Chapter 37

In Situ Hybridization with Radiolabeled cRNA Probes, Using Tissue Sections and Smears

Carolin Adams, Qutayla A. Hamid, and Julia M. Polak

1. Introduction

Immunocytochemistry, together with biochemical procedures, has ensured rapid and accurate identification of tissue components. This has led to a better appreciation of cellular events, particularly storage and secretion of products. However, such techniques have certain drawbacks, in particular the impossibility of monitoring the intracellular processes concerned with protein synthesis. Thus, there was a need for a method that would provide more detailed information about the functional morphology and gene expression in tissues at cellular level. Advances in molecular biology allowed the development of *in situ* hybridization, a procedure that localizes specific nucleotide sequences (DNA or RNA) in tissue preparations using labeled complementary probes (DNA or RNA).

Initial *in situ* studies (*1,2,3*) involved the visualization of ribosomal DNA, using radiolabeled cRNA probes. Since then, the technique has expanded to include the hybridization of both DNA and cytoplasmic RNA with a variety of nucleic acid probes (*4,5*).

For localization of RNA, there are four types of nucleic acid probes: double-stranded DNA probes (*5*), single-stranded cDNA probes (*6*), cRNA probes (*7*), and finally, synthetic oligonucleotide probes (*5*). Double-stranded probes require "melting" and may reanneal during hybridizations, thus reducing the amount of probe available for reaction with the mRNA. The advantages of cRNA probes include the high thermal stability and affinity of RNA–RNA hybrids, a constant probe size, no vector sequences, and the availability of RNase to remove unhybridized probes (*8*). In addition, a probe with an identical sequence to the mRNA can be prepared and used as a sense probe.

The probes can be labeled with radioactive or nonradioactive tags. The most successful of the nonradioactive methods uses biotin-substituted nucleotides. However, at present, autoradiography appears to be the most sensitive detection method available for hybridization histochemistry. In the following pages, we will outline the method we follow using radioactive labeled riboprobes and give some examples of *in situ* hybridization for the investigation of the diffuse neuroendocrine system (*9,10,11*).

2. Materials

2.1. Transcription

1. 5 x Transcription Buffer: 0.2*M* Tris-HCl, pH 7.5, 30 m*M* $MgCl_2$, 10 m*M* Spermidine.
2. 100 m*M* dithiothreitol (DTT).
3. RNasin (Human Placental Ribonuclease Inhibitor): 25 U/μL.
4. Nucleotide Mixture: 2.5 m*M* each of ATP, GTP, and UTP.
5. 100 μ*M* cytidine triphosphate (CTP).
6. 1 mg/mL linearized plasmid template DNA in water or Tris-EDTA Buffer.
7. Cytidine (α–^{32}P triphosphate) 10 mCi/mL.
8. SP6 RNA Polymerase, T7 RNA Polymerase, or T3 RNA Polymerase: 10 U/μL. These enzymes are very labile and should be out of the –20°C deep freeze for a minimal time.
9. DNase (RNase free): 1 μg/μL.
10. t RNA: 10 μg/μL.

11. 4*M* NaCl.
12. Phenol: Melt the solid at 68°C, add 8-hydroxy-quinoline (antioxidant) to a final concentration of 0.1%. Extract several times with an equal volume of buffer (1.0*M* Tris HCl, pH 8.0, followed by 0.1*M* Tris HCl, pH 8.0 with 0.2% β mercaptoethanol) until the pH of the aqueous phase is 7.6.
13. Chloroform:isoamyl alcohol (24:1).
14. 7*M* Ammonium Acetate.
15. Absolute Ethanol.
16. 10% TCA (Trichloroacetic acid).
17. Bovine Serum Albumin: 10 µg/µL.

Store solutions 1–10, 15, 17 at –20°C, and 12 at 4°C. All other solutions may be stored at room temperature.

