to analyze readily over a thousand samples, and, where necessary, to acquire quantitative information on both hybridization and RNA retention for each sample.

The work presented here improves and extends the methodology of *in situ* hybridization. Optimization of a number of technical variables has resulted in a simplified hybridization protocol which is highly sensitive and yet is non-destructive to cellular RNA and morphology. In addition, the approach implemented to evaluate hybridization protocols may itself be of interest in that it extends the potential utility of *in situ* hybridization to situations in which a single-cell analysis is not required. Because this approach is rapid, quantitative and requires less than a hundred thousand cells, in some instances it could provide an appropriate alternative or complement to conventional filter or solution hybridizations.

METHODS

Cell Culture

Skeletal myoblasts were isolated from the pectoral muscle of twelve-day chicken embryos and cultured by standard techniques. Cells were plated at a density of 2 X 10^6/100 mm plate into plates containing glass coverslips (22

mm^2) which had been previously autoclaved in 0.5% gelatin. Culture medium consisted of Minimum Essential Medium, 10% fetal calf serum and 2% chick serum. For most experiments cells were fixed after three days of incubation, when 20-30% of the cells had differentiated into multinucleated myofibres. Where indicated primary fibroblasts isolated from whole chicken embryos were used in lieu of muscle cells.

For experiments in which retention of total cellular RNA was monitored, H^3-uridine (38.4 Ci/mMol, NEN) was added to cultures three hours prior to fixation, at a final concentration of 10 uCi/ml. Coverslips containing cells were rinsed twice in Hanks Balanced Salt Solution (HBSS) and fixed for the times indicated in either 4% paraformaldehyde (Fisher) in phosphate buffered saline (PBS) containing 5mM $MgCl_2$, 4% glutaraldehyde in PBS plus $MgCl_2$, 3 ethanol:1 acetic acid, or Carnoy's fixative (1 acetic acid:6 ETOH:3 chloroform). After initial experiments, cells were fixed in paraformaldehyde routinely, except where specified. After fixation, coverslips were placed in 70% ETOH and stored at 4°C until further use.

From each isolation of cells large numbers of coverslips (60-100) with cells of uniform density were prepared, so that samples within and between experiments could be directly compared provided samples were from the same cell preparation. Just prior to hybridization coverslips were cut in half using a diamond pencil, and one half was hybridized with the actin probe while the other half was hybridized with the control probe (pBR 322 lacking actin insert). An additional two coverslips from each cell preparation were stained with the DNA fluorochrome DAPI (4'-6-diamidino-2-phenylindole) for ten minutes at 1 ug/ml, and were viewed under a Zeiss ICM microscope with epifluorescence optics for determination of cell numbers and percent of nuclei in multinucleated myotubes. From different cell preparations, the total RNA was isolated from one culture plate with triton and subsequent phenol extraction (17), and was quantitated by optical density at 260 nm.

Cell Pretreatments

In the course of these studies we determined that extensive cell pretreatments are not favorable for either RNA retention or hybridization. Hence, unless otherwise noted, the only cell treatments prior to hybridization were removal from 70% ETOH and rehydration in phosphate buffered saline plus 5 mM $MgCl_2$ for ten minutes, followed by ten minutes in 0.1M Glycine, 0.2M TrisHCl pH 7.4. Coverslips were then kept in 50% formamide, 2XSSC for at least ten minutes prior to hybridization.

For those experiments in which proteolytic cell pretreatments were performed, cells were rehydrated in PBS and then incubated in 5 ug/ml freshly prepared Proteinase K (Boehringer Mannheim) in PBS for the times indicated. The reaction was terminated by immersion in 2% paraformaldehyde for 30-60 seconds. Cells were then placed in 0.2M Tris, 0.1M Glycine for ten minutes and hybridized as usual.

In experiments which used acetic anhydride cell treatment, prior to hybridization cells were immersed for ten minutes in freshly prepared 0.25% acetic anhydride in triethanolamine buffer, pH 8.0 at room temperature (7, 18).

Hybridization Conditions

Unless otherwise indicated the amount of probe used was 6 ng/10 ul/sample (0.6 ug/ml). Although higher signals could be obtained with greater probe concentrations (see Results), 6 ug/ml was chosen for routine use as a means of conserving probe, since this concentration generally produced signals 40-50 fold above background. For each sample, the probe, 5 ug sonicated salmon sperm DNA (Sigma) and 20 ug *E. coli* tRNA (Boehringer) were suspended in 5 ul of 100% formamide and heated to 70-80°C for approximately ten minutes. After heating, the DNA in formamide was mixed with an equal volume of hybridization buffer, so that the final concentration of the hybridization solution consisted of 50% formamide, 2XSSC (0.3M sodium citrate buffer), 1% BSA, 10mM

Vanadyl sulfate (19), and 10% dextran sulfate (Sigma). Formamide used routinely was from Fluka or MCB, and was deionized for 30 minutes using analytical Mixed Bed Resin (AG-501-8X Biorad). Half-coverslips were placed cell side down onto the ten ul drop of the hybridization mixture on parafilm. Care was taken that the hybridization mixture was applied to the cells immediately after its preparation, while still warm. Coverslips were then covered loosely with parafilm and incubated at 37°C in a humidified chamber for the times indicated.

After hybridization, coverslips were placed in 10 ml volume Coplin staining jars (VWR) and put through three rinses of at least 30 minutes each in 2XSSC, 50% formamide at 37°; 1XSSC, 50% formamide at 37°C; and 1XSSC on a shaker at room temperature. More extensive rinsing was found to be unnecessary (see Results). For quantitation of hybridization, samples were placed in PBS in scintillation vials cell-side up and the results obtained immediately from a Beckman LS9800 scintillation counter (Cerenkov radiation). For experiments in which retention of H^3-labelled cellular RNA was monitored, samples were dehydrated in 100% ETOH, air dried, placed in scintillation fluid (liquifluor, NEN) and counted.

