0022-1554/95/$3.30
The Journal of Histochemistry and Cytochemistry

Vol. 43, No. 5, pp. 481–488, 1995
Printed in U.S.A.

Original Article

Quantification of ANP mRNA in Primary Cultures of Adult Rat Atrial Myocytes by Image Processing: In Situ Hybridization to Multiple Parallel Samples Using Single-Stranded cDNA Probes[1]

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Received for publication June 17, 1994 and in revised form December 28, 1994; accepted January 13, 1995 (4A3407).

In primary cultures of adult rat atrial myocytes, we quantified the accumulation of atrial natriuretic peptide (ANP) mRNA in parallel with ANP secretion. ANP mRNA was quantified by image analysis of myocytes hybridized in situ with single-stranded cDNA probes generated by two successive thermal cycling procedures. In situ analysis permitted measurement of many small experimental samples in tandem while avoiding the possibility of differential extraction and processing of mRNA from sample to sample. The single-step application of ^{32}P-labeled probes allowed processing of many parallel samples and generated intense punctate autoradiographic signals that were readily countable by image processing. Biotin-labeled probes, in conjunction with gold-labeled anti-biotin antibodies and silver intensification, gave an apparently equivalent specific signal but presented more difficulty in uniform processing of many samples and was harder to quantify by our image processing system. Measurement of ANP mRNA during atrial myocyte culture showed that ANP mRNA accumulated from undetectable levels after 1 day of culture to maximal levels by Day 8. In contrast, secretion of ANP (which is stored in atrial granules) slowly decreased, but was not abolished, during the first 5 days of culture. Subsequently, ANP secretion increased, with the increase trailing ANP mRNA accumulation by at least 24 hr. (*J Histochem Cytochem 43:481–488, 1995*)

KEY WORDS: In situ hybridization; Primary culture; Rat atrial myocytes; Single-stranded cDNA probes; Image processing; ANP; Multiple mRNA samples; PCR.

Introduction

In the absence of immortalized cell lines, primary cultures of cardiac myocytes have become a widely used model system for heart cell research. However, relative to cell lines, primary cultures provide the investigator with limited material with which to work. Quantification of the levels of specific messenger RNA (mRNA) expression can be a particular problem when small sample sizes are being handled. For this reason, single experiments involving many small samples to be processed in parallel require specialized techniques to quantify biochemical events, such as changes in mRNA levels, as a function of time or in response to pharmacological or physiological challenges.

In this report we describe an assay that utilizes image processing of labeled cDNA probes hybridized to mRNA in situ in multiple (20 or more) individual cell culture samples. We have also developed a method for generating single-stranded, isoform-specific, uniformly labeled cDNA probes by two successive thermal cycling steps. The cDNA probes were labeled with radiosiotope (^{32}P) or with biotin. With these methods we were able to quantify the time-dependent content of mRNA in individual cultured atrial myocytes.

To illustrate the usefulness of these techniques, we have measured the levels of atrial natriuretic peptide (ANP) mRNA in parallel with measurements on the secretion of ANP as a function of culture age in primary cultures of adult rat atrial myocytes. ANP is a member of a family of peptides that regulate renal sodium excretion, blood volume homeostasis, and blood pressure (18). The expression and secretion of ANP, in turn, are regulated in the atria by stretching of the atrial wall and by neurotransmitters and hormones. Primary cultures of atrial myocytes are commonly used in the investigation of the molecular mechanisms of the regulation of ANP secretion (1,5). A portion of this work has been presented previously in abstract form (10).

Materials and Methods

Hybridization Probes. To produce isoform-specific, single-stranded cDNA hybridization probes labeled either isotopically (with ^{32}P) or non-

[1] Supported by an American Heart Association–Metropolitan Chicago Senior Fellowship (SKA) and by USPHS grants HL 44004 (DDD), HL 10503, and HL 20592 (Ernest Page).

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isotopically (with biotin), we employed two sequential thermal cycling reactions. In the first reaction we used as template a cDNA clone of the translated region of ANP mRNA, kindly provided by Dr. Christine Seidman, Harvard University (20). Sense and anti-sense 24-mer primers spaced about 160 bases apart were used to make by PCR a double-stranded (ds) template of about 200 bases. The primers chosen here annealed to the 3′ end of the translated region of ANP mRNA:

sense AAGTCAACCCGTCTCAGAGAGATG, beginning at BP 318, and anti-sense TATCTTCGGTACCGGAAGCTGTTG, beginning at BP 522.

This region of ANP mRNA had about 40–45% homology with BNP (9) and CNPmRNA (8). PCR in 40 μl contained 1 μg ANP clone, 0.5 μM (100 ng) each primer, 200 μM each dA, C, G, and TTP, 10 mM KCl, 2 mM $MgSO_4$, 20 mM Tris-HCl, pH 8.8, 0.1% Triton X-100, and 2 U Vent DNA polymerase (New England Biolabs; Beverly, MA). The PCR consisted of 1 min at 94°C (denaturation), 40 sec at 55°C (annealing), and 40 sec at 72°C (extension) for 35 cycles. The 200-BP product was isolated from other reactants, especially primer pairs that could compromise the following single-strand synthesis, by agarose gel electrophoresis and was eluted electrophoretically (Hoefer), followed by phenol, phenol–chloroform, and chloroform extraction and Na-acetate–ethanol precipitation. The reliability and yield of ^{32}P labeling in the subsequent reaction was dependent on the purity of the ds product. DNA concentrations were determined by absorbance at 260 nm, with corrections made for absorbance at 280 and 320 nm for protein and background, using a Beckman microcell in a Beckman DU-65 instrument. Greater than 1 μg of template was routinely made in a single PCR tube.

