

## Use of Oligodeoxynucleotide Probes for Quantitative *in Situ* Hybridization to Actin mRNA

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We have employed an analytical approach for the development of an *in situ* hybridization methodology using synthetic oligodeoxynucleotide probes for actin messenger RNA detection in cultures of chicken fibroblasts and myoblasts. The methodology developed shows that oligonucleotides can complement the use of nick-translated probes in specific situations. Since they can be made to specific nucleic acid regions independent of restriction enzyme sites, they may be the most convenient approach for analysis of gene families among which sequences are highly conserved. However, it was found that oligonucleotides synthesized to different regions of a messenger RNA behave *in situ* with differing efficiencies, indicating that not all target sequences are equivalent. Therefore it was necessary to screen several oligonucleotide probes to a target molecule to find the optimal one. The convenience of using synthetic DNA probes makes it worthwhile to explore some of these characteristic properties so as to increase the sensitivity of this approach beyond its application to targets in high abundance. © 1987 Academic Press, Inc.

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Over the last few years the technique of *in situ* hybridization has become popular for the detection and, particularly, the localization of nucleic acids in cells, tissues, or genomes. A number of investigators have contributed to the current methodology beginning with the first work on *Drosophila* polytene chromosomes and more recently using recombinant DNA probes (1-18). In the last few years, synthetic DNA probes have been introduced to *in situ* hybridization methodology (19-29). Use of oligodeoxynucleotides has several advantages over the use of cloned probes. These advantages have been detailed elsewhere (19,23,24) and will be expanded upon here. The first is that oligonucleotides are easily made. Commercial establishments (e.g., our DNA Synthesis Center at the University of Massachusetts Medical School) can make custom oligodeoxynucleotides rapidly and inexpensively. The use of oligodeoxynucleotides may be the

most convenient way to generate probes with which to investigate the expression of members of a gene family where the sequences of the messenger RNAs are known and are found to be highly conserved. Such a case exists with the actin gene family (30). Furthermore, the probes are more stable than RNA, are single stranded, and are therefore not subject to competition from the complementary strand (although the double-stranded nature of nick-translated DNA can amplify signals by formation of "networks" (16)). These features, combined with their short length, may affect the ability of synthetic DNA probes to penetrate tissue farther than larger RNA probes or double-stranded cDNA probes. However, because the oligonucleotide probe is generally targeted to a significantly smaller sequence than a nick-translated probe, it will have less mass for detection upon hybridization. Finally, during their synthesis, oligonucleotides can

be conjugated directly to reporter groups such as enzymes or fluorochromes by means of modified bases, thereby allowing the production of simplified hybridization reagents.

We wished to analyze the use of oligonucleotides as quantitatively as possible in order to derive optimized protocols for our system of interest. Therefore, we used the approach previously employed to evaluate nick-translated probes for *in situ* hybridization (16–18). This allows a critical analysis of methodology using oligonucleotides *in situ*, which may clarify some of its strengths and weaknesses. The cell culture system, chicken differentiating myoblasts, was employed since much is known about the quantitative expression of actin mRNA in these cells (31) and with respect to cell morphology (32). The expression of the members of the chicken actin family has been characterized by nucleic acid sequence (33–35) and with respect to muscle differentiation as well (30, 36,37). This provides background information with which to characterize the hybridization of oligonucleotides *in situ*.

## MATERIALS AND METHODS

*Synthesis and purification of DNA probes.* DNA probes were synthesized on an Applied Biosystems DNA Synthesizer 380A by the phosphoramidite method. The probes were purified on a 15% polyacrylamide sequencing gel; the DNA band was eluted from the gel and dried in a Speedvac. DNA was labeled using [ $\gamma$ - $^{32}$ P]ATP (Amersham) and T4 polynucleotide kinase (Boehringer-Mannheim). Alternatively, the probe was labeled at the 3' end using [ $\alpha$ - $^{35}$ S]dCTP (Dupont/NEN) and terminal deoxynucleotidyltransferase (Boehringer-Mannheim).