2.2. *Fixation*

1. 0.1*M* phosphate-buffered saline (PBS): Dissolve 87.9 g NaCl, 2.72 g KH_2PO_4 and 11.35 g Na_2HPO_4 (or 23.9 g $Na_2HPO_4 \cdot 12H_2O$) in 10 L of distilled water.
2. 4% Paraformaldehyde: Dissolve 4 g of paraformaldehyde in 100 mL of hot PBS (50–60°C) with stirring. Continue stirring until the solution is clear. If necessary, add 10*N* NaOH dropwise until the solution clears. Cool, check pH (7.2), and use immediately.
3. 15% Sucrose in phosphate-buffered saline.
4. Poly-L-lysine-coated glass slides: Soak slides in detergent overnight. Wash in running tap water for 4–6 h. Rinse in several changes of double-distilled H_2O. Bake at 250°C for 4 h. Coat slides with 0.01% poly-L-lysine (Sigma, mol wt 300,000) (stored at –20°C). Air dry.

2.3. In Situ *Hybridization with cRNA Probes*

1. Phosphate-buffered saline.
2. 0.1*M* Glycine in phosphate-buffered saline.
3. 0.3% Triton X 100 in phosphate-buffered saline.
4. Proteinase K solution: 1 µg/mL, 0.1*M* Tris HCl pH 8.0, 500 m*M* EDTA pH 8.0 (Stock proteinase K: 500 µg/mL, store at –20°C).
5. 4% Paraformaldehyde (freshly prepared).
6. Acetylation solution: 0.25% (v/v) Acetic Anhydride, 0.1*M* Triethanolamine pH 8.0. Use immediately.
7. Formamide.

8. 10 x standard sodium citrate (10 x SSC) stock: 1.5*M* NaCl, 0.15*M* Trisodium citrate. Dilute as required.
9. Hybridization solution: 50% deionized formamide, 12.5 x Denhardts, 10% dextran sulfate, 250 m*M* Tris HCl pH 7.5, 0.5% sodium pyrophosphate, 0.5% sodium dodecyl sulfate (SDS) and 250 µg/mL denatured salmon sperm DNA. This is prepared freshly from the following stock solutions, which are stored at –20°C:
   a. 100% deionized formamide.
   b. 100x Denhardts: 2% bovine serum albumin, 2% polyvinylpyrrolidone (PVP-360), 2% Ficoll 400.
   c. 5*M* Tris-HCl, pH 7.5.
   d. 30x standard sodium citrate (SSC).
   e. 20% sodium dodecyl sulfate (SDS).
   f. 50% dextran sulphate.
   g. Salmon sperm DNA: 20 mg/mL.
10. RNAase A solution: 20 µg/mL in 0.5*M* NaCl, 10 m*M* Tris-HCl pH 8.0, 1 m*M* EDTA pH 8.0 (Stock RNase A: 10 mg/mL, store at –20°C).
11. Dimethyl-dichlorosilane-coated coverslips: Dip coverslips in 5% dimethyl-dichlorosilane in chloroform. Rinse several times with ddH_2O. Dry.
12. 70, 90, and 100% ethanol containing 0.3*M* ammonium acetate.
13. Autoradiography emulsion: Kodak NTB-2 or Ilford K-5 diluted 1:1 with double-distilled H_2O.
14. Kodak D19 developer.

3. Methods

3.1. Synthesis of High Activity Single-Stranded cRNA Probes (Transcription)

The following protocol is a modification of that given by Promega Biotec for synthesis of RNA probes (*see* Notes 1–4; Figs. 1 and 2).

1. To a sterile microfuge tube, at room temperature, add in the following order: 4.0 µL 5 x Transcription Buffer, 2.0 µL 100 m*M* dithiothreitol (DTT), 0.8 µL RNasin, 4.0 µL Nucleotide Mixture, 2.4 µL 100 µm cytidine triphosphate (CTP), 1.0 µL linearized plasmid template DNA (1 µg), 5.0 µL α–^{32}P-CTP (50 µCi), 0.5–0.8 µL SP6 RNA polymerase, T7 RNA Polymerase or T3 RNA Polymerase, and DEPC-treated water to 20 µL final vol.