The single-stranded (ss) probe was generated in a second reaction in which the double-stranded product served as the template when added to sense or anti-sense primer (same sequences as above) in the presence of nucleotides and appropriate enzymes. One of these nucleotides was labeled with isotope or biotin, and a labeled sense probe or a labeled anti-sense probe was produced by a thermal cycling reaction. In this reaction, we employed 60 cycles and used Vent (exo-) DNA polymerase (New England Biolabs) because of its pronounced thermal stability and absence of exonuclease activity. For ^{32}P labeling, we adopted the method of Stürzl and Roth (21), in which we used 50 ng ds template, 700 ng (3.5 μM) primer, 200 μM each dA, G, and TTP, 18.75 μM dCTP, and 6 μl (3.3 μM) [^{32}P]-dCTP (3000 Ci/mmol) (Amersham; Poole, UK) in 40 μl volume containing Vent buffer (augmented to contain 5 mM $MgSO_4$). Cycling was done at 95°C for 1 min, 37°C for 2 min, and 72°C for 3 min. For biotin labeling, we used 100 μM of all four nucleotides where 25–75% of dATP contained biotin-14 (Gibco BRL; Gaithersburg, MD). Typical cycling was at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, although biotin labeling was so reliable that it could be done in parallel with ^{32}P labeling, under the cycling conditions described above, with good results. Generated probe was separated from unreacted nucleotides and primer by double ammonium acetate precipitation.

Northern Hybridization. Pieces of heart (ventricle plus atria) and liver were excised from adult rats, then frozen and pulverized in liquid nitrogen. Poly(A) mRNA was isolated from the tissue powder using a Micro-Fasttrack mRNA isolation kit (Invitrogen; San Diego, CA) according to the manufacturer's instructions. mRNA was fractionated on a horizontal 1% agarose–formaldehyde gel and transferred to 0.22-μm nitrocellulose membrane (Schleicher & Schuell; Dassel, Germany) by capillary action (19). The filters were pre-hybridized in 50% formamide, 6 × SSC, 5 × Denhardt's solution (19), 200 μg/ml sheared salmon sperm DNA, and 0.5% SDS at 42°C. Filters were hybridized in 6 ml fresh pre-hybridization containing 1.0 × 10^6 DPM/ml either sense or anti-sense probe. Filters were washed at room temperature (RT) in 1 × SSC, 0.1% SDS for 30 min with one change, followed by washing at 68°C in 0.2 × SSC, 0.1% SDS for 30 min with one change. Filters were exposed to Hyperfilm-MP film (Amersham) at −70°C.

Primary Cultures of Atrial Myocytes. Atrial myocytes were isolated by collagenase digestion of both left and right atria obtained from six to nine 300–350-g adult male Sprague–Dawley rats per preparation and were cultured by our previously published methods (1,6). Briefly, the isolated myocytes were plated on laminin-coated 13-mm coverslips in 16-mm wells (four-well multidish) (Nunc; Rostilde, Denmark) at a density of approximately 12 × 10^4 rod-shaped myocytes per culture well and maintained in a humidified 95% air/5% CO_2 atmosphere at 37°C. The culture medium consisted of Gibco Medium 199 (M199) supplemented with 10% fetal bovine serum, 20 μg/ml cytosine β-D-arabinofuranoside (ARA-C), 100 U/ml penicillin, and 100 μg/ml streptomycin. Unattached cells were removed from the wells on Day 5 of culture. Experiments were performed on 1–10-day-old cultures that contained 7–9 × 10^4 atrial myocytes per culture well.

Atrial myocyte density in a sample well was determined for each culture preparation to standardize the individual measurements of different preparations. For this purpose, three wells from a four-well dish of cultured myocytes were fixed on the day of the experiment with 3% glutaraldehyde and then stained with Toluidine Blue. The myocytes were directly counted by projecting their image onto the screen of a Nikon Profile Projector to determine the number of myocytes in three 4-mm^2 areas, to calculate the number of myocytes per well. The average number of myocytes per well was defined as the mean obtained from the three individual wells.

Measurement of ANP Secretion. Secretion of ANP was measured in the same atrial myocytes that were subsequently used for in situ hybridization. The secretory interval was initiated by sequentially removing the growth medium, washing three times with 1 ml M199, and adding 0.5 ml M199 to each of four culture wells (each containing myocytes on a coverslip) from a single four-well dish. After 1 hr of incubation at 37°C in a 5% CO_2 incubator, the supernatant was collected and the myocytes fixed for in situ hybridization as described below. Aprotinin and EDTA were added to the supernatant samples (final concentrations 2 μg/ml and 4 mM, respectively) and the samples were stored at −70°C until further use. The concentration of immunoreactive ANP (pmol/ml) was determined by radioimmunoassay of diluted aliquots of the samples (1,6). Unless otherwise noted, the data were normalized to the number of atrial myocytes in each culture well and were expressed as the amount of immunoreactive ANP (in attomoles) per myocyte.