Probes and Nick-Translation

The actin probe consisted of pBR 322 into which the full-length transcript coding region of chicken beta-actin (2kb) had been inserted at the pst 1 site (20). Under the hybridization conditions used this probe hybridizes with the mRNAs of different actin isoforms. The control probe used was pBR 322 without insert. Plasmid DNA was nick-translated with P^{32}-dCTP (Amersham) using standard techniques (21). The specific activity of the probes ranged from 0.8-2.2 x 10^8 cpm/ug (Cerenkov Counts). Nick-translations utilized endonuclease-free DNA polymerase I(Boehringer Mannheim) and the fragment size range of the probe was controlled by varying the amount of DNAse (Worthington) in the reaction from 1 ng/ml to 300 ng/ml. For each nick-translation the probe sizes were determined by 1.5% alkaline agarose gel electrophoresis with appropriate molecular weight markers. Unless otherwise indicated, the probe size used routinely was 300-500 nucleotides. It was found that better hybridization was generally achieved when probes were used within a few days after nick-translation. For hybridization to poly A, an ^{3}H-labelled poly U probe was obtained from NEN with a specific activity of 5.1 Ci/mMol. The control was ^{3}H-labelled poly A and hybridization was for one hour.

RESULTS

Fixation

The ideal fixative for hybridization to RNA *in situ* is one which not only preserves cellular RNA and morphology, but does so in such a way that diffusion of probe throughout the cytoplasmic matrix is maximized. In past work (9) our laboratory used fixation in 4% paraformaldehyde, which provides excellent morphology as well as minimal autofluorescence. For this reason we were interested in optimizing paraformaldehyde fixation and, therefore, tested increasing paraformaldehyde fixation times to determine if there was an effect on either RNA retention or hybridization of probe. We also evaluated the three fixatives most commonly used by other investigators, 3:1, ethanol:acetic acid (3, 4, 5, 6, 11, 22), glutaraldehyde (7, 8, 14, 23, 24), and Carnoy's fixative (Enzo Biochemical, protocols for Bioprobe). In order to monitor pre-

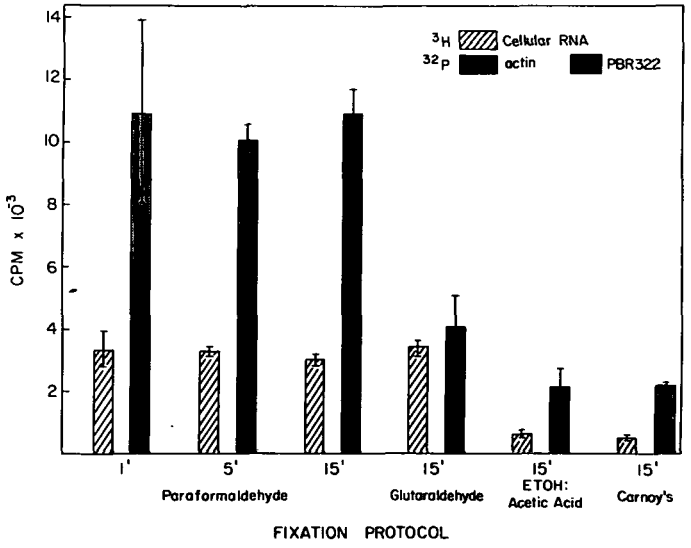


FIGURE 1: EFFECTS OF FIXATION PROTOCOL ON CELLULAR RNA RETENTION AND ON HYBRIDIZATION Cells were fixed for the times indicated in either 4% paraformaldehyde in PBS plus $MgCl_2$, 4% Glutaraldehyde in PBS plus $MgCl_2$, 3 ETOH:1 Acetic acid, or Carnoy's fixative (1 acetic acid:6 ETOH:3 choloform). Hybridization was for 16 hours as described under Methods. Results are the average of two experiments, each of which used triplicate samples. Bars indicate standard deviations.

servation of total RNA, cultures were incubated with H^3-Uridine immediately prior to fixation. The extent of hybridization achieved was evaluated using a P^{32}-labelled pBR-322 actin probe, whereas non-specific "background" was assessed using P^{32}-labelled pBR322 lacking the actin insert.

The results of these analyses are presented in Figure 1. The H^3-U results on the series of paraformaldehyde-fixed samples demonstrate that RNA retention remains essentially constant with increasing paraformaldehyde fixation times of 1-15 minutes. Although there is some internal scatter in the extent of hybridization of actin probe (^{32}P results), there is no consistent or significant difference between samples fixed for different times. Hence a treatment as brief as one minute in paraformaldehyde is adequate for both RNA preservation and for hybridization.

Under the conditions tested, results for cells fixed in 3:1 ethanol:acetic acid, glutaraldehyde, or Carnoy's fixative are all dramatically inferior to results for paraformaldehyde fixed cells. Use of either 3:1 ethanol:acetic acid or Carnoy's fixative results in loss of 75% or more of total cellular

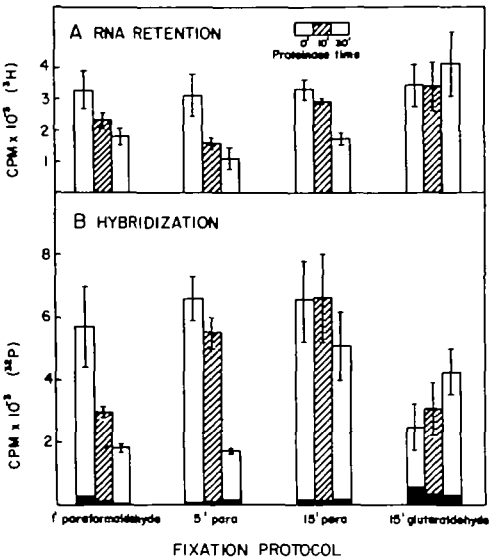


FIGURE 2: EFFECT OF PROTEINASE DIGESTION ON HYBRIDIZATION AND RNA RETENTION
Black bars in B represent hybridization of the control pBR 322 probe. Prior to hybridization, samples were incubated for varying times in 5 ug/ml Proteinase K in PBS plus $MgCl_2$. Hybridization was as described under Methods. Results presented are the average of two experiments each of which utilized duplicate samples.

