determination of antigens. J. biol. Chem. *251* (1976) 4172–4177.
29 Van Weemen, B.K., and Schuurs, A.H.W.M., Immunoassay using hapten-enzyme conjugates. FEBS Lett. *24* (1972) 77–81.
30 Voller, A., and Bidwell, D.E., Enzyme immunoassays, in: Alternative Immunoassays, pp. 77–86. Ed. W.P. Collins. John Wiley & Sons Ltd, Chichester 1985.
31 Waldmann, H., and Milstein, C., Monoclonal antibodies, in: Clinical Aspects of Immunology, 4th edn. Eds P.J. Lachmann and D.K. Peters. Blackwall Scient. Publ. CRC Press, New York 1982.
32 Weeks, I., Sturgess, M., Siddle, K., Jones, M.K., and Woodhead, J.S., A highly sensitive immunochemiluminometric assay for human thyrotrophin. Clin. Endocr. *20* (1984) 489–495.
33 Wood, W.G., Luminescence immunoassays: problems and possibilities. J. clin. Chem. clin. Biochem. *22* (1984) 905–918.
34 Yalow, R.S., and Berson, S.A., Immunoassay of endogenous plasma insulin in man. J. clin. Invest. *39* (1960) 1157–1175.

0014-4754/87/070734-08$1.50 + 0.20/0

Hybridization histochemistry

by J.D. Penschow, J. Haralambidis, P.E. Darling, I.A. Darby, E.M. Wintour, G.W. Tregear and J.P. Coghlan

Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne Parkville, Victoria, 3052 (Australia)

Summary. The location of gene expression by hybridization histochemistry is being applied in many areas of research and diagnosis. The aim of this technique is to detect specific mRNA in cells and tissues by hybridization with a complementary DNA or RNA probe. Requirements for optimal specificity, sensitivity, resolution and speed of detection may not all be encompassed in one simple technique suitable for all applications, thus appropriate procedures should be selected for specific objectives. With reference to published procedures and our own extensive experience, we have evaluated fixatives, probes, labels and other aspects of the technique critical to the preservation and hybridization in situ of mRNA and detection and quantitation of hybrids.
Key words. Hybridization histochemistry; hybridocytochemistry; in situ hybridization, gene expression; histochemical hybridization; nucleic acid hybridization; histocytochemistry.

Introduction

Hybridization histochemistry is a technique for the location of gene expression in histological sections[54]. Intracellular messenger RNA (mRNA) is hybridized with a labelled specific complementary DNA (or RNA) probe and the RNA-DNA hybrids in the tissue located by the probe label. The principle is analogous to the location of peptides or proteins by specific labelled antibodies, a technique which has proven extremely useful in diagnosis and research. However, the presence of an intracellular protein antigen does not necessarily indicate whether the gene is being expressed or whether the antigen is a product of the cell in which it is located. A major area of current interest is the regulation of gene expression. For many such studies it is important to identify the cell types in which particular genes are expressed and to observe any cellular changes in the expressing cell population which may occur under varying physiological circumstances and which may affect the level of expression per cell, though not necessarily the level for the tissue as a whole.

The historical time-frame for development of hybridization histochemistry was influenced directly by the availability of specific probes from recombinant DNA technology[42, 46, 54, 89, 108]. Particular mRNA populations could be located only if the probe was known to have a specific complementary nucleotide sequence.

The pioneers of nucleic acid hybridizations[50] annealed in solution denatured chromosomal DNA, usually with labelled ribosome fractions and recovered the hybrids by density gradient centrifugation. Immobilization of the DNA component on a nitrocellulose membrane[43] paved the way for many subsequent advances including the first hybridizations in situ. In these studies denatured intracellular DNA in cell squashes[38], cell cultures[60], paraffin sections[20] and ultrathin glycol methacrylate sections[57] was hybridized with radiolabelled ribosomal RNA (rRNA) fractions to determine the nuclear location of rRNA genes. Further progress was hindered in most fields by inadequate probe purification procedures resulting in probes of very limited specificity. However, purified viral nucleic acids were recognized as useful probes for the location of specific virus[80] and poly (U) as a probe for total mRNA[61], which was used mainly in developmental biology.