In Situ Hybridization. Four coverslips of cultured myocytes per time point were fixed for 10 min at RT with 4% paraformaldehyde in PBS (pH 7.2). The myocytes were subsequently washed and dehydrated in a graded series of increasing concentrations of ethanols containing 50 mM ammonium acetate, and were stored desiccated under vacuum at 4°C. Specimens from a single experiment were processed for in situ hybridization together to enable comparisons of message levels from multiple time points or treatments. For hybridization, the myocytes on two separate coverslips were rehydrated in a series of decreasing concentrations of ethanols and rinsed in PBS (pH 7.2) for 2 min. Cells were then permeabilized by the following protocol: 0.05% Triton X-100 in PBS for 10 min, two 5-min PBS washes, 0.2 N HCl for 5 min, two 5-min PBS washes (all at RT), digestion with 1 μg/ml proteinase K in 100 mM Tris-HCl, 50 mM EDTA (pH 8.0) for 10 min at 37°C, and two 5-min PBS washes at RT. Samples were post-fixed in 4% paraformaldhyde (pH 7.2) for 5 min, washed in 200 mM Tris-HCl, 100 mM glycine (pH 7.6) for 10 min, and acetylated with 0.25% (v/v) acetic anhydrate in triethanolamine buffer (pH 8.0) for 10 min. After a rinse in 2 × SSC, mRNA in the cells was denatured for 10 min at 60°C in 1 × SSC, 50% formamide. Coverslips were placed on 25-μl droplets of pre-hybridization solution on Parafilm and incubated for 30 min at 37°C. The pre-hybridization solution contained 50% formamide, 0.3 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 × Denhardt's solution, 500 μg/ml yeast tRNA, 500 μg/ml poly(A). The coverslips were transferred to 25-μl droplets containing hybridization solution (pre-hybridization solution plus 10% dextran sulfate and 0.2 ng/μl ^{32}P- or biotin-labeled ssDNA probes) and in-

cubated at 37°C overnight. One coverslip was hybridized with the anti-sense probe and the other coverslip hybridized with the sense probe. After hybridization, coverslips were washed at RT in a large volume of 2 × SSC for 1 hr, followed by 50% formamide, 2 × SSC for 40 min, then 50% formamide, 1 × SSC for 20 min, both at 37°C. A final wash in 1 × SSC was performed at RT. The coverslips were dehydrated through a series of ethanols (70–95%) containing 50 mM ammonium acetate and then air-dried. For specimens hybridized with ^{32}P-labeled probes, hybridization signals were localized by autoradiography. Coverslips were mounted cell side out on microscope slides with double-adhesive tape and the slides were dipped in Kodak NTB-2 nuclear track emulsion with a Pelco emulsion coater (Ted Pella; Tustin, CA). After air-drying, samples were exposed for 15 days at −70°C to ensure that the signal was fully developed for those conditions in which the mRNA content was small (e.g., culture Day 3). The samples were submitted to routine photographic procedures to develop the exposed silver grains. For biotin-labeled specimens, coverslips were blocked with 10% goat serum in PBS, incubated with 5-nm gold-labeled goat anti-biotin antibodies, and intensified with silver for 10–15 min according to the manufacturer's instructions (Ted Pella). Finally, with either type of labeling, coverslips were stained wtih hematoxylin and eosin, dehydrated with a series of ethanol concentrations, and mounted on microscope slides with Permount.

Image Processing. Measurements were made using the Cue-2 Image analyzer, Version 3.0 (Olympus), interfaced with the Olympus BH-2 microscope via closed circuit television (Sony Galai CCD Video Camera, Sony Trinitron Color Video Monitor). As previously described (11), the Cue-2 system consisted of a video chain, a high-speed digital image processor, and a general purpose computer (Zeos 386, Zeos International, St Paul, MN). The video signal from the television camera was transferred simultaneously to the digital image processor and to a television monitor. The image processor transformed the signal from the camera into a matrix of 512 × 512 pixels. The intensity of the signal at each pixel was encoded as one of 128 shades of gray ranging from black to white. The processed image was then displayed on the Sony monitor as well as transferred to the computer for quantitative analysis. Brightfield optics or darkfield optics plus UV side illumination were used for locating and orienting the specimen. A darkfield condenser with a × 40 Olympus SPlan objective lens was used for the actual measurement of the silver precipitates.