RNA. Therefore, it is not surprising that hybridization is also drastically reduced, to approximately the same degree. Indeed, microscopic observation of the cells confirms that they do not appear to be well preserved morphologically. This decrease in RNA in samples fixed in ethanol:acetic acid or Carnoy's was not due to loss of cells from the coverslips, since good cell retention was obtained with all of the fixation protocols tested. Although 3:1 ethanol:acetic acid is the most widely used fixative, our results indicate that it is not the most appropriate for in situ hybridization to cellular RNA. The results on cells fixed in 4% glutaraldehyde also reveal a striking dimunition in signal relative to paraformaldehyde fixed cells, with hybridization reduced by 60-70% in glutaraldehyde fixed cells. Not only is hybridization of the actin probe markedly reduced, but the background of the pBR322 control is consistently elevated, in some experiments as much as three fold higher than the paraformaldehyde fixed cells (see background data in Figure 2 also). Hence, the signal:noise ratio for glutaraldehyde fixed cells is approximately 10:1, as contrasted with up to 70:1 for paraformaldehyde

fixed cells in the same experiment. The explanation for this lower signal, however, is not that cellular RNA has been removed, as was the case with 3:1 ethanol:acetic acid and Carnoy's fixations. Our data indicate that glutaraldehyde preserves RNA to the same extent as does paraformaldehyde. The reduced signal and increased background observed with glutaraldehyde fixed cells are consistent with the explanation that the cellular matrix is tightly bound, so as to impede both penetration of the probe and effective rinsing away of non-specific binding. Experiments below involving proteinase digestion corroborate this view.

Proteolytic Cell Pretreatment

A standard part of almost all published in situ hybridization procedures, including that previously used in our own laboratory, has been post-fixation treatments of cells with proteinase (4, 5, 7, 9, 10, 11, 13, 15, 25). The rationale for this is that partial removal of proteins would allow greater penetration of the probe through the cellular matrix and greater accessibility of mRNA for hybridization. In Figure 2 we examine the necessity for, and effects of proteinase treatments, varying proteinase treatment time at a constant concentration of 5 ug/ml proteinase K. Our results demonstrate that with paraformaldehyde fixation of 1 - 15 minutes we find no advantage to proteolytic digestion of the cells. To the contrary, Figure 5A illustrates that incubations as short as ten minutes in 5 ug/ml of proteinase K can cause loss of more than half of total cellular RNA. This loss of RNA is most marked with the 1 minute and 5 minute paraformaldehyde fixation times and was not a consequence of cells being lost from the coverslip. If one examines the results of the glutaraldehyde fixed samples, a different pattern emerges. Glutaraldehyde protects RNA (Figure 2A), but apparently crosslinks the cytoplasm such that mRNA is less accessible for hybridization (Figure 2B). If the cells are digested with proteinase, hybridization is seen to increase. In these experiments, however, after extensive digestion of glutaraldehyde fixed cells, the hybridization obtained is substantially lower and the background higher than for paraformaldehyde fixed cells with no proteolytic pretreatment. It should be noted that these experiments utilized probe fragments approximately 300 nucleotides in length which may not be optimal for each of the fixatives tested. However, we have observed that with glutaraldehyde-fixed cells proteolytic digestion is necessary for optimal signal even if smaller probe fragments or less extensive fixation (1% vs. 4% glutaraldehyde) are used. As expected, microscopic examination of these cells after proteolysis indicates that the digestion is detrimental to the cell morphology. Hence, we conclude

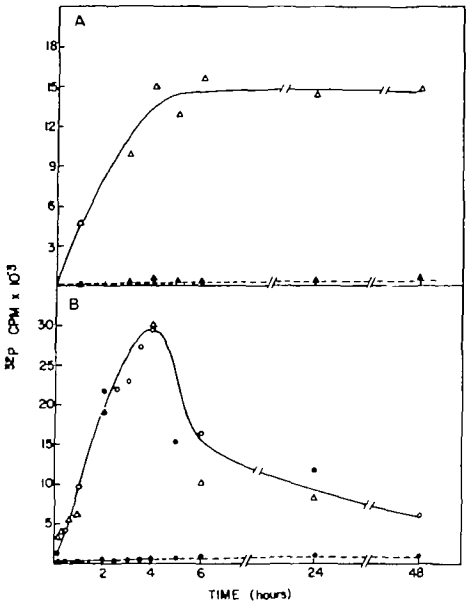


FIGURE 3: TIME COURSE
Hybridization was for the times indicated and was performed as described under methods. Open circles or triangles denote actin probe, closed circles or triangles denote pBR 322 control probe. (A) Each point represents average of six samples. (B) Triangles and circles represent data from two experiments, each of which used triplicate samples. Curves are drawn to facilitate visualization of the data and do not represent theoretical fit. For each set of data, some samples were fixed in paraformaldehyde for one minute and some for 15 minutes. Since there was no consistent difference between these, results were averaged.

that use of paraformaldehyde fixation is highly advantageous not only because it provides excellent hybridization while preserving RNA, but because it achieves this with much less degradative treatment of the cell. This can be is especially important for in situ hybridization methodology, since one major reason for this technique is to provide a means of obtaining molecular information while preserving cellular detail.

Time Course

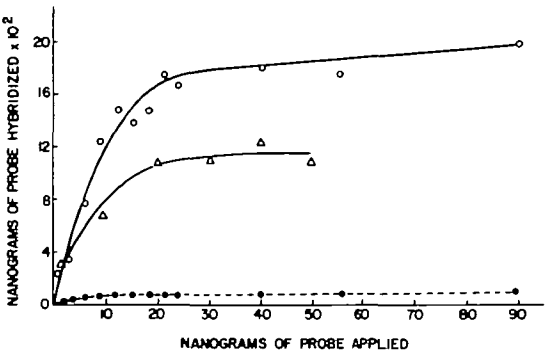
Experiments were undertaken to describe the kinetics of in situ hybridization in order to determine the optimal length of time for the hybridization reaction. Most investigators use in situ hybridization times ranging from one to four days (8, 9, 11, 13, 15, 22, 26, 27). However, results of our experiments consistently demonstrated that hybridization is readily detectable within ten minutes and is complete within the first three to four hours. Results of representative experiments are presented in Figures 3A and 3B, both of which illustrate that hybridization increases sharply and reaches a maximum within the first four hours of incubation. Because the probe DNA (0.6 ug/ml) drives the reaction, this time course should be independent of the cellular mRNA concentration.