*Cell culture.* Pectoral muscle cells from 12-day chick embryos were plated out at a density of  $2 \times 10^6$  in 100-mm plates which contained 0.5% gelatin-coated, autoclaved glass coverslips. Cells were grown in mini-

mum essential medium (MEM),<sup>1</sup> 10% fetal calf serum, and 2% chick serum. After 3 days of incubation, when approximately 40% of the cells had differentiated into multinucleated myofibers, coverslips containing cells were rinsed twice with Hanks' balanced salt solution (HBSS) and fixed with 4% paraformaldehyde or 4% glutaraldehyde in phosphate-buffered saline (PBS; 2.7 mM KCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , 0.137 M NaCl, 8 mM  $\text{Na}_2\text{HPO}_4$ ) and 5 mM  $\text{MgCl}_2$ . After fixation, the coverslips were stored at 4°C in 70% EtOH. Sample coverslips were taken from each cell batch for cell counts. Cell densities were found to vary from 3 to  $7 \times 10^5$  cells per full (uncut, see below) coverslip.

*Hybridization and washing.* Prior to hybridization, the cells were rehydrated with PBS containing 5 mM  $\text{MgCl}_2$  for 10 min at room temperature and then with 0.1 M glycine and 0.2 M Tris-HCl, pH 7.4, for 10 min at room temperature, followed by 10 min at 65°C in 50% formamide,  $2 \times \text{SSC}$ . Two nanograms of the oligodeoxynucleotide, 5  $\mu\text{g}$  of sonicated salmon sperm DNA (Sigma), and 10  $\mu\text{g}$  of *Escherichia coli* tRNA were suspended in 5  $\mu\text{l}$  of 100% deionized formamide, pH 7.0. The solution was heated at 70°C for 5 min. This solution was mixed with 5  $\mu\text{l}$  of hybridization buffer consisting of  $4 \times \text{SSC}$ , 2% BSA (molecular biology grade, Boehringer-Mannheim), 20 mM vanadyl sulfate, and 20% dextran sulfate. The total hybridization solution (10  $\mu\text{l}$ ) was put on the parafilm and the coverslip (22-mm square cut in half with a diamond pencil) was placed cell side down on the solution for hybridization. The coverslips were sealed with parafilm and incubated at 37°C in a humidified incubator for the appropriate times.

After hybridization, the coverslips were washed successively with 50% formamide,  $2 \times \text{SSC}$  at 37°C; 50% formamide,  $1 \times \text{SSC}$  at

<sup>1</sup> Abbreviations used: MEM, minimum essential medium; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; BSA, bovine serum albumin; EtOH, ethyl alcohol; SSC, standard saline citrate.

37°C; 1× SSC and 0.5× SSC at room temperature, each step for 30 min. The coverslips were counted in 1× PBS solution in a scintillation counter. In some experiments autoradiography of the cells was performed by ethanol drying of the coverslips, then attaching the slips to a glass slide with permount, coating them with NTB-2 emulsion (Kodak), and exposing them for several days. Slides were then developed and Giemsa stained.

## RESULTS

### *Oligoprobe Synthesis and Cell Samples*

Four oligodeoxynucleotide probes were made to various regions of the DNA sequence of  $\beta$ -actin (35), shown in Table 1. One oligonucleotide (51 nucleotides) was made to the coding region of amino acids 2 through 18 of  $\alpha$ -cardiac actin (33,34). One oligonucleotide was made as a control and had no sequences in common with RNA of the cultured cells. The sequence was chosen from prokaryotic sequences and has a base composition similar to that of the  $\beta$ -actin probe. For purposes of quantitation, the

method of Lawrence and Singer (16) was followed wherein the probe (end-labeled using [ $\gamma$ - $^{32}$ P]ATP and polynucleotide kinase) was hybridized to coverslips containing differentiating myoblasts fixed on the third day of culture and the amount of hybridization was quantitated by Cerenkov radiation in a scintillation counter. This allows a rapid and accurate method for analysis of many samples where a number of parameters can be varied. The results of this approach are detailed below.

### *Saturation Curve*

To determine the optimal probe concentration, coverslips were cut in half with a diamond pencil and a control probe was used to determine nonspecific hybridization to the other half. The probe concentration was varied and hybridization was for 3 h, a time determined as optimal for nick-translated probes (16). Figure 1 shows the results of this experiment using an oligonucleotide probe of 54 nucleotides to the coding region (Exon 3) of chicken  $\beta$ -actin from nucleotides 548 through 602 (35). This probe has 95%