Before quantification, each specimen from a single experiment was assigned a randomly selected code number to ensure impartial selection of the counting regions. The number of silver grains within each counting region (100 × 100 μm^2), chosen and calibrated by the operator, was automatically counted by the computer. The counting region generally contained fractions of two or three myocytes. Resolution of probe-generated silver grains from non-probe-generated noise was maximized by establishing a threshold value in a randomly selected counting region within each specimen. The threshold value was determined by visual inspection of the image. The Cue-2 system enabled the operator to examine and compare any choices of threshold. When a threshold value was chosen, pixels in the graphic area with gray values less than threshold were designated as black by the computer. Pixels at or above threshold were designated as white. Subsequently, the computer designated each cluster of white pixels as a single spot, or silver grain. The threshold level set for the randomly selected counting region was automatically retained and employed by the computer for all regions counted in the specimen. To increase the accuracy of our measurement, 10 separate regions, selected from the specimen by moving systematically across or down the field in one direction, were counted for each specimen and averaged.

Results

cDNA probes for hybridization were made using two sequential thermal cycling reactions. The probes were labeled on a single strand and isoform specificity was determined by the judicious choice of primers. Agarose gel electrophoresis of anti-sense and sense probes showed that both probes were uniformly full length, and that labeled strands of length other than 200 BP were not produced (Figure 1). In addition, the 60 cycle thermal reaction produced a large surplus of labeled strands relative to the unlabeled template cDNA. For the labeling reaction depicted in Figure 1 (in which 50 ng of ds DNA served as template), 980, 864, and 224 ng of DNA were collected in the anti-sense, sense, and control (polymerase omitted) reactions, respectively, after double ammonium acetate–ethanol precipitation. The anti-sense and sense products contained 3.27 and 1.91 × 10^7 DPM/μg, respectively, whereas the control product contained 1.26 × 10^4 DPM/μg. Because even two successive ammonium acetate–ethanol precipitations do not remove all unlabeled primer, and labeled and unlabeled free nucleotides from the samples, the purpose of our control reaction was to determine how much of the radioactivity in the enzyme-containing reactions was actually associated with labeled strands. This enabled us to determine more accurately the specific activities of the probes. By subtracting the control DNA value from the DNA values of the two probes, and neglecting the residual amount of labeled free nucleotide remaining in the control, we calculated a specific activity of 4.24 × 10^7 and 2.58 × 10^7 DPM/μg for the anti-sense and sense probes. In addition, our determination of DNA and radioactive content in our samples allowed us to estimate the proportion of full-length strands in our probe mixtures that were actually labeled. By subtracting the control DNA value from the DNA values for the two probes, and assuming that all unlabeled template was recovered in the final products, we calculated a lower bound for the ratio of labeled anti-sense or sense (i.e., probe) strands to unlabeled template anti-sense or sense strands of 30.2 and 25.6 for the anti-sense and sense probe mixtures, respectively. For five separate labelings, anti-sense and sense labeled strands had specific activities of 2.68 ± 1.16 (mean ± SD) and 2.17 ± 0.93 × 10^7 DPM/μg, and labeled to unlabeled strand ratios of 26.7 ± 4.0 and 23.6 ± 4.6, respectively. In the subsequent quantification of in situ hybridization signals with ^{32}P-labeled probes presented in this study (Figure 5), we corrected for differences in the intensities of the anti-sense and sense labeled probes employed (see Discussion).

For biotin-labeled probes we were similarly able to estimate the yield and relative proportion of labeled DNA. However, the relative degree of labeling of anti-sense and sense probes, by blotting equivalent amounts of anti-sense and sense probe onto nitrocellulose, probing with reporter-labeled streptavidin, and comparing staining intensities densitometrically, was not determined in this study.

In a Northern analysis, the ^{32}P-labeled anti-sense probe synthesized by the methods described above hybridized to a single band of heart (ventricle plus atrium) poly(A) mRNA of the appropriate size, 850 bases (14, 20), but did not hybridize to poly(A) mRNA from liver (Figure 2). The sense probe did not hybridize to poly(A) mRNA from either rat heart or liver (Figure 2). Similar results were obtained for sense and anti-sense probes labeled with biotin (data not shown).

Figure 3 compares the results obtained with biotin-labeled (Figures 3A and 3B) versus ^{32}P-labeled (Figure 3C–3H) anti-sense and sense cDNA probes hybridized in situ to ANP mRNA in pri-

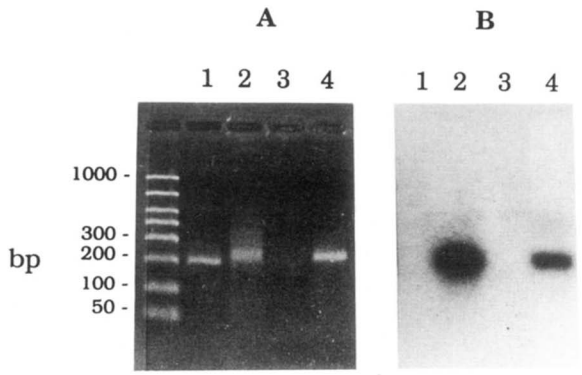

Figure 1. Production of single-stranded ^{32}P-labeled cDNA probes. (**A**) Analysis of the PCR products by electrophoresis in a 2% non-denaturing agarose gel. Lane 1, 30 ng of ds 200 BP product made by PCR from ANP mRNA clone using sense and anti-sense primers and subsequently used as template for synthesis of single-stranded probes. Lane 2, 98 ng of ss product (10% of the total product) made by thermal cycline using anti-sense primer and 200 BP ds template. Lane 3, 10% of the total product of a companion reaction as in Lane 2, except that thermal stable DNA polymerase was omitted. Lane 4, 86 ng of ss product (10% of the total) made using sense primers. (**B**) Autoradiograph of dried gel shown in **A**. Exposure time was 5 min.