We have observed two different results for longer hybridization times. In some experiments, as represented in Figure 3A, the signal remains relatively

constant for two days after achieving a plateau by three and one half hours. In contrast, in experiments such as the one presented in Figure 3B, we observed the less expected result that hybridization peaks in samples incubated for three to four hours, but then decreases by 50% or more in samples incubated one to two days. Results essentially equivalent to those presented in Figure 3B were obtained in three separate experiments using duplicate or triplicate samples in each experiment. We felt it important to note that this decrease in hybridization with time did occur in approximately 20-25% of experiments, and that its occurrence could not be consistently correlated with several technical parameters examined, such as the fixation time, formamide lot (see below), use of vanadyl to inhibit RNase, or use of hybridization buffer for humidifying the incubation chamber. For prolonged hybridization times the decrease in signal may be explained by degradation of RNA, since in two of three experiments there was also a significant decrease in total cellular RNA in samples hybridized for 1-2 days (not shown). However, the main conclusion to be drawn from this time course study is that, the in situ hybridization reaction is complete in as little as three hours. Hence, use of brief hybridization times may be optimal, not only for the sake of efficiency but because longer incubations introduce the risk of decreased signal as well as degradation of cell morphology through prolonged exposure of the cell to the hybridization buffer. Lastly, it should be noted that in most experiments, as illustrated by Figure 3B, the background contributed by the control pBR 322 probe increases gradually with time, an observation further favoring the use of brief hybridizations.

Concentration Curve

To determine the amount of actin mRNA detected at saturation, and to evaluate the effect of DNA concentration on signal and noise, samples were incubated with increasing concentrations of the actin or the pBR322 (control) probe. Figure 4 presents the concentration curves obtained for fibroblast cultures and for muscle cultures in an early stage of in vitro differentiation. Both concentration curves show a sharp increase in signal with increasing probe concentration up to approximately 2 ug/ml, after which a much more gradual increase or plateau is reached. In contrast, background hybridization with the control probe shows only a gradual increase with increasing DNA concentration. Because our probes are double-stranded, two competing reactions must occur simultaneously: probe-cellular mRNA hybridization and probe reannealment (see Discussion). Hence, at any DNA concentration, only a fraction of total probe hybridizes to RNA within the cell.



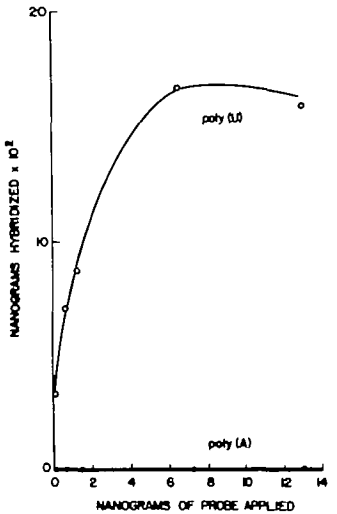


FIGURE 4: CONCENTRATION CURVE Upper panel: Hybridization with actin probe was for three hours and results presented are the average of three samples. Circles represent hybridization to muscle cultures. Open circles represent the actin probe, closed circles represent the pBR 322 control. Triangles represent hybridization of actin probe to fibroblast cultures. Hybridization of the control pBR322 to fibroblast cultures was essentially the same as for myotube cultures. Lower panel: Hybridization with poly-U and poly-A (control) probes was for one hour.

In the experiment presented on fibroblast cultures, the average number of actin mRNA molecules hybridized per cell can be calculated from the amount of probe hybridized at saturation and the number of cells per sample, as determined by cell counts:

$$\frac{2 \times 10^{3} \text{ nucleotides/molecule actin mRNA}}{6 \times 10^{23} \text{ molecules/mole}} \times 330 \text{ gms/mole} = 1.1 \times 10^{-9} \text{ng/molecule}$$

$$\frac{1.17 \times 10^{-1} \text{ng/sample}}{1.1 \times 10^{-9} \text{ng/molecule}} = 1.06 \times 10^{8} \text{ molec/sample of actin}$$

$$\frac{1.06 \times 10^{8} \text{ molec/sample}}{1.1 \times 10^{5} \text{ cells/sample}} = 9.64 \times 10^{2} \text{ molec of actin/cell}$$

Hence, at saturation we detect approximately 964 copies of the 2 kb beta-actin messenger RNA per fibroblast. The reproducibility of results from saturation experiments was very high, with two other experiments on fibroblasts yielding essentially equivalent results (range 900 - 1,080).

Another experiment was performed on a three day culture of chicken embryonic myoblasts in which 28% of the cells had differentiated into multinucleated myofibres and the remainder of the cells were fibroblasts or undifferentiated myoblasts. In this experiment, we hybridized 0.2 ng of actin probe at saturation in samples containing 8.6×10^4 cells, corresponding to 2,114 messages per cell. The amount of actin mRNA contributed by the differentiated cells is indicated by comparing these results to results above for cultures in which none of the cells were differentiated into myofibres. The average number of molecules per cell is 2.3 fold higher in the differentiating culture, which contained a relatively small fraction of differentiated muscle cells (28%). As considered further in the Discussion, this indicates that the number of molecules contributed by each muscle fibre nucleus is actually much higher than the average 2,100 molecules/cell.

Figure 4 also shows a saturation curve for poly A RNA using ^{3}H-labelled poly U probe on samples from three-day muscle cultures. With this probe we detect approximately 0.18 ng of poly A sequence per sample at saturation. Since the poly A sequence comprises approximately 5% of total poly A mRNA (28, 29) this corresponds to 20 x 0.18 ng = 3.6 ng of poly A mRNA detected per sample. Equivalent results were obtained in three separate experiments. Consideration of the relative hybridization efficiencies of the actin and poly U probes will be dealt with in the Discussion.