Although hybridization histochemistry accompanied the technology explosion occasioned by gene cloning, application of the technique was restricted by the limited number and availability of cloned cDNA probes. Even now, access to the numerous probes for growth factors, peptide hormones, enzymes, oncogene products, cell surface receptors, viruses and other cell proteins for which genes have now been cloned is limited. The successful utilization of synthetic oligodeoxyribonucleotide probes in the hybridization histochemistry technique[22] removes the difficulties arising from cDNA probe usage, as functional probes may be synthesized easily, quickly and accurately from published nucleic acid sequences. The other major advance in probe design is the single-stranded RNA probe generated by the SP6 vector system[75]. There are several different labelling systems which incorporate a variety of isotopically or nonisotopically labelled nucleotides into the probe which should be matched to the probe type and to the relevant detection system.

The recent proliferation of in-situ hybridization techniques address many questions in a diversity of areas. The choice of technique depends ultimately on the particular application however, in our experience, the simplest procedures with the minimum manipulations are most likely to succeed. We have developed a simple, reliable, reproducible method for the location of specific intracellular mRNA populations which is applicable to a wide range of tissues. Frozen sections of whole small animals[22, 86], human tumours and biopsies[114], sheep brains[21, 24, 25, 82], plants[7] and numerous other sheep[31, 112], rodent[23, 25, 26, 30, 58] and human tissues[24, 26] have been hybridized successfully. Also, cell cultures[26], smears and paraffin embedded tissues[24] can be also used routinely. Tissue preparation methods may be adapted to enable immunohistochemistry[24] or receptor localisation to be performed on adjacent sections.

Following a brief outline of the method, which is described in

detail elsewhere[82], we shall discuss our experiences and those of others in optimizing procedures for specific applications.

General procedure

Fresh tissue is freeze-embedded in OCT compound by immersion in hexane cooled to −70 °C with dry ice. 5-µm cryostat sections are cut at −15 to −20 °C, thaw-mounted onto gelatinized slides, laid immediately on dry ice and left for 15–30 min. Sections are fixed at 4 °C for 5 min in 4% glutaraldehyde in 0.1 M phosphate pH 7.2 with 20% ethylene glycol, rinsed twice in hybridization buffer [600 mM sodium chloride, 50 mM sodium phosphate pH 7, 5 mM EDTA, 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% poly (vinlypyrrolidone), 0.1% DNA (degraded free acid) and 40% deionized formamide], left to soak for 10 min–2 h at 40 °C in a fresh change of buffer, rinsed in ethanol and left to dry.

The ^{32}P-end-labelled oligodeoxyribonucleotide probe[82] is diluted to 0.4 ng/µl in hybridization buffer, heated to 90 °C for 1 min then a drop placed on a coverslip (20 µl for 22 × 22 mm) which is applied to the sections on the slide. Slides are incubated at 30–40 °C in a humidified chamber for 1–3 days (the temperature is variable depending on the probe length and homology with target mRNA)[82].

After hybridization slides are immersed in 2 × SSC until cover-slips dislodge (2 × SSC is 0.3 M sodium chloride and 0.03 M sodium citrate), rinsed in fresh 2 × SSC, washed at 40 °C for 30 min in 1 × SSC then rinsed in ethanol and allowed to dry. Slides are taped to blotting paper in an X-ray film cassette and overlaid with a sheet of X-Omat AR-5 (Kodak) which is left to expose for 24 h (for ^{32}P). The film is developed and fixed and the results evaluated. Slides for liquid emulsion autoradiography are selected, dipped at 40 °C in K5 (Ilford) diluted 1:2 with distilled water and left for 1–14 days (for ^{32}P) over silica gel to expose.

Autoradiographs are developed for 2 min at 15 °C in D19 (Kodak), rinsed in distilled water, fixed in Hypam (Ilford) diluted 1:4, washed and stained.

Methodology

Tissue preparation. The choice of tissue preparation methods for hybridization histochemistry is limited by the need to preserve and retain intracellular mRNA such that it is accessible to the probe and available for base-pairing. Whilst good morphological preservation is a high priority, many fixation and processing regimes are incompatible with these requirements.