TABLE 1  
OLIGODEOXYNUCLEOTIDE PROBES SYNTHESIZED FOR QUANTITATIVE EXPERIMENTS

Probe to	Sequence
$\beta$ -Actin (35)	
548-602 (Coding)	5' AGC TCA TTG TAG AAA GTG TGG TGC CAG ATC TTC TCC ATA TCA TCC CAG TTG GTG 3'
571-593 (Coding)	5' GTA GAA AGT GTG GTG CCA GAT CT 3'
2644-2698 (3' Untranslated)	5' TTG TTT TAT GCG CAT TTA TGG GTT TTG TTT CAT CAC AGG GGT GTG GGT GTT GGT A 3'
3006-3060 (3' Untranslated)	5' GGA TCA AGT GTG TTT TGT TTT TAA GGC GGA AGA TAC AAA AAG ACA CTT GTT GGG T 3'
$\alpha$ -Actin, cardiac (33,34)	
1630-1680 (Coding)	5' AGG CGC GAG CCG TTG TCG CAC ACC AGC GCG CTG GTC TCC TCG TCG TCA GAC 3'
$\mu$ -Phage sequence	5' AAA ACT TCA GCT TTC GCG CTT CAA ACG AGA CAG CTG TAT TAG TTA CTG CTT TTT GG 3'

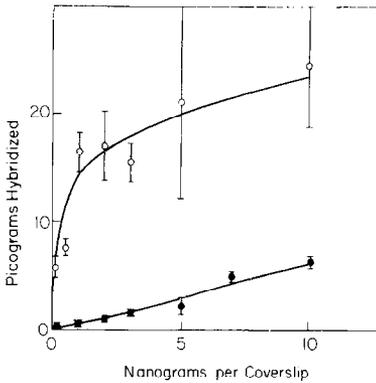


FIG. 1. The hybridization of an actin-specific oligonucleotide probe as a function of probe concentration. Increasing amounts of probe ( $\beta$ -actin, 548-602) were added per coverslip and hybridized for 3 h. The open circles represent the average of duplicate samples. The closed circles represent the average of duplicate samples for the control probe.

homology with  $\alpha$ -cardiac actin and should hybridize to all actin species in the cell. The amount of probe hybridized was calculated from the specific activity of the probe and the counts hybridized per sample. It can be seen from this data that the cellular target sites become mostly saturated at a probe concentration of 2 ng per coverslip (0.2  $\mu$ g/ml). Even at probe concentrations tenfold less, hybridization is easily detectable. Although the amount of probe hybridized appears to saturate the target sequences at between 15 and 18 pg per coverslip, it continues to slope upward with increasing probe concentrations.

When a control probe was used, which contained a sequence of similar length and base composition, the nonspecific sticking increased slightly with increased probe concentration. Therefore, it is evident that probe concentrations greater than 0.2  $\mu$ g/ml will increase nonspecific background. In order to differentiate between the amount of nonspecific sticking from the bona fide hybridization, a competition experiment was done where the hybridization was performed in the presence of increasing amounts of a homologous and a heterologous sequence. The

heterologous sequence used was a 55-nucleotide synthetic DNA made to Exon 6 in the untranslated region of  $\beta$ -actin (nucleotides 3006-3060). It can be seen in Fig. 2 that addition of the heterologous probe did not decrease the hybridization of the labeled probe used in Fig. 1. The unlabeled, identical probe decreased the hybridization approximately 90% when compared to the duplicate coverslip to which the nonhomologous probe was added. The reduction of the cell-associated label was not complete even at a 50-fold excess of unlabeled homologous probe. The residuum of labeled probe associated with the cell we considered to represent the nonspecific sticking. When this background was subtracted from the signal, approximately 12 pg was determined as bona fide hybridization to target sequences. Hence, the mass of probe hybridized ( $M_r$  18,463) yields  $0.66 \times 10^{-3}$  pmol hybridized or about  $4 \times 10^8$  molecules per coverslip. Since the coverslip was found to contain approximately  $2 \times 10^5$  cells, this represents approximately 2000 copies of actin message per cell. This work correlates well with values obtained in a previous work (16) using a nick-translated probe for the entire  $\beta$ -actin sequence (2 kb) where we obtained a value of

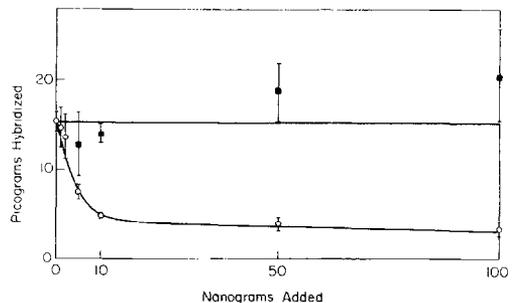


FIG. 2. The inhibition of hybridization of the actin oligonucleotide probe by the addition of increasing amounts of heterologous and homologous probes. Two nanograms of probe in Fig. 1 was hybridized to the sample and competed with increasing amounts of either that identical probe (open circles) or another probe ( $\beta$ -actin, 3006-3060) synthesized to the 3' untranslated region of the actin mRNA (closed squares). The circles and squares represent the average of duplicate samples.