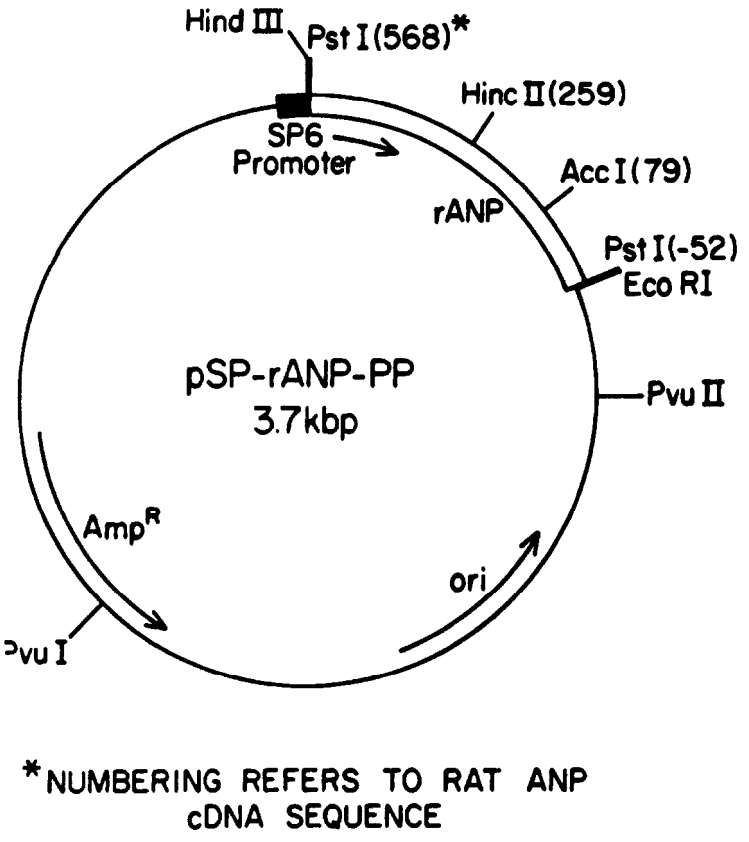

Fig. 1. Schematic diagram of the SP6 plasmid (ANP-cDNA) used for in vitro transcription.

2. Incubate for 1–1-1/2 h at 37–40°C.
3. To terminate transcription, add 1 µL of RNase-free DNase and 1 µL of RNasin. Incubate for 10 min at 37°C.
4. Add: 1 µL of tRNA, 175 µL of DEPC-treated water, and 5 µL of 4*M* NaCl. Extract with an equal vol (200 µL) of phenol/chloroform (1:1 v/v). Mix by vortexing. Separate the phases by centrifugation in a microfuge (5 min).
5. Remove the upper aqueous phase (200 µL) and extract this with an equal vol (200 µL) of chloroform. Mix and spin as above.
6. To the upper aqueous layer add 100 µL of 7*M* ammonium acetate (2.5*M* final concentration), 750 µL of absolute ethanol (2.5 vol, –20°C). Mix and leave at –20°C overnight.
7. Spin in a microfuge for 30 min. Discard the supernatant. Dry the RNA pellet under vacuum. When dry, dissolve the pellet in 20 µL of DEPC-