Probe Fragment Size

The fragment size of the probe after nick-translation proved to be an important technical parameter. Studies using *in situ* hybridization to chromosomes suggest that use of large probe fragments may enhance signal (30). In contrast, several studies using cellular *in situ* hybridization indicate that use of small probe fragments, below 200-300 nucleotides, is important for successful hybridization to nucleic acid sequences within cells or tissues (5, 7, 15, 22). To resolve the effect of the probe fragment size on signal and noise in our system using paraformaldehyde fixed cells, this parameter was controlled by varying the amount of DNase added during the nick-translation reaction, as indicated under Methods. For a given experiment several nick-translations were performed and the sizes determined by radioautography on alkaline agarose gels with appropriate marker DNA. With little DNase added

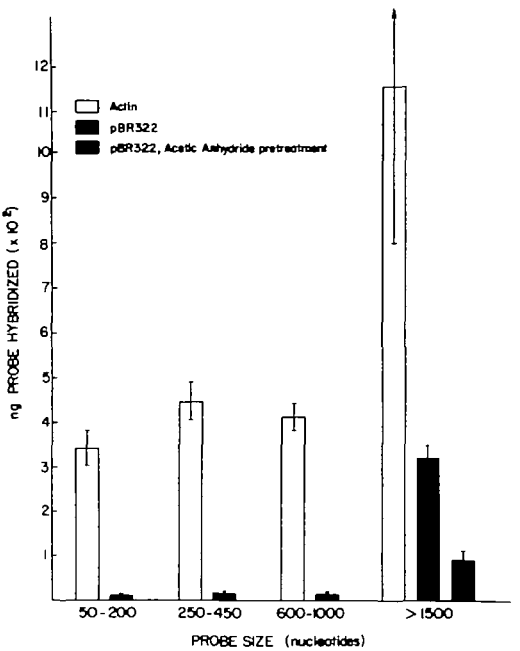


FIGURE 5: EFFECT OF PROBE SIZE ON HYBRIDIZATION AND BACKGROUND
Results presented are from two experiments each of which utilized duplicate samples, bars indicate standard deviations.

to the nick-translation reaction the probe fragment size range was above 1,500, whereas higher DNase concentrations produced fragments sizes as low as 50-200 nucleotides. Probes used in a given experiment were nick-translated at the same time, and their specific activities generally did not differ more than two fold despite up to ten fold differences in average fragment size.

Several experiments were performed in which probes of different average fragment size were evaluated, and representative results are presented in Figure 5. Within the limits of the probe sizes tested, we did not see an increase in hybridization with decreasing probe size in paraformaldehyde fixed cells. There is a slight increase in signal as the probe fragment size is increased over 200 nucleotides, however a striking increase comes when most of the probe molecules are very large, above approximately 1,500 nucleotides. In the experiment presented, hybridization obtained with probe molecules over 1,500 nucleotides is on the average 3-4 fold higher than hybridization obtained with molecules below 1,000 nucleotides, using equivalent weights of DNA (6 ng/sample, 0.6 ug/ml). This is representative of the average increase in hybridization observed in most experiments using very large probes. When one examines the individual samples within an experiment, however, much larger increases in hybridization are frequently observed. As suggested by the large

standard deviation in the data for probe fragments over 1,500 nucleotides, the increase in signal varied in samples having identical treatments. In some experiments individual samples had up to 25 fold higher signal than that consistently obtained using smaller probe fragments. Using only 6 ng DNA per sample, it was occasionally possible to obtain up to 1 ng hybridized per sample (1/2 coverslip) from a myotube culture and 0.32 ng hybridized for a fibroblast culture. These results greatly exceed the maximum estimates of hybridization expected for 100% hybridization efficiency (see Discussion). These extremely high signals were not observed with small probe fragments or with the control pBR322 probe, and hybridization on these samples was not reduced by longer rinsing. These results suggest that double-stranded probes with large fragment sizes can significantly amplify signal via formation of probe networks at the mRNA target site. This conclusion is supported further by results presented below.

While the average hybridization obtained with probe fragments below 1,000 nucleotides is lower than that with very large probes, the variability in the data is reduced, showing some scatter which is largely attributable to differences in the number of cells or myotubes per sample. The noise level, as judged by non-specific sticking of pBR322, is also greatly reduced by a decrease in probe size. At lengths greater than 2,000 nucleotides there is high background, whereas background is relatively negligible for probe sizes below 1,500 nucleotides. However, we found that the very high backgrounds observed with large probes can be effectively reduced by pretreatment of cells in acetic anhydride (7, 18). As illustrated in Figure 5, the acetic anhydride pretreatment reduced the background of large probe fragments by an average of 70%. This pretreatment had no significant effect on either hybridization with the actin probe or on the already low background obtained with probe fragments below 1,500 nucleotides (results of two experiments, not shown).

Formation of Probe Networks

The experiment outlined in Figure 6 was designed to determine if the formation of probe networks (31) contributes significantly to the hybridization obtained. Since our probe consists of approximately 4.5 kb of pBR322 sequences and 2 kb of actin sequences (20), the pBR322 sequences would constitute a substantial part of any probe networks that may form. The rationale for this experiment was to use the restriction enzyme Pst 1 to cut the actin sequences from the pBR322, thereby eliminating the possibility that the pBR322 sequences participate in network formation by removing "junction pieces" (Figure 6). Hence, if substantial networking does occur, signals obtained

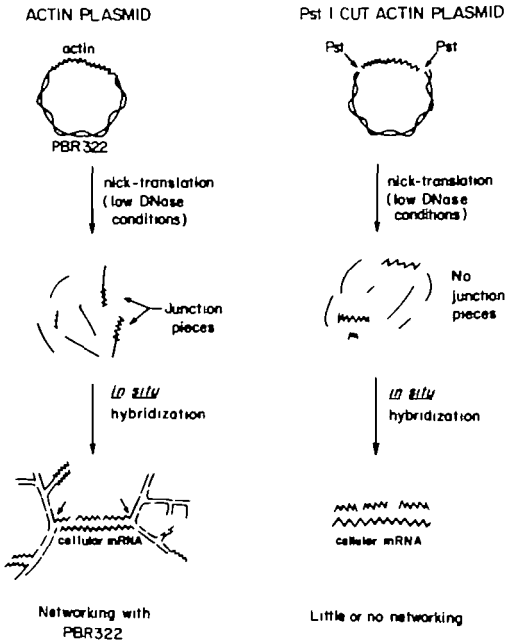


FIGURE 6: EXPERIMENTAL RATIONALE Due to the presence of "junction pieces", containing both actin and pBR 322 sequences, the pBR 322 sequences may contribute significantly to signal via the formation of probe networks. Elimination of junction pieces by Pst-1 digestion prior to nick-translation will greatly reduce network formation. Hence, if the very high signals obtained with large double-stranded probes are due to the contribution of "junction pieces" to probe networks, these signals will be markedly reduced by Pst-1 digestion of the probe.

with the Pst 1 cut probe should be considerably reduced relative to the uncut probe. As can be seen in Table 1, with relatively small probe molecules of an average 450 nucleotides there is no significant difference in the amount of probe hybridized between Pst 1 cut and uncut actin probe. This indicates that, for the probe fragment size routinely used in our experiments (approximately 300-500 nucleotides), the formation of probe networks does not contribute significantly to the signals obtained. In contrast, with probe

Table 1: Effect of pst 1 Digestion on Hybridization of Actin Probe. For explanation see Figure 6.