200 pg hybridized, which also represented 2000 molecules per cell. This would appear to indicate that the use of this particular oligonucleotide probe is similar in its efficiency to nick-translated probes when corrected for mass. However, other considerations prevent a conclusive comparison of oligonucleotide probes with nick-translated probes (see Discussion).

An alternative method of determining the signal-to-noise level is by using the same probe on expressing and nonexpressing cells, rather than by comparing different probes on the same cells. For this purpose we synthesized a probe to the cardiac form of sarcomeric actin (33,34) and hybridized it to two different cultures, a chick muscle culture in which differentiated cells express this message (36,37) and a nonexpressing human fibroblast culture (W138). The kinetics of hybridization revealed by Fig. 3 shows the difference in hybridization between the two cultures. The level of saturation at 2 ng per coverslip (0.2  $\mu\text{g}/\text{ml}$ ) is essentially the same as that of the  $\beta$ -actin probe, as is the signal-to-noise level (about 10:1). The saturation

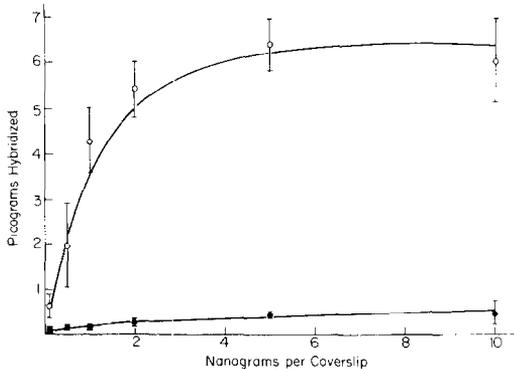


FIG. 3. Detection of a specific gene product in expressing and nonexpressing cells. A probe for chick cardiac actin (1630–1680) was hybridized with cultures of 3-day chicken myotubes (open circles) and human W138 cells (closed circles). Increasing amounts of probe were used to establish saturation value. The W138 cells were at a density of  $7 \times 10^4$  cells per coverslip. The myotube culture was at a density of  $2 \times 10^5$  cells per coverslip with 43% of the nuclei in differentiated myotubes.

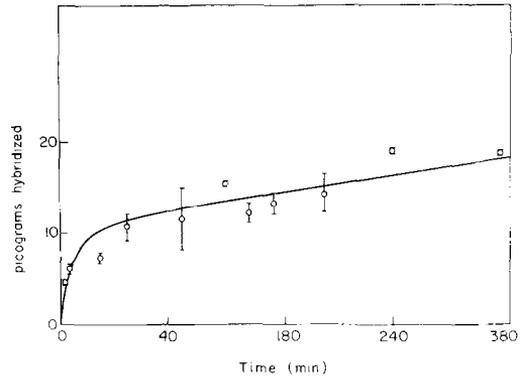


FIG. 4. Time course of hybridization of the actin oligonucleotide probe. Two nanograms of oligonucleotide probe ( $\beta$ -actin, 548–602) were hybridized per coverslip and samples were removed at increasing times. The open circles represent the average of duplicate samples.

level indicates that, at this period in the differentiation of the culture, cardiac actin is expressed at a level approximately equal to that of  $\beta$ -actin (6 pg of cardiac versus 12 pg of  $\beta$  plus cardiac). Hence, using this approach, oligonucleotide hybridization can rapidly, accurately, and conveniently determine the message levels of a particular gene or of members of a gene family.