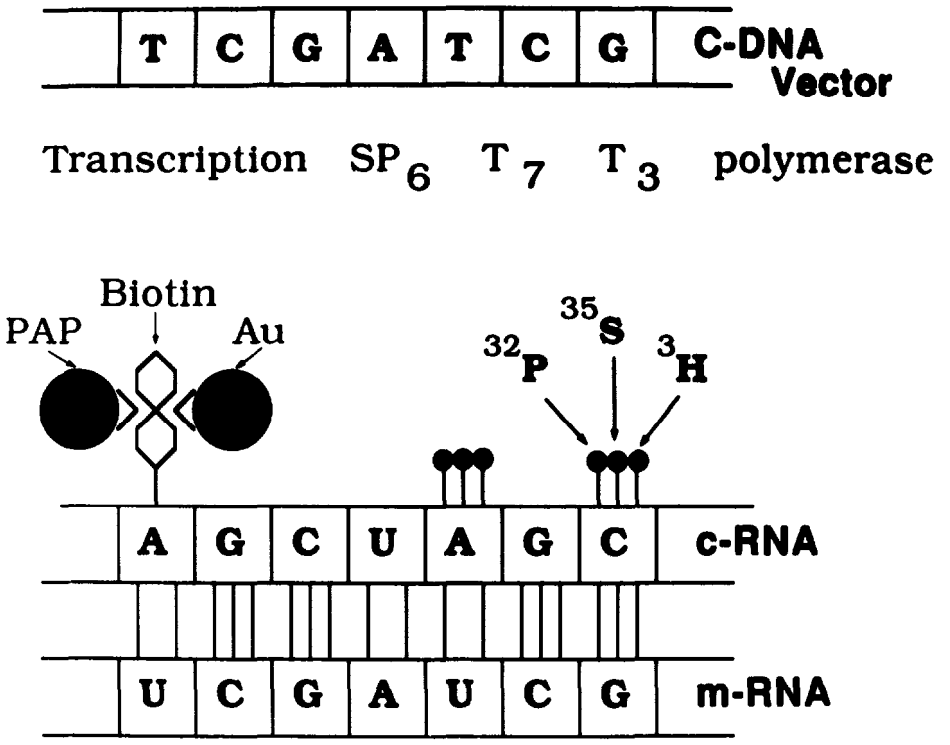

Fig. 2. Diagrammatic representation of cRNA synthesis.

treated water. Remove 1 µL for assessment of incorporation of radioactivity. Store ^{35}S probes at –70°C, ^{32}P probes at –20°C. The maximum storage time will depend on the radioisotope used. However, background increases with storage time.

8. Incorporation of radioactivity is estimated by determination of trichloroacetic acid (TCA)-precipitable counts. Mix 1 µL labeled RNA probe, 50 µL Bovine Serum Albumin, and 100 µL 10% trichloroacetic acid. Vacuum filter on GF/C (Glass microfiber paper, Whatman). Wash the filter twice with 10% TCA and twice with absolute ethanol. Dry the filters. Count the radioactivity on the filters and from this determine the percent of incorporation of radioactivity.

3.2. *Fixation of Material* (See *Note 5*)

3.2.1. Tissue

We have evaluated the use of various fixatives, including 10% formalin, Bouin's solution, 2.5% glutaradehyde/paraformaldehyde mixtures,

acetic acid/alcohol, and 4% paraformaldehyde. Formalin and Bouin's gave the best morphology but poor RNA retention, whereas glutaraldehyde fixation retained more RNA, but the resultant morphology was poor. Thus, we compromised with 4% paraformaldehyde, which gave the best results when considering both RNA retention and cellular morphology.

1. Tissues for hybridization must be collected as fresh as possible. Fix small pieces of tissue (1 x 1 x 0.5 cm) by immersion in freshly prepared 4% paraformaldehyde for 4 h at 4°C (prolonged fixation reduces the hybridization signal). To avoid RNase contamination, wear gloves, use sterile equipment, and use DEPC treated water.
2. Transfer the tissue to phosphate-buffered saline containing 15% sucrose and store at 4°C (maximum 2 mo).
3. After washing the tissue, prepare cryostat blocks, cut sections (15 µm), thaw-mount on poly-L-lysine (PLL)-coated glass slides, and allow to dry at 37°C overnight before processing for hybridization. Use these slides as soon as possible; otherwise store in a container with desiccant at –70°C.