Glutaraldehyde or paraformaldehyde-fixed cryostat sections of fresh frozen tissues give the strongest hybridization signals[99], which in our experience are approximately equivalent, although glutaraldehyde provides superior morphological preservation. Receptor localisation studies may be performed on adjacent unfixed sections which have been treated appropriately[76]. Paraformaldehyde-perfused, frozen tissues are used widely for hybridization, although perfusion is not always applicable and the hybridization signal is generally lower and less consistent than with fresh, post-fixed cryostat sections[99]. Paraformaldehyde-perfused tissues may be used for immunohistochemistry, which permits the location of mRNA and protein antigens on adjacent sections[40]. Both procedures have been performed sequentially on the same frozen section[98] but with a reduced hybridization signal resulting from the preceding immunohistochemistry. Similarly viral RNA and protein has been colocalised in the same paraffin sections[18, 63].

Parameters for hybridization of fixed, embedded tissues are difficult to evaluate as the size of the tissue sample, ribonuclease content, type and rate of fixation and subsequent processing and embedding procedures must be considered in relation to the hybridization signal and the quality of morphological preservation.

Immersion fixation of tissues followed by processing for paraffin[49, 70, 108, 110] or methacrylate[59] embedding instead of freezing[84, 107], has been used effectively for small samples where target mRNA's are abundant and some hybridization signal can be sacrificed for improvements in morphology. As prolonged exposure to fixatives tends to reduce the hybridization signal[19, 111], large samples ideally are frozen and post-fixed[15, 82, 99] or dissected from perfusion-fixed animals then frozen[98, 111] or processed immediately for embedding. Paraffin-embedded tissues perfused with paraformaldehyde, glutaraldehyde or a combination of both have been used successfully for hybridization of viral RNA[18, 77]. In our experience using perfused embedded tissues for location of mRNA, glutaraldehyde gave a stronger signal than Karnovsky's fixative[62] or paraformaldehyde, provided that tissues were rinsed briefly in buffer and processed immediately through absolute ethanol to paraffin.

We favour an alternative method for embedded tissues, which causes little or no reduction in hybridization signal compared to fresh frozen glutaraldehyde-fixed sections. Tissues up to 2-mm-thick are frozen in liquid propane, freeze dried at −45 °C and 10^{-2} Torr, fixed under vacuum at 37 °C for 1 h in paraformaldehyde and vacuum-embedded in paraffin. This is an ideal technique for the location of peptide hormone mRNA's[24] as intracellular storage granules are retained, preservation of antigenicity is excellent for immunohistochemistry and tissue morphology is good.

Several studies using cell culture systems where various types of fixatives were compared[8, 41, 44, 68, 77] have shown that extensive cross-linking of proteins is advantageous for retention of mRNA but can cause poor penetration of probes through the fixed tissue matrix. Glutaraldehyde was found to give the best morphological preservation and RNA retention but probe penetration was poor, especially for longer probes. mRNA is more accessible to short probes and proteolytic treatment of fixed cells may be necessary for effective hybridization with longer probes, especially where vigorously cross-linking fixatives are employed[8, 68]. A loss of cellular RNA may result from proteoloytic treatments which are not carefully monitored[8, 19, 41, 68] or in tissues where proteins are not extensively cross-linked[44, 68]. Penetration with non cross-linking ethanol-based fixatives is less of a problem[77] but morphological preservation is poor and low hybridization signals may result from loss of intracellular RNA[8, 44, 68].

Tissue preparation procedures for the location of genomic viral nucleic acids may differ markedly from those appropriate for hybridization of mRNA, as virus particles are stable for prolonged periods in post-mortem[39, 46, 47] and immersion-fixed[19] tissues where mRNA survival is poor. Deproteination of the viral capsid is necessary for probe penetration and denaturation of double-stranded viral nucleic acids to permit hybridization[14, 19, 46].