#### Time Course

The time necessary for the hybridization to reach a plateau was determined by removing samples from the solution at appropriate times. An optimal concentration of probe for  $\beta$ -actin at 0.2  $\mu\text{g}/\text{ml}$  was used, as determined from Fig. 1. It can be seen in Fig. 4 that hybridization increased rapidly for the first half hour and then more slowly for the remaining time. The initial leveling off of the curve at 30 min occurs at the 12-pg level, the same amount as was detected with the saturation curve after subtraction of background determined by the noncompeted sticking. The amount of probe associated with the cells continues to rise, presumably representing an increase in nonspecific binding of the probe to the cell, as shown in Fig. 1. This nonspecific binding, when investigated by

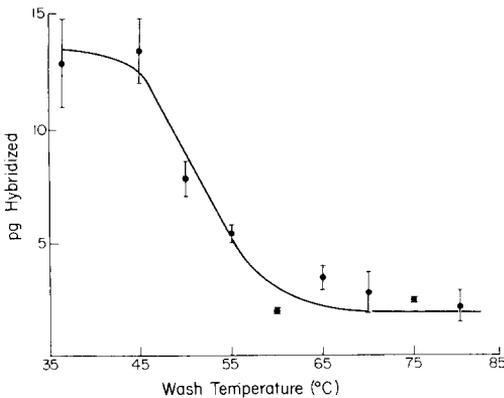


FIG. 5. Melting the hybridization of the actin oligonucleotide probe. *In situ* hybridization was performed for 3 h with 2 ng of the probe per coverslip. Subsequent to the standard washing procedure, the coverslips were placed in 50% formamide,  $1\times$  SSC for an hour at the indicated temperatures and then washed again. The closed circles represent the average of duplicate samples.

autoradiography (data not shown), appears to be the result of trapping in the cell nucleus, and also shows increased sticking to glass. Therefore, these results suggest that the more rapid phase represents true hybridization and less background will be obtained in experiments with this shorter time.

#### Melting of Probe

Another method of determining the bonafide hybridization was to perform a melting curve to see how much of the label is removed at a temperature consistent with a true double strand (Fig. 5). After hybridization with  $\beta$ -actin probe, the cells were exposed to increasing temperatures in a solution of 50% formamide,  $1\times$  SSC. The coverslips were further washed for 30 min with  $1\times$  SSC and 30 min with  $0.5\times$  SSC at room temperature to remove formamide. The cells were shown to bind approximately 14 pg, of which 12 pg were shown to melt with kinetics and temperature consistent with base composition and probe size. A temperature of  $60^\circ\text{C}$  is sufficient to remove all the probe, which is stable at  $45^\circ\text{C}$ . This work independently confirms the level of specific hybrid-

ization (12 pg) which was obtained in the previous figures, and demonstrates that use of higher rinse temperatures is not useful for reduction of nonspecific sticking.

#### Fixation

We wished to determine if the use of paraformaldehyde was necessary when oligonucleotides were used as probes. Several laboratories have suggested that smaller probes are necessary for glutaraldehyde-fixed cells, and our results suggest that this fixative may crosslink the cell so extensively that probe penetration is inhibited (16). However, when cells fixed in each fixative were hybridized to the  $\beta$ -actin-specific 54-nucleotide probe, glutaraldehyde-fixed cells were found to be less efficiently hybridized than paraformaldehyde-fixed cells (Fig. 6), which is consistent with previous results using larger probes (16). Therefore, it may be that glutaraldehyde makes messages less available for hybridization possibly by crosslinking proteins tightly to the nucleic acid. However, it should be considered that the hybridization of this specific probe may be unique to a particular target region of the messenger RNA and may not be representative of results obtained using other oligonucleotide probes.

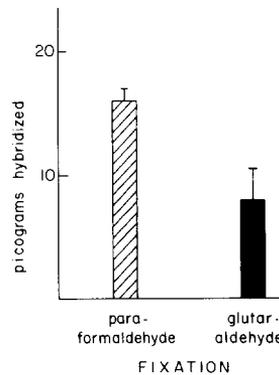


FIG. 6. Effect of fixation on hybridization efficiency. Cells were fixed with either 4% paraformaldehyde or 4% glutaraldehyde in PBS and 5 mM  $\text{MgCl}_2$  for 15 min. Hybridization was for 3 h with 2 ng of probe per coverslip. Each bar represents an average of 10 samples.