Actin Probe	Approximate Fragment Size	Ng. Probe Hybridized (range)
Uncut	450	.032 (.028-.039)
Pst-1 cut	450	.034 (.028-.045)
Uncut	1,500	.302 (0.101-1.05)
Pst-1 cut	1,500	.045 (0.03-0.07)

molecules of an average 1500 nucleotides in length, there is a large difference in hybridization between the Pst 1 cut and uncut actin probes. The large increase in hybridization generally observed with probes of this size, as described above, was eliminated in this experiment when the probe was digested with Pst 1 prior to nick-translation. This result substantiates our interpretation that the unusually high signals obtained with large probes represent hybridization to actin mRNA that has been amplified by probe reannealment at the mRNA target site. Although a number of experiments were directed towards making probe network formation more reproducible, the extent of signal enhancement achieved with large probes continued to vary among samples.

From this analysis we conclude that probe molecules of several hundred nucleotides or less, which do not promote networking, are appropriate for quantitation of the amount of mRNA detected. Large probe molecules enhance signal to a variable extent via network formation and, hence, are more appropriate for non-quantitative applications in which greater sensitivity of detection is desired.

Other Variables Examined

A number of other technical parameters were examined, for which the results will be only briefly stated. Unless otherwise indicated our conclusions are based upon two or more independent experiments, each of which utilized duplicate or triplicate samples.

(a) Formamide. One parameter which we found to be of unexpected importance was the particular lot of formamide used for the hybridization reaction. Three experiments were conducted in which formamides from several different sources were tested in the hybridization protocol. We consistently observed that different formamides yield up to a twenty fold variation in signal:noise ratios. This is due primarily to dramatic differences in the amount of background contributed by the pBR322 control that occurs with different formamides. The extent of hybridization achieved is also affected in some cases, but to a lesser degree. Some relatively small differences in cellular RNA retention are observed with the different formamides, however more data would be necessary to determine if these differences are significant. Our results indicate that the specific lot of formamide used is more important than the commercial producer or, in some cases, whether or not the formamide had been deionized (see Methods). It was further noted that backgrounds tend to increase with formamides kept at $4^{o}C$ for more than a few weeks.

(b) Sample dehydration. The hybridization protocols used by several investigators (3, 5, 6, 7, 13) involve dehydration of the cell or tissue sample prior to application of the hybridization solution, in order to enhance penetration of probe into the cell. We examined the effectiveness of this technique on cultured muscle cells and on frozen sections of muscle. Samples were processed as above (see Methods) except that they were dehydrated through a 70%, 95%, 100% ethanol series and air dried just prior to applying hybridization solution. Under the conditions tested, we found that this approach increases background two to three fold and, therefore, a dehydration step was not included in our hybridization protocol.

(c) Isolation of actin sequences. Another parameter that we evaluated was whether it is advantageous to use probe consisting of the actin gene sequences isolated from the plasmid, as compared to use of the entire plasmid consisting of both actin and pBR322 sequences. As expected we found that a higher concentration of pBR322-actin probe is required to achieve the same level of hybridization (below saturation) as obtained with the isolated actin sequences. However, since there was no significant difference in the background observed with the two probes, we concluded that it is more efficient simply to use the entire pBR322-actin plasmid than to isolate the actin sequences. Moreover, the presence of the pBR322 sequences can be advantageous, since in some cases they allow amplication of signal (see section on Probe Size and Networks).

(d) Dextran sulfate is known to increase hybridization, presumably by increasing the effective DNA concentration of the hybridization solution (31, 32). Using probe molecules of less than 500 nucleotides at several non-saturating DNA concentrations, we found that 5-10% dextran sulfate in the hybridization buffer results in up to a three fold increase in hybridization. In addition, we found that in the absence of dextran sulfate the retention of total cellular RNA was reduced by more that 50% in three of four experiments. Therefore, use of dextran sulfate may be important for protecting messenger RNAs as well as for maximizing signal.

(e) Rinse procedure. It is frequently assumed that very extensive rinsing of samples is necessary after in situ hybridization, presumably because unhybridized probe is trapped within the cellular matrix. In one experiment we tested several different rinse procedures and determined that no advantage is obtained by increasing the rinse time, the volume of rinse solutions, or the rinse temperature beyond the three 30 minute rinses specified under Methods. Background was not reduced by more stringent washes, but in some cases

hybridization was. Use of brief rinses (five minutes each) resulted in only slightly increased background.

(f) Prehybridization. Standard filter hybridization procedures call for prehybridization of filters in hybridization solution lacking probe DNA to reduce backgrounds. This approach was tested for hybridizations in situ, using 2-4 hr. prehybridization at 37°C in hybridization solution containing salmon sperm DNA and E. coli tRNA, but lacking probe DNA. Results from three experiments showed no consistent decrease in non-specific binding of the pBR322 control probe as a result of prehybridization. This was true with both large and small probe molecules.

In situ hybridization using tritium or biotin-labelled probes

All of the above analyses utilized ^{32}P-labelled probes as a means of rapidly quantitating results. However, many applications of in situ hybridization would require a single-cell analysis, for which tritium or biotin labelled probes are more appropriate. Experiments were performed to verify that the methodology described above is applicable to use with these types of probes and that comparable results can be obtained.