3.2.2. Culture

1. Cultured cells can be grown on coverslips, slides, or in suspension. Rinse coverslips or slides, with attached cultures, in cold phosphate-buffered saline, and fix by immersion in 4% paraformaldehyde for 60 min at 4°C.
2. Rinse several times in phosphate-buffered saline and finally in double-distilled H_2O.
3. Dry the cultures for 6 h at 37°C and store in a dessicated box at –70°C.
4. Cell lines, grown in suspension, are cytospun onto poly-L-lysine-coated slides, which are then air-dried for 5–10 min before being fixed and stored as above.

3.3. In Situ *Hybridization with cRNA Probes*

Precautions should be taken to avoid RNase contamination until hybridization is complete. Coverslips with cultured cells grown on them should be attached to slides with paper clips (*12*).

3.3.1. Tissue Preparation

1. Rehydrate in phosphate-buffered saline (PBS) (pH 7.2) for 5 min.
2. Immerse in 0.1*M* glycine/PBS (5 min).

3. Permeabilize by immersion in 0.3% Triton X 100 in PBS for 15 min.
4. Wash with PBS (2x 3 min).
5. Deproteinize by incubation with 1 µg/mL Proteinase K solution for 20 min at 37°C.
6. Stop deproteinization by immersion in 4% paraformaldehyde/PBS (5 min).
7. Immerse in freshly prepared acetylation solution for 10 min to reduce nonspecific binding.
8. Prehybridize in 50% formamide, 2x SSC (37°C at least 15 min) to enhance signal to noise ratio.

3.3.2. Hybridization

1. Drain the slides briefly (do not dry).
2. Apply 20 µL of hybridization mixture preheated to 37°C, containing 2–3 ng radiolabeled cRNA probe (5×10^5 cpm/section) diluted in hybridization buffer.
3. Cover the sections with suitably sized dimethyl dichlorosilane-coated coverslips.
4. Incubate at 37–43°C for 16 h.

3.3.3. Posthybridization Washing

1. Remove the coverslips by immersion in 4 x standard sodium citrate (SSC).
2. Wash the slides with 4 x SSC (37°C, 3 x 20 min) with gentle shaking.
3. Remove unhybridized single-stranded cRNA probe by treating preparations with RNase A solution for 30 min at 37°C.
4. Wash the slides with 2 x SSC (37°C, 30 min), with gentle shaking.
5. Wash the slides with 0.1 x SSC (37°C, 30 min), with gentle shaking.
6. Dehydrate in 70, 90, and 2 x 100% ethanol containing 0.3*M* ammonium acetate (10 min each at room temperature).
7. Air dry (60–90 min).

3.3.4. Autoradiography

1. Dip the slides in emulsion.
2. Air dry for 1–2 h.
3. Store in light box for 2–5 d, depending on the radioisotopes used (2–3 d for ^{32}P, 5 d for ^{35}S).
4. Develop in Kodak D19 developer prepared and used according to manufacturer's instructions. Fix as appropriate.
5. Wash well with water.

3.3.5. Counterstaining

1. Preparations are usually lightly counterstained with hematoxylin or hematoxylin/eosin. However, eosin may cover fine grains. Other counterstains may be used where appropriate (e.g., Coomassie blue for myocytes, Toluidine blue for brain preparations).
2. Dehydrate, clear, and mount with DPX. Examples of *in situ* hybridization results are shown in Figs. 3–6.