### Autoradiography

The conditions for optimal hybridization determined in this work were then used to hybridize cells for autoradiography. In order to evaluate autoradiography critically for signal-to-noise ratio, a probe was used which would hybridize only to some cells in a culture, giving positive and negative cells in the same microscopic field. For purposes of improved resolution, the probe was labeled with sulfonated nucleotides by terminal deoxynucleotidyltransferase. The experiments reported in Figs. 1 through 3 were repeated with sulfonated probes with essentially identical results although sulfonated probes are more variable in background levels. We used a probe for the cardiac-actin, protein-coding region from nucleotides 1630–1680 (33,34) as reported in Fig. 3. This message is expressed in differentiating muscle myotubes in larger abundance, but much less so in single cells (see (17,36,37)).<sup>2</sup> It can be seen in Fig. 7 that the oligonucleotide probe hybridizes to the differentiated myotubes as expected, but not to single cells in the field. The signal-to-noise ratio of 8:1 as determined by grain counts was roughly equivalent to that determined by scintillation counting. This ratio was determined by dividing the number of grains over myotubes expressing cardiac action by the number of grains over fibroblasts and normalized for cellular area. This more accurately reflects the increase in the expression of this gene.

### DISCUSSION

Previous work using synthetic DNA for *in situ* hybridization has shown that these probes are suitable for this purpose (19–29). Recent work (19,24) has analyzed the hybridization of an oligonucleotide probe for

proopiomelanocortin mRNA quantitatively on tissue sections. This work showed clearly that synthetic probes could be used to assess mRNA levels in pituitary tissue sections. In the work presented here, we present data that quantitatively analyze oligodeoxyribonucleotide probes by means of methodology established to test the effect of various parameters on *in situ* hybridization using nick-translated, double-stranded DNA probes (16). We investigated fixation, concentration of probe, hybridization time, and quantitative considerations resulting from their optimization. We found that variation in efficiency between oligonucleotides used as hybridization probes requires that several probes be tested to determine which is optimal under our conditions. The optimized probe used in this work was found to be a useful and convenient reagent for the analysis of actin gene expression in cultures by scintillation counting and in single cells by autoradiography.

In these investigations, one aspect, the choice of sequence, was found to be of primary importance. One sequence chosen ( $\beta$ -actin, 2644–2698) gave unusually high levels of hybridization (24 pg) and a comparison of the sequence with other known sequences showed homology (75% between nucleotides 23–37) with 18-S ribosomal RNA. Other sequences chosen, most often in the 3' untranslated region, showed very poor hybridization efficiency. Hybridization of oligo(dT) probes, which would be expected to hybridize to all polyadenylated messages, was not efficient either, as was found when poly(U) was used in previous work (16). It is known that poly(A) is extensively bound with protein (38). Of the oligonucleotide probes synthesized, the coding region was found to be best for hybridization efficiency despite the presumed presence of ribosomes. A comparison of the efficiency of the nick-translated actin cDNA clones with oligonucleotide probes is difficult because of the variation in efficiency along a message molecule. This variability among sequences may

<sup>2</sup> Note added in proof. We have found that single myoblasts express cardiac actin early in culture before their fusion into myotubes; however the experiment shown here is a later (3 day) culture when the single cells are much less prevalent (manuscript in preparation).