Actin and pBR322 plasmids were nick-translated with ^{3}H-labelled nucleotides, and in situ hybridization was performed as described above. For autoradiography, coverslips were mounted cell side up on microscope slides and dipped into Kodak NTB-2 emulsion. An exposure of three months was chosen arbitrarily, after which the grains over cells hybridized with actin probe were extremely dense, with grains over the actin rich myofibres being so dense that quantitation was difficult. Figure 7 compares the label observed in samples reacted with pBR322 and with actin probe. Grain counts performed on mononucleated cells yielded a signal:noise ratio of 59:1. (If no subtraction was made of the density of grains over bare glass then the signal:noise ratio was 20:1.) This experiment confirms that the methodology described above is extendable to use with tritium-labelled probes and autoradiography, yielding roughly comparable results.

A more complex picture emerged when probes were labelled with biotinated nucleotides (9, 33). Experiments were performed in which probes nick-translated with both biotin d-UTP and ^{32}P-dCTP were compared to probes labelled with only ^{32}P-dCTP. Initial experiments indicated that introduction of the biotin moiety into the pBR322 probe results in large but variable increases in background, ranging from 3-20 times above the background of non-biotinated probes. Further analysis revealed that both hybridization and the background obtained with biotinated probes is more dependent upon probe

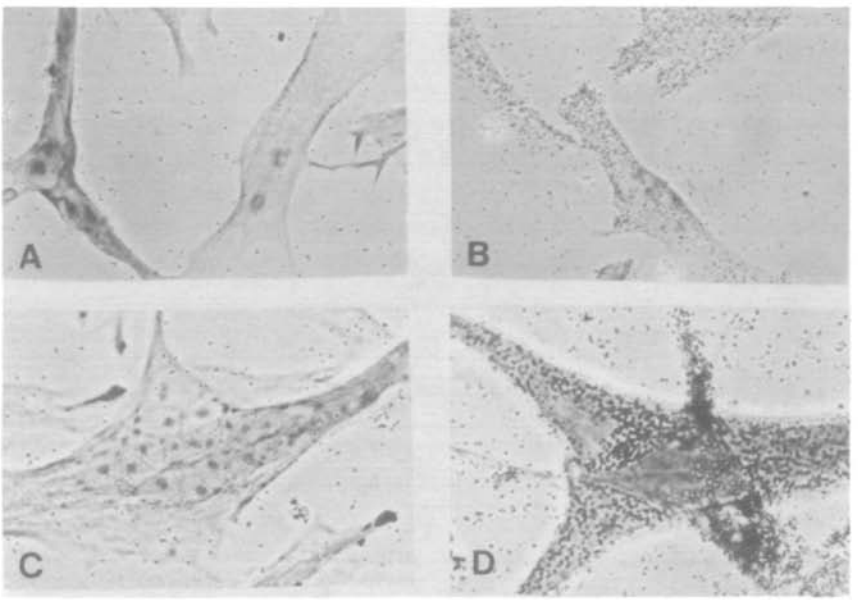


FIGURE 7: AUTORADIOGRAPHY ON CELLS HYBRIDIZED WITH ^{3}H LABELLED PROBES A. pBR322 control probe on single cells. B. Beta-actin probe on single cells. C. pBR322 control probe on large myofibre from same sample as A. D. Beta-actin probe on two overlapping myofibres exhibiting extremely dense label, same sample as B. Specific activities were 3.7 x 10^{6}cpm/ug for the control probe and 4.4 x 10^{6}cpm/ug for the beta-actin probe. Magnification = 800X standard phase optics

size than is the case for non-biotinated probes on paraformaldehyde fixed cells. Non-specific sticking of pBR322 was extremely high and hybridization of the actin probe relatively low unless the fragment size range of the biotinated probes was below approximately 150 nucleotides. In some cases it was possible to reduce the non-specific adhesion of larger probe molecules by post fixation treatment of cells in 0.5% triton, 1 mM biotin in PBS for ten minutes prior to hybridization. However, even in the absence of this extra step, appropriately sized biotinated probes routinely yielded signal:noise ratios of at least 10:1. A more complete analysis of parameters which specifically affect hybridization of biotinated probes is currently in progress.

DISCUSSION

Our goal in this work was to analyze, as quantitatively as possible, the combination of variables affecting hybridization of nick-translated DNA probes

to messenger RNA in situ in order to derive a method which, within the limits of our analysis, yields optimal hybridization, minimal background, and maximal preservation of cellular RNA and morphology. To achieve this we implemented a simple but novel approach for rapid quantitation of in situ hybridization utilizing scintillation counting by Cerenkov radiation. This investigation has allowed us to develop an in situ hybridization technique which is highly sensitive and reproducible, requires few steps, and is not disruptive to cellular RNA or morphology. Furthermore, this "user friendly" technique is directly applicable to a single cell analysis wherein messenger RNA can be resolved within the context of cell structure. It is not our intention to suggest that the technical protocol derived represents the only right way to detect RNA within cells by in situ hybridization. Our results contribute to a clearer definition of in situ hybridization methodology, however, it is clearly not feasible to test all the different variables and permutations possible in a given study. The analysis we present pertains specifically to detection of mRNA in tissue culture cells using nick-translated probes. Although this protocol has also been used successfully on sectioned tissues and with single-stranded RNA probes (unpublished observations), the optimal conditions for different experimental applications may vary. The methodology will continue to be improved in our laboratory as well as others.

Our results show that the fixative used determines the other steps in the protocol that will be required. The ideal fixative is one which crosslinks the cellular matrix enough to preserve cellular RNA and morphology, but not so much that the probe cannot penetrate easily. Paraformaldehyde fixation was initially emphasized in our analysis because of our interest in the use of fluorescent detection methods with biotinated probes (9) and because paraformaldehyde fixation offers the advantage that cells exhibit minimal autofluorescence. Analysis of different fixatives revealed other advantages of paraformaldehyde fixation, i.e. good RNA retention, ability to use large probe fragments, and good hybridization without proteolytic digestion or acetic anhydride cell treatments. Our results clearly demonstrate that with paraformaldehyde fixed cells proteolytic digestion is not only unnecessary, but detrimental. Our ability to obtain good hybridization with probe fragments as large as 1.5 kb, while others report fragments below 200 (mass average of 50) nucleotides should be used (5, 7), is apparently related to our use of paraformaldehyde fixation. In contrast, recent experiments on glutaraldehyde fixed cells have shown an increase in hybridization when probe fragments below 150-200 nucleotides are used, although proteolytic digestion is still required

(not shown). The decrease in signal observed in some experiments using long hybridization time may also be related to our use of paraformaldehyde fixation. Because glutaraldehyde appears to fix the cell more extensively, it may provide better RNA retention for hybridizations requiring long incubation times. Our results indicate that the primary advantage of paraformaldehyde is that it fixes the cellular matrix in a more open configuration, and thus renders the cellular RNA more accessible to probes of various sizes without the need for cellular proteolysis.