4. Notes

1. Control experiments are very important to assess the specificity of the hybridization and should include the following:
   a. Sense probes: Probes identical to the coding strand of the mRNA under investigation are transcribed and hybridized as above.
   b. Ribonuclease treatment: Sections or cultures are treated with RNase A (20 μg/mL, 37°C, 30 min) before the prehybridization step. A remnant of the ribonuclease could result in probe degradation and thus invalidates the results.
   c. Inappropriate probe for the tissue in question.
   d. Inappropriate tissue for the probe in question.
   e. Northern Blot Analysis: The presence of the particular mRNA in the tissue may be confirmed by Northern Blot hybridization.
   f. Several probes, coding for different regions of the same gene.
   g. Immunocytochemistry: The correlation of immunocytochemistry results with those obtained by *in situ* hybridization is one of the most useful indications of the specificity of the signal.
2. Signal/Background Ratio: Although probes labeled with ^{35}S give better subcellular resolution than those labeled with ^{32}P, there is an increase in background. The background may be reduced by the following manipulations:
   a. Decreased autoradiography time.
   b. Minimize the amount of probe used for hybridization.
   c. The inclusion of dithiothreitol (50 m*M*) in one or all the following: prehybridization solution, hybridization mixture or post-hybridization washings. This is particularly true with the use of ^{35}S-labeled probe.
   d. The addition of "cold" cytidine triphosphate to the prehybridization buffer.

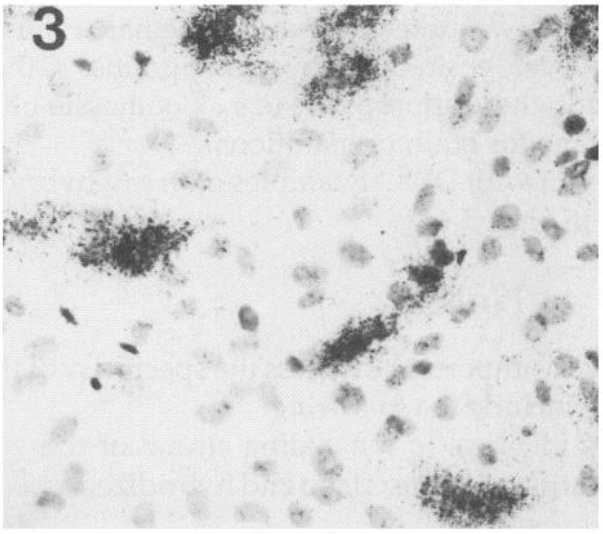

Fig. 3. *In situ* hybridization of ANP-mRNA in cultured myocytes of rat atrium and ventricle using ^{32}P-labeled ANP-cRNA probes.

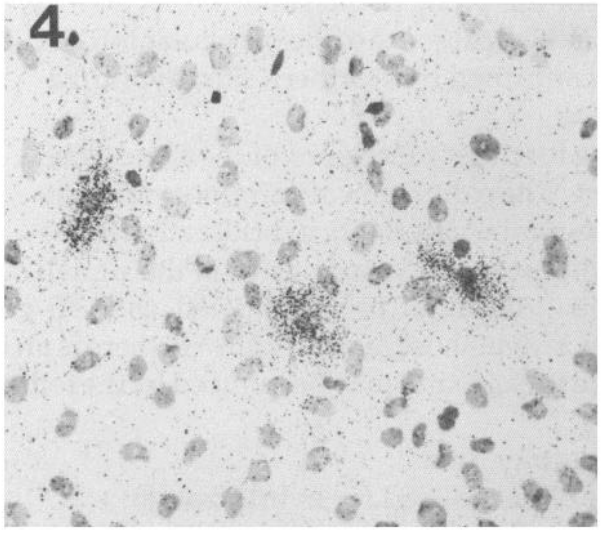

Fig. 4. Autoradiographic preparations of cultures, fixed in 4% paraformaldehyde, and counterstained with hematoxylin x 250.