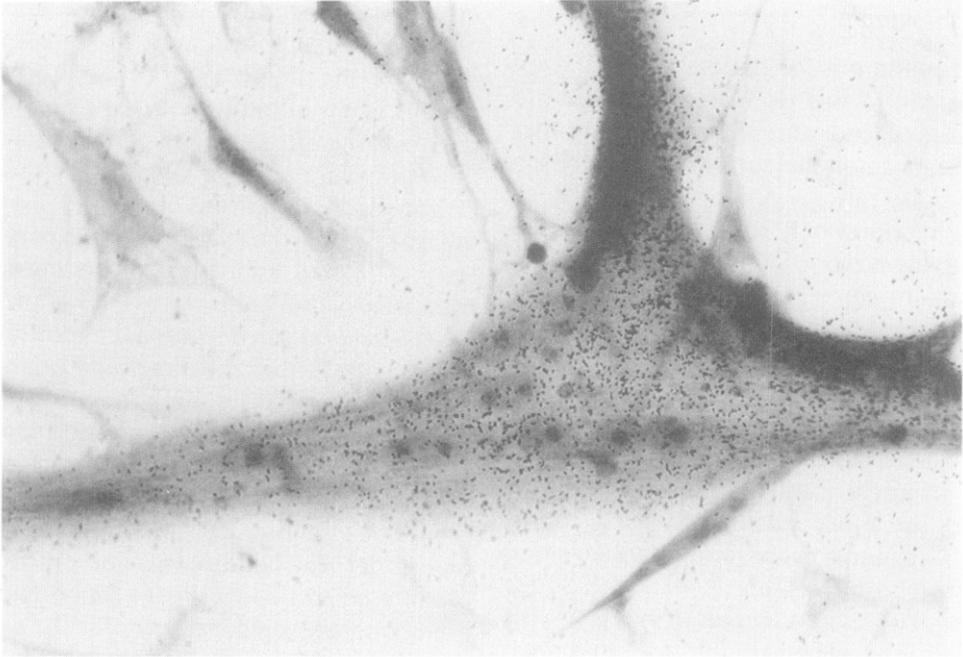


FIG. 7. Autoradiography of cells using a probe specific for cardiac actin. The probe for  $\alpha$ -cardiac actin (1630–1680, see Fig. 3) was labeled using sulfonated nucleotides and hybridized at 2 ng per coverslip for 3 h. The samples were then washed, dehydrated, coated with emulsion, and developed after 7 days. The expression of cardiac actin can be seen in the myotube in the field but not in the single cells.

be indicative of domains within messages which are rich in secondary structure or contain protein-binding sites. We are currently exploring this possibility further by using a series of oligonucleotide probes targeted to different regions of the actin molecule. Preliminary results corroborate the above conclusions.

Some differences in behavior between oligonucleotide probes and nick-translated probes are worth some detailed consideration. It has been suggested (14) that single-stranded RNA probes have the ability to hybridize more efficiently than double-stranded probes because of the lack of competitive reannealing of the message-like strand. In fact, our calculation of the efficiency of the particular oligonucleotide used gave roughly the same hybridization as was calculated using double-stranded probes (16). The value obtained may be indepen-

dent of the nature of the probe in paraformaldehyde-fixed cells. It is possible to increase the hybridization efficiency of double-stranded probes (unpublished data) when ribosomes are released from the message by drugs, such as puromycin, indicating that ribosome binding interferes with the efficiency of hybridization. Although it has been reported that smaller probes are more efficient than larger probes (14), we found in previous work that probe size is not an important factor in cells fixed in paraformaldehyde for short periods but may be so when glutaraldehyde-fixed material or material extensively fixed by paraformaldehyde are used (16). Nonetheless, the same decreased efficiency of *in situ* hybridization was obtained with glutaraldehyde-fixed cells independent of whether the probe was an oligonucleotide or nick-translated, double-stranded DNA (16). This suggests that penetration of the cellular

material from tissue culture, expected to be more efficient with the smaller probes, is not rate-limiting for hybridization. Rather, the physical nature of the message, or its cross-linking to ribosomes or other cytosolic components, may be the limiting factor.

When quantitatively analyzing the signal-to-noise ratios of a 54-nucleotide probe, we found it to yield a ratio as high as 10:1. A smaller oligonucleotide probe (23 nucleotides, see Table 1) to the same region had higher background levels, often nuclear associated. A number of attempts to reduce the background further were not consistently successful. For instance, the following did not reproducibly reduce nonspecific sticking of a particular oligonucleotide: addition of oligonucleotides of different sequence but identical base composition as nonspecific competitors, addition of mono-, di-, or oligonucleotides of single-base composition, pre-hybridization to saturate nonspecific binding sites, different batches of formamide, or variations in salt concentrations and temperature of hybridization and washes. It is evident, however, that the signal-to-noise ratio can be increased by probe concentrations below saturation level or by times of hybridization which are in the range of 20 min. at saturating level of probe.

The value of synthetic probes is most apparent when the expression of specific members of a multi-gene family is investigated. In the case of the actin genes, we have found this approach to be highly useful (see Fig. 7) since the messages vary little except for discrete regions, usually in the untranslated region. The probe used to characterize the expression of the cardiac form of actin was made to a unique region in the coding sequence to compare it directly with a probe which would recognize all forms of actin. We found that the synthesized probes evaluated for their hybridization effectiveness were very valuable in detailing the extent of gene expression of the specific actin isoforms as well as all actin isoforms, both quantitatively, by scintillation counting and *in situ* by

autoradiography. Another advantage (see, for instance, (19,24)) of the use of oligonucleotide probes may be on tissue samples where penetration of probe may be more important than with cultured cells, since there is much more cellular material present. Further work to evaluate some of these points discussed above as well as methods to increase the sensitivity of nonisotopic detection of the probes is currently in progress.

#### ACKNOWLEDGMENTS

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