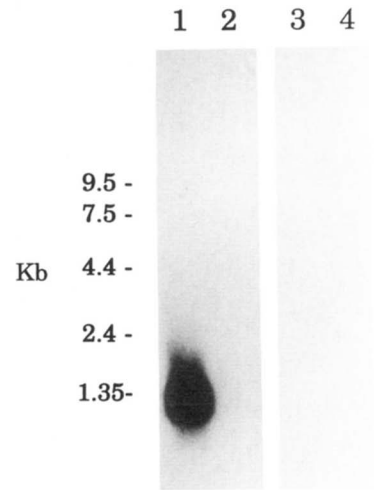

Figure 2. Northern blot analysis of ANP mRNA probe specificity. Poly(A) mRNA from (Lane 1) rat heart or (Lane 2) rat liver, 2 μg each, was separated by agarose gel electrophoresis and transferred to nitrocellulose membranes. The membranes were hybridized with anti-sense ^{32}P-labeled probe, made as described in Figure 1, then exposed to autoradiographic film. In Lanes 3 and 4, equivalent poly(A) mRNA samples from either rat heart (Lane 3) or liver (Lane 4) were hybridized with sense ^{32}P-labeled probes. Autoradiographic exposure time was 3.5 hr.

mary cultured atrial myocytes. Darkfield micrographs of representative areas (Figures 3A–3F) showed silver precipitates as bright spots, which in brightfield micrographs (Figures 3G and 3H) appeared as dark spots. The signal generated with either the biotin- or the ^{32}P-labeled probe was primarily localized over the cytoplasm of the myocytes; labeling was not very evident over the nuclear region (Figures 3A, 3E, and 3G). The spots obtained from silver enhancement of the gold-labeled anti-biotin antibodies showed a continuous spectrum of sizes and intensities. In contrast, the sizes and intensities of the spots obtained from autoradiography of ^{32}P-labeled cDNA probes were much more uniform.

Figure 4 shows histograms of the distribution of shades of gray vs the number of pixels of each shade in computer-generated images of the micrographic areas displayed in Figures 3A and 3C. The shades of gray correspond to the intensity of silver precipitation within the silver-enhanced gold-labeled specimen or in the emulsion overlying the ^{32}P-labeled specimen. Note the biphasic distribution of the data displayed in Figure 4B (^{32}P-labeled probe), which is not discernible in Figure 4A (biotin-labeled probe). The intensity of the large punctate clusters of pixels generated by the silver precipitates made it relatively easy to distinguish ^{32}P-generated signal from the less intense background. In the example presented in Figures 3C and 4B, the imaging system detected 264 clusters of pixels (spots or grains) with gray values equal to or greater than 112, the operator-chosen threshold. Similar analysis of the specimen shown in Figure 3D (hybridization with the sense probe) detected only 18 suprathreshold silver grains in an area of the same size (not shown).

As shown by the histogram in Figure 4A, no threshold value could be assigned to the micrographic region displayed in Figure 3A that would approximate a distinction between probe- and non-probe-generated signal. To determine a specific signal for silver-enhanced gold-labeled specimens, it would be necessary to measure the entire non-black area within the specimen hybridized with the sense probe and to subtract it from a similar determination for the anti-sense probe-hybridized specimen. This would result in a generally less favorable signal-to-noise ratio than for the ^{32}P-labeled specimens. The gold-labeled specimen displayed here may be overexposed with silver, but we found it necessary to provide the specimens with adequate exposure to ensure visualization of the gold at the light microscopic level. Optimization of development for a single specimen was difficult, and the prospect of coordinating optimal development for 20 or more specimens was compounded by the short times for silver intensification.

Examination of the expression of ANP mRNA in the cultured atrial myocytes showed that the levels of ANP mRNA varied with the duration of the culture period (Figure 5A). Each filled circle represents the average count of silver grains in the photographic emulsion overlying myocytes hybridized with ^{32}P-labeled anti-sense probe, and open circles represent values obtained from parallel cultures of myocytes hybridized with the sense probe to determine the nonspecific probe-generated signal. In the experiment depicted in Figure 5A, the specific activities of the anti-sense and sense probes used were 3.16 and 2.47 $\times$ 10^7 DPM/μg, respectively. The number of silver grains measured in the sense-exposed specimens was multiplied by 1.28 to reflect this difference in specific activities. During the first 3 days in culture, ANP mRNA could barely be detected above background levels. However, continued culture of the atrial myocytes resulted in the reaccumulation of ANP message. Maximal levels of ANP mRNA were attained after 7–9 days of culture.