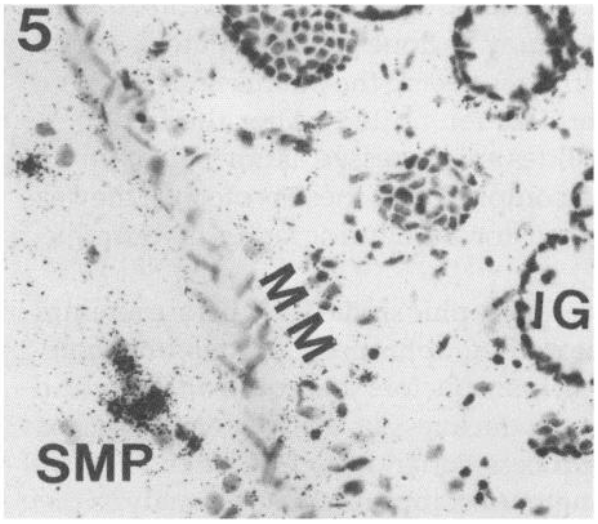

Fig. 5. *In situ* hybridization of CGRP-mRNA in rat colon (fixed by perfusion with 4% paraformaldehyde) using a ^{32}P-labeled cRNA probe. Positive hybridization signal in submucous plexus (SMP) x 300. MM = muscularis mucosa, IG = intestinal glands.

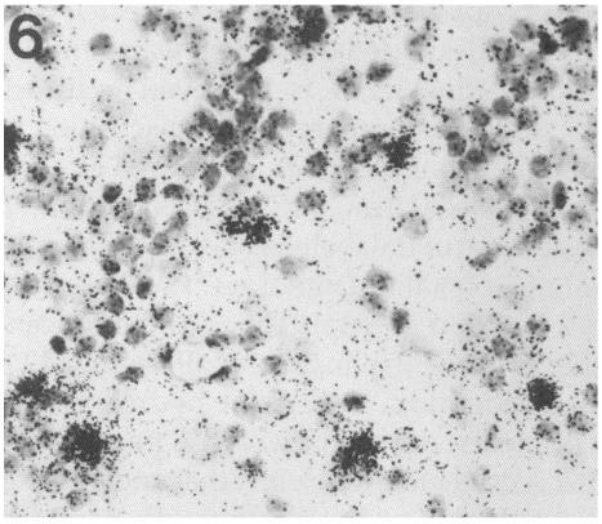

Fig. 6. The localization of bombesin-mRNA in culture of small cell carcinoma of the lung, cytospun onto slides, using ^{32}P bombesin-cRNA probe.

3. *In situ* hybridization:
   a. Combined immunocytochemistry and *in situ* hybridization. Immunocytochemistry may be done subsequent to *in situ* hybridization (*8*), the difference being the ommission of dextran sulfate from the hybridization buffer. After the final post-hybridization wash, slides are processed as normal for immunocytochemistry. On completion of these protocols, they are dehydrated and dipped for autoradiography as described above.
   b. Quantitation of autoradiographic signal (*12*). Before attempting to quantify the autoradiographic preparations, one should take into consideration many factors that could affect hybridization signal such as the thickness of tissue or emulsion, loss of mRNA in tissue processing, and the efficiency of *in situ* procedure. Densitometry or computer imaging analysis give semi-quantitative estimates.
4. Transcription: A major problem when working with mRNA preparations is RNase contamination. Thus, gloves should be worn throughout the transcription and hybridization protocols. Glassware should be baked at 250°C for 4 h; batches of plasticware should be set aside exclusively for RNA work and autoclaved where appropriate before use. All solutions should be prepared with DEPC-treated water. DEPC (0.1%, final concentration) is added to distilled water and left at room temperature for 12 h. Residual DEPC is destroyed by autoclaving this water for 15 min. Solutions prepared for transcription with this water are then aliquoted into sterile tubes and stored at –20°C.

   Other labeled rNTPs can be used (10 mCi/mL, 400 Ci/mmol). This reaction can be run in the absence of unlabeled cytidine triphosphate. For a 20 µL reaction, 100 µCi of 400 Ci/mmol α-^{32}P-CTP is 12 µm. However, the yield of full-length transcripts drops as the concentration of limiting nucleotide cytidine triphosphate falls below 12 µm. The size of the probes may be reduced by alkaline hydrolysis (*7*).
5. Fixation: In animal experiments, perfusion with 4% paraformaldehyde gives the best results: Anesthetize rats with ether and perfuse intracardially with PBS followed by 4% paraformaldehyde. Remove the appropriate tissue and continue fixation in 4% paraformaldehyde for 1 h as above. Human tissue could also be perfused, for example, perfusion of the bowel through mesenteric vessels and the brain through the Circle of Willis.