Probes

The selection of the type of probe and label depends largely on the specificity, sensitivity and resolution required by the experiment. The original and most commonly used specific probes for hybridization histochemistry are recombinant cDNA, which are double-stranded, generally long (unless reduced in size by restriction enzymes) and usually labelled by nick translation[88], although the random primer method[104] is simpler and more efficient. By these methods, labelled copies of both strands are produced and exist with the unlabelled template in the hybridization mixture. Some or all of the four deoxyribonucleotide components of the probe may be labelled with ^{3}H[14, 49, 98, 108, 111], ^{35}S[33, 77, 115], ^{32}P[24, 37, 54, 58] or less commonly ^{125}I[70, 83]. Incorporation into the probe of a

biotin molecule[19, 72, 100] has been the main approach in developing non-radioactive labels and other types of cDNA derivatives[36, 53, 66] have been designed as probes but the sensitivity of detection methods has not yet been proven superior to autoradiography for hybridization histochemistry. The effective probe concentration of double-stranded probes is reduced by self-annealing and competition with unlabelled strands, thus single stranded probes of equivalent specific activity are more efficient.

Oligodeoxyribonucleotides up to approximately 100mer can be prepared in the current generation of DNA synthesizers[82] using the solid phase phosphoramidite technology[2] developed by Beaucage et al.[13]. These have proven very efficient as probes for hybridization histochemistry[9, 21, 22, 24, 97, 114] and have an advantage in specificity, as they may be designed for a particular application to exploit minor differences in nucleotide sequences and may thus discriminate closely homologous mRNA's[24, 82, 106, 112]. These single-stranded probes are labelled enzymically by attachment of a single ^{32}P-labelled phosphate from γ-[^{32}PATP] at the 5′ end using T4 kinase[74] or a number of ^{35}S or ^{3}H-labelled nucleotides at the 3′ end using terminal transferase[105]. A variety of other labelling methods for addition of enzymes[56], fluorophores[4, 101] or biotin[56, 78] have been reported but not yet demonstrated as effective for hybridization histochemistry. Preparation of overlapping, complementary oligodeoxyribonucleotides to produce a primed, double-stranded probe enables a greater number of labelled nucleotides to be incorporated.

RNA probes prepared in the SP6 vector system are now being used extensively for hybridization histochemistry[27, 51, 63, 93]. These are single-stranded copies of a DNA insert which must be in the correct orientation to ensure transcription of the required sequence. The SP6 promotor in the presence of SP6 polymerase can generate multiple copies of the insert, the limitation being the concentration of each ribonucleotide in the reaction mixture. At present ^{32}P, ^{35}S and ^{3}H-labelled ribonucleotides are available, and although expensive, probes of very high specific activity can be produced by using sufficient concentrations of each labelled base[75]. The use of non-optimal concentrations of labelled ribonucleotides often results in short transcripts. However, ^{3}H probes of high specific activity are liable to degradation by radiolysis.

Other types of probes include single-stranded cDNA which has been used for hybridization histochemistry whilst incorporated in a biotin-labelled M13 vector[107] and probes prepared from purified viral nucleic acids[17, 19, 80], or mRNA[73, 85] which were developed in the prerecombinant era and have largely been supplanted by cloned sequences.

In our experience there is no single probe and label which is optimal for all requirements of specificity, sensitivity, resolution and speed of detection.

Hybridization

There is a general belief that parameters which apply to hybridization of denatured mRNA deposited and dried on nitrocellulose are relevant for mRNA in fixed tissues, which has a secondary structure and is trapped with associated ribosomes in a matrix of cross-linked proteins. Although there are some parallels, recovery and hybridization of specific mRNA from tissue homogenates is subject to a different set of problems and conditions than preservation of the same mRNA in a hybridizable form in whole tissue samples. Problems which may be encountered include loss of RNA by enzymic degradation, which is somewhat tissue-specific and is more rapid in homogenates, reduced penetration of long probes through fixed tissues[8, 17, 41, 68, 77] or loss of RNA from cells which may occur after certain fixation protocols[8, 44, 68]. Probes may fail to hybridize in tissues where directed against regions of the mRNA molecules which may be inaccessible because of its secondary structure[35] or masked by association with proteins[3].

The similar relationship to probe specificity of salt, formamide concentration and hybridization temperature applies equally to hybridization of mRNA in situ, on filters or in solution, although optimal hybridization and washing conditions may vary considerably for a given interaction[17, 27].