The majority of ANP in rat atrial myocytes is stored highly con-

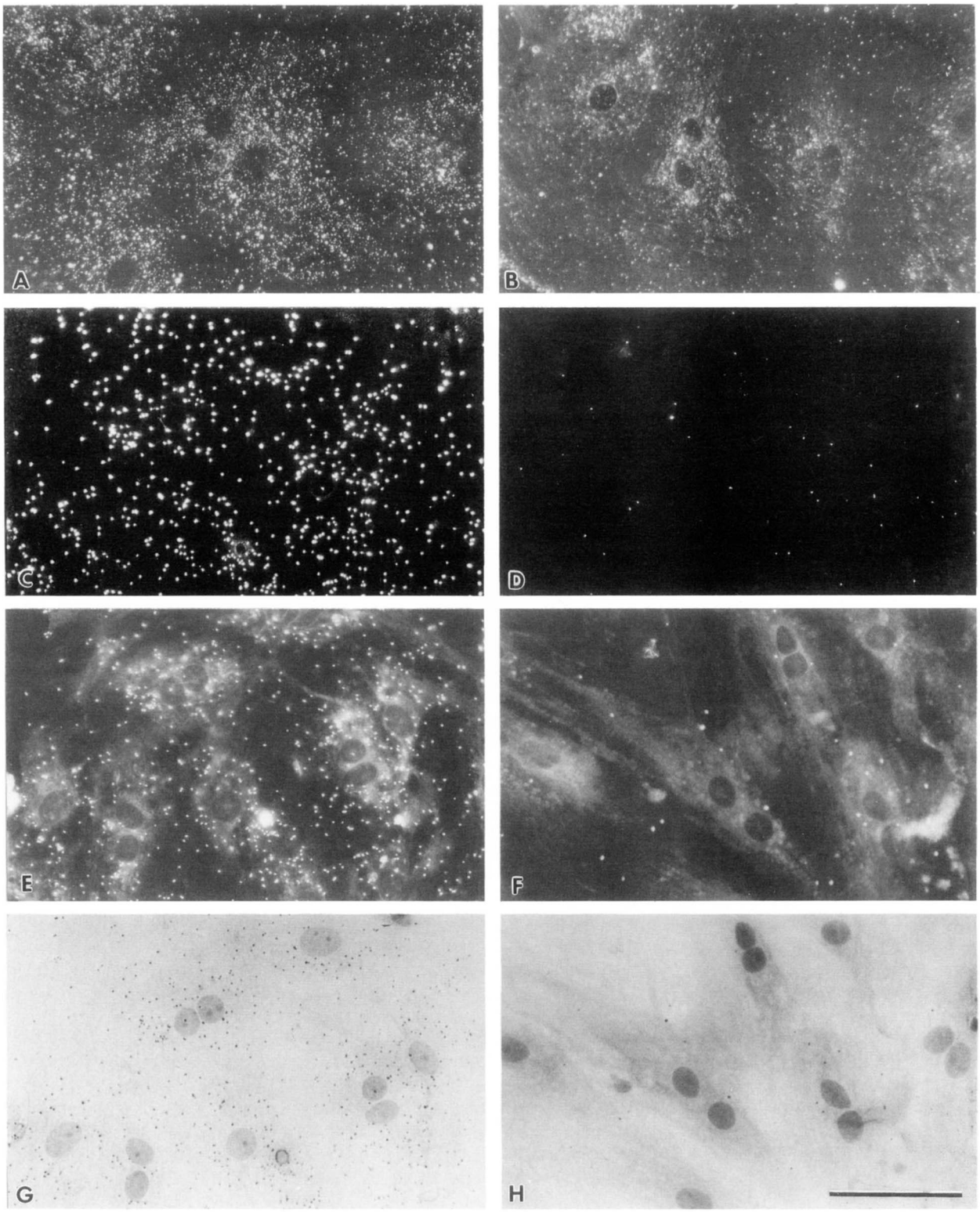

Figure 3. In situ hybridization of anti-sense and sense cDNA ANP probes in primary cultures of atral myocytes. Hybridization of ANP cDNA to atrial myocytes cultured for 8 days was visualized by (**A,B**) silver enhancement of gold-labeled anti-biotin antibodies or by (**C–H**) autoradiography of ^{32}P. Darkfield micrographs of representative areas (**A–F**) show silver precipitates as bright spots, which in brightfield micrographs (**G,H**) appear as dark spots. Cells were lightly stained with hematoxylin and eosin y; cell nuclei appear as dark oval areas in the brightfield micrographs. Anti-sense probe was hybridized to samples shown in **A,C,E,** and **G**; sense probe to those shown in **B, D,F,** and **H**. Note that **C,E,** and **G** and **D,F,** and **H** display the same fields of view but with different forms of illumination. In **E** and **F**, in addition to the regular darkfield condenser, UV illumination was used to show cytoplasmic detail of the myocytes under darkfield conditions. The UV illumination was not used during image processing, as represented by **C** and **D**. Bar = 50 μm.