Acknowledgments

The authors are grateful to J. Dixon, Purdue University, West Lafayette, USA; E. Spindel, Harvard Medical School, USA; and S. Amara, Yale University, New Haven, USA, for supplying ANP, bombesin, and CGRP, cDNA, respectively. This work was supported by Amersham International and the Upjohn Company.

References

1. Gall, J. and Pardue, M. (1969) Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc. Natl. Acad. Sci. USA* **63,** 378–383.
2. John, H. A., Birnstiel, M. L., and Jones, K. W. (1969) RNA-DNA hybrids at the cytological level. *Nature* **223,** 582–587.
3. Buongiorno-Nardelli, S. and Amaldi, F. (1970) Autoradiographic detection of molecular hybrids between RNA and DNA in tissue sections. *Nature* **225,** 946–948.
4. Coghlan, J. P., Aldred, P., Haralambidis, J., Niall, H. D., Penschow, J. D., and Tregear, G. W. (1985) Hybridization histochemistry. *Analyt. Biochem.* **149,** 1–28.
5. Penschow, J. D., Haralambidis, J., Darling, P. E., Darby, I. A., Wintour, E. M., Tregear, G. W., and Coghlan, J. P. (1987) Hybridization histochemistry. *Experientia* **43,** 741–750.
6. Varndell, I. M., Polak, J. M., Sikri, K. L., Minth, C. D., Bloom, S. R., and Dixon, J. E. (1984) Visualisation of messenger RNA directing peptide synthesis by *in situ* hybridization using a novel single-stranded cDNA probe. *Histochemistry* **81,** 597–601.
7. Cox, K. H., De Leon, D. V., Angerer, L. M., and Angerer, R. C. (1984) Detection of mRNAs in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. *Devl. Biol.* **101,** 485–502.
8. Hoefler, H., Childers, H., Montminy, M. R., Lechan, R. M., Goodman, R. H., and Wolfe, H. J. (1986) *In situ* hybridization methods for the detection of somatostatin mRNA in tissue sections using antisera RNA probes. *Histochem. J.* **18,** 597–604.
9. Terenghi, G., Polak, J. M., Hamid, Q. A., O'Brien, E., Denny, P., Legon, S., Dixon, J., Minth, C., Palay, S. L., Yasargil, G., and Chan-Palay, V. (1987) Localisation of neuropeptide Y-mRNA in neurones of the human brain cortex using *in situ* hybridization and cRNA probes. *Proc. Natl. Acad. Sci. USA* **84,** 7315–7318.
10. Hamid, Q. A., Wharton, J., Terenghi, G., Hassalle, C. J., Aimi, H., Taylor, K. M., Nakazato, H., Dixon, J. E., Burnstock, G., and Polak, J. M. (1987) Localization of atrial natriuretic peptide mRNA and immunoreactivity in rat heart and human atrial appendage. *Proc. Natl. Acad. Sci. USA* **84,** 6760–6764.
11. Hamid, Q. A., Bishop, A. E., Springall, D. R., Adams, C., Giaid, A., Denny, P., Ghatei, M., Legon, S., Cuttitta, F., Rode, J., Spindel, E., Bloom, S. R., and Polak, J. M. (1989) Detection of human probombesin mRNA neuroendocrine (in small) cell carcinoma of the lung: *in situ* hybridization with cRNA probe. *Cancer* **63,** 266–271.
12. McCabe, J. T., Morrell, J. I., and Pfaff, D. W. (1986) *In situ* hybridization as a quantitative autoradiographic method: Vasopressin and oxytocin gene transcription in the Brattleboro rat, in *In situ Hybridization in Brain* (Uhl, G. R., ed.), Plenum, New York, Ch. 5, pp. 73–97.