The ability to use large probe molecules (1-2 kb) with paraformaldehyde fixed cells allowed us to investigate the possibility of probe networking for amplification of signal. Our results show that the presence of "junction pieces", containing both pBR322 and actin sequences, make it possible for signal to be enchanced as much as twenty five fold. Because of variability, this approach, while improving detection, is not useful for quantitation of mRNA. However, this enhancement in hybridization is potentially advantageous for the detection of mRNAs present in low copy number or in instances in which recombinant probes with small gene inserts are used. Smaller probe fragments, which minimize or eliminate networking are more appropriate for reproducible quantitation of cellular mRNA levels.

The sensitivity limit of this technique may be extrapolated from the signal obtained from fibroblasts at saturating DNA concentrations when compared with the range of noise. We find that the DNA probe when incorporated with 10^8 Cerenkov counts per microgram gave an optimal signal corresponding to 110 picograms of probe hybridized per 10^5 cells. At this level of specific activity, the background noise in various experiments ranged from 0.5 to 3 picograms. We, therefore, feel that our limit of detection with current methods is in the range of a few picograms per sample. For a sample consisting of 10^5 cells, this would correspond to 20 copies of a 2 kilobase message per cell. It should be noted that this estimation refers to results with smaller probe fragments which do not promote amplification of signal.

In a fibroblast culture, we detect approximately 1,000 copies per cell of actin mRNA. For muscle cultures we calculate 2,100 molecules detected on the average per nucleus. Assuming that this increase is attributable to the expression of the alpha actin messenger RNA (the skeletal muscle-specific actin expressed with myofibre formation) in the 28% of the cells which had differentiated, then it can be calculated that we detect approximately 9,500 copies of actin mRNA per differentiated nucleus (1,100 nucleotides of alpha

actin mRNA will cross hybridize because of conservation in the coding region, 16).

Based on the work of Schwartz and Rothblum (16), two types of evidence allow an estimation of our hybridization efficiency. These investigators quantitated actin mRNAs during differentiation in chicken muscle cultures based on Rot curves using cloned recombinant probes. They detected approximately 1,800 actin mRNA molecules per cell in undifferentiated myoblast or fibroblast cultures. By 70 hours of culture, they detect approximately 8,000 copies per differentiated nucleus, which increased rapidly to 15,000 copies per nucleus in muscle fibres at 75 hours. Our results obtained by hybridization in situ are consistent with these data from solution hybridizations. The fact that we detect 9,500 copies of actin mRNA per differentiated nucleus in cultures incubated for 60-65 hours and 1,000 copies per cell in fibroblasts suggest that we detect most, possibly all, of the actin messages present. This conclusion is also supported by an alternative method of estimating the amount of actin mRNA per sample based on measurements of total RNA in our cultures and on Schwartz and Rothblum's calculations of actin as a percent of total RNA. In contrast, hybridization efficiency appears to be lower with the poly U probe. We detect approxmately five fold less poly A per sample than expected based on our measurements of total RNA per culture and estimations of poly A mRNA as 3% of total RNA in myoblast or myofibre cultures (34). Therefore hybridization efficiency to poly A is in the range of 20%. A low hybridization efficiency (22%) with a poly U probe was also observed by Angerer and Angerer (7). These observations are consistent with reports that the poly A sequence of mRNA is heavily bound with protein (35, 36).

In a recent, extensive study from Angerer's laboratory (14), quantitative autoradiography was employed to evaluate single-stranded RNA probes for detection of histone mRNAs in sectioned sea urchin embryos. Our results are in agreement with this laboratory that four hours of hybridization time is optimal and that ethanol:acetic acid fixation is insufficient for RNA preservation (7). Single-stranded probes offer the advantage that there is no reannealing in solution to compete with hybridization to cellular mRNA. However, our results show that the ability of nick-translated, double-stranded probes to reanneal at the mRNA target site can be exploited to amplify signal up to twenty five fold. In addition to this, the stability of DNA and the general availability of pBR 322 recombinants may make double-stranded DNA probes sufficient for most applications. Currently, we are evaluating single-stranded DNA and RNA probes for in situ hybridization to cellular actin

mRNA using the methodology reported here.

The method described here may be applicable to a wide variety of experimental situations. Results from our laboratory and others indicate that this method can be employed to investigate mRNA expression in a variety of cultured cell types with different probes, as well as in tissue sections. It is understood that the goal of in situ hybridization is an analysis of single cells and the results presented here on biotinated probes and autoradiography illustrate that this technique can be used to maximize and quantitate parameters previous to further analysis at the cellular level.

The rapid quantitative approach implemented for this study may make it feasible to use in situ hybridization in ways not previously considered. In instances in which a single-cell analysis is not required, the efficient quantitation afforded by scintillation counting on cultured cells or on tissue sections can provide significant advantages as an alternative or complement to nitrocellulose hybridizations ("dot blots"). The benefits of the in situ approach are that as few as ten thousand cells per sample are required, the effort of isolating RNA from each sample is avoided, hundreds of samples can be evaluated simultaneously, and results are obtained in quantitative form. After all of this, an analysis of single cells by autoradiography or non-isotopic methods can be done on the same samples. Surprisingly, the availability of messenger RNA to hybridize in situ appears to be about the same if not better as for RNA bound to filters, despite the fact that cellular mRNA is enclosed within a matrix and crosslinked to the protein synthesis machinery. Furthermore, there are diverse applications for which rapid quantitation of relative mRNA levels by this technique could substitute for solution hybridization. For instance, because the mRNA is essentially on solid support, and the hybridization is in such a small volume, labelled DNA excess experiments are easily done (in a few hours) to determine copy number. Reannealed probe is washed away and does not complicate the assay. Lastly, other investigators may use this approach as a fast, objective means of verifying that in situ hybridization conditions appropriate for their particular experimental situation have been achieved.

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