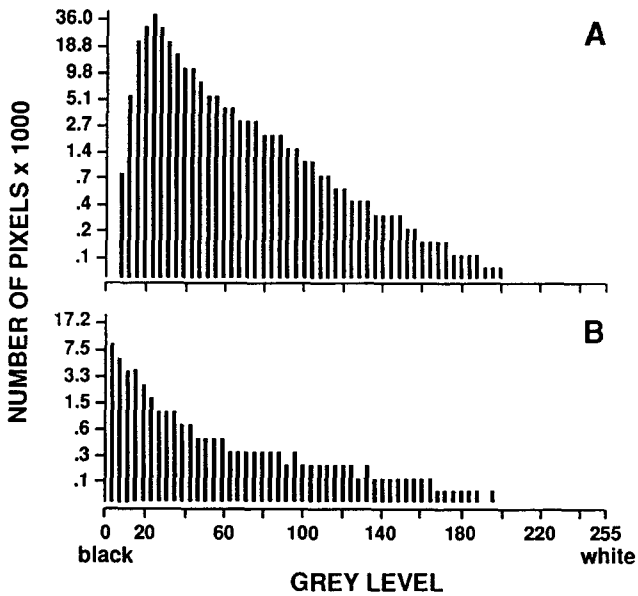

Figure 4. Distribution of pixel intensities in computer-derived images of in situ hybridized specimens. **(A)** Histogram of the number of pixels of each shade of gray for 128 shades ranging from black to white. The pixel matrix was 512 × 512. Data were obtained from image analysis of the micrograph shown in Figure 3A, in which silver intensification of gold-labeled antibodies against biotin was employed. **(B)** Gray level histogram obtained from image analysis of micrograph of atrial myocytes hybridized with ^{32}P-labeled anti-sense cDNA probe shown in Figure 3C.

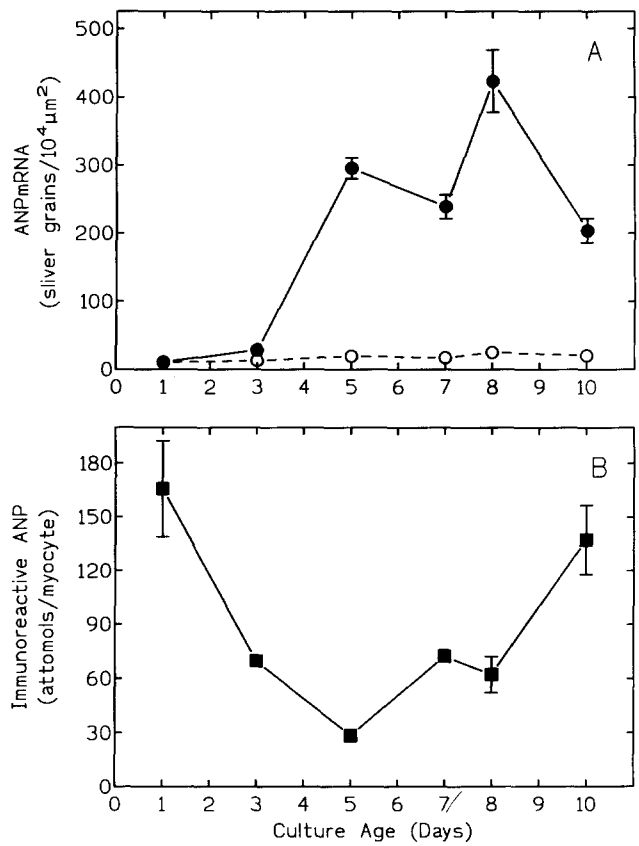

Figure 5. Expression of ANP mRNA and ANP secretion in atrial myocytes as a function of culture age. **(A)** Levels of ANP mRNA in atrial myocytes as determined by image analysis of in situ hybridized ^{32}P-labeled cDNA probes. Filled circles, average ± SE counts of silver grains in the photographic emulsion overlying myocytes hybridized with anti-sense probe; empty circles, average ± SE obtained from myocytes hybridized with sense probe as a control. Each point represents the average number of silver grains obtained from 10 100 × 100 µm^2 areas in a single coverslip. **(B)** ANP secretion by atrial myocytes as quantified by radioimmunoassay. Each symbol represents the mean ± SE obtained from triplicate culture wells. The data were obtained from atrial myocytes that were subsequently fixed and then analyzed to generate the data displayed in **A**. The entire figure is representative of results obtained from three separate culture preparations.

centrated in atrial granules (18). ANP secretion by the atrial myocytes during the culture period displayed a biphasic pattern as a function of culture age (Figure 5B). ANP secretion declined from approximately 165 amols/myocyte on Day 1 to a minimal value of 30 amols/myocyte by Day 5 of culture. ANP secretion began to recover by Day 7 of culture and reached maximal values (140 amols/myocyte) 8–10 days after the myocytes were placed in culture. The increase in the amount of ANP secretion occurred at least 24 hr after the renewed accumulation of ANP mRNA. The myocyte density remained relatively constant throughout the culture period. In parallel studies, we have shown that ANP secretion is proportional to cellular ANP content. Furthermore, the secretion of ANP throughout the culture period, even in the absence of ANP mRNA, agrees with electron micrographic observation of abundant atrial granules in the atrial myocytes after 1 or after 8 days of culture, but relatively fewer atrial granules after 4 days of culture (S. K. Ambler, D. D. Doyle, L. Kordylewski and E. Page, data not shown).

Discussion

The problem addressed in the present study is the quantification of the relative content of a particular mRNA species in many small samples of cultured cells. In lieu of extracting RNA from each individual sample, with all the concomitant problems of differential RNA degradation and of transfer loss from sample to sample, we chose to quantify mRNA levels in intact primary cell cultures by in situ hybridization.

The method of producing probes described here allows both non-isotopic and isotopic labeling. Our approach is therefore amenable to the wide spectrum of read-out choices available with non-isotopic labeling with which we might quantify our signal. There are many advantages to using non-isotopic labeling, including ease of probe production, reduced cost, long-term stability of the probes during storage, and avoidance of environmental problems. Analog signals produced by non-fluorescent and fluorescent substrates of alkaline phosphatase complexed with non-isotopically labeled probes have been quantified using internal standards for each experiment (12,13). Chemiluminescent substrates of horseradish peroxidase produce a highly sensitive read-out, especially for low-abundance messages (15). Silver intensification of horseradish peroxidase non-chemiluminescent product has the potential to be digitized by a computer-driven imaging system by virtue of generating

a dark enough signal in relation to background (17). However, because non-isotopic read-outs involve immunolabeling followed by histochemical staining, they necessarily require multiple steps for fruition, each of which may contribute nonspecific background to the signal. Each of these steps requires fine tuning to optimize the signal-to-noise ratio. In this study, we have investigated non-isotpyically labeled probes that were subsequently tagged with silver-intensified gold-labeled antibodies. Although we may not have fully optimized the procedure, our results exemplify the central problem of the situation at hand: the parallel processing, in a single batch, of 20 or more specimens from a single experiment so that mRNA quantities in each of the specimens will be equivalently assessed. As an alternative to non-isotopic labeling, we found that the essentially single-step method of isotopic labeling was easy to regulate and therefore allowed uniform processing of each specimen. Moreover, the intense, punctate ^{32}P signal was easily interpretable. The recently developed technique of ^{33}P labeling (3,16) was not tried in this study, but can presumably readily substitute for ^{32}P.

Considerable progress has been made in the past decade in the production of cDNA hybridization probes utilizing thermally stable polymerases with isotopic and non-isotopic labeling. An et al. (2) reported production of non-isotopically labeled single-stranded cDNA hybridization probes by two sequential thermal cycling steps. They used two sets of primers: the first set was employed to generate a double-stranded template; the second, a nested set, was used to generate labeled sense and anti-sense strands. Stürzl and Roth (21) used thermal cycling to label single-stranded cDNA with ^{32}P in a run-off reaction that required convenient restriction sites in the plasmid used as template for the reaction. In this study, we have combined sequential thermal cycling using a single set of primers with carefully chosen reaction conditions and ratios of labeled to unlabeled nucleotides (21) to generate ^{32}P-labeled ss cDNA probes. The efficiency of incorporation of non-isotopically labeled nucleotides into extended strands, although reportedly less than for unlabeled nucleotides (22), did not require such carefully chosen reaction conditions.

The specific activities of the sense and anti-sense probes were predictably non-identical and varied somewhat from one labeling reaction to another. However, because our method of probe synthesis allows accurate determination of the specific activity of each probe, one can adjust background values to reflect the difference of the specific activities of the anti-sense and sense probes. An alternative method to assess background levels is to include a 100-fold excess of unlabeled anti-sense probe in a control sample.

Using the methods of cDNA probe synthesis and image analysis of isotope-labeled hybridization products as developed in this study, we have measured changes in the levels of ANP mRNA in atrial myocytes during primary culture. ANP mRNA levels initially dropped, from levels in intact myocytes of between 1 and 3% of total cell mRNA (20), to levels indistinguishable from nonspecific hybridization by Day 1 of culture. ANP mRNA levels subsequently recovered by Day 5, reaching maximal values between Days 8–10 of culture. In contrast, ANP secretion by the cultured myocytes remained measurable, albeit decreasing, during the first 5 days of culture. Since the bulk of ANP in rat atrial myocytes, in cells in culture as well as cells in intact tissue (4,7), is found in long-term storage granules, these granules are presumably the source of the ANP secreted in the first few days of culture despite the absence of measurable ANP mRNA. Subsequently, recovery of ANP secretory levels after Day 5 paralleled changes in ANP mRNA levels, although the changes in the recovery of secretory values lagged behind the changes in the recovery of mRNA levels by at least 24 hr.

We conclude that characterization of the time-dependent changes in ANP mRNA and ANP secretion helps to reveal the complexity of the interrelationship between molecular retention and storage and dedifferentiation, redifferentiation, and growth of atrial myocytes adapting to culture conditions. In addition, the methods developed and applied in this study to characterize these relationships form the basis of an assay with which to monitor physiological and pharmacological perturbations of mRNA content and peptide secretion in model systems of limited sample size.

Acknowledgments

We thank Drs Jack Kyle and Toshihiko Toyofuku for advice on using PCR to generate hybridization probes. We are grateful to Mr Jeff Winterfield for assistance with probe generation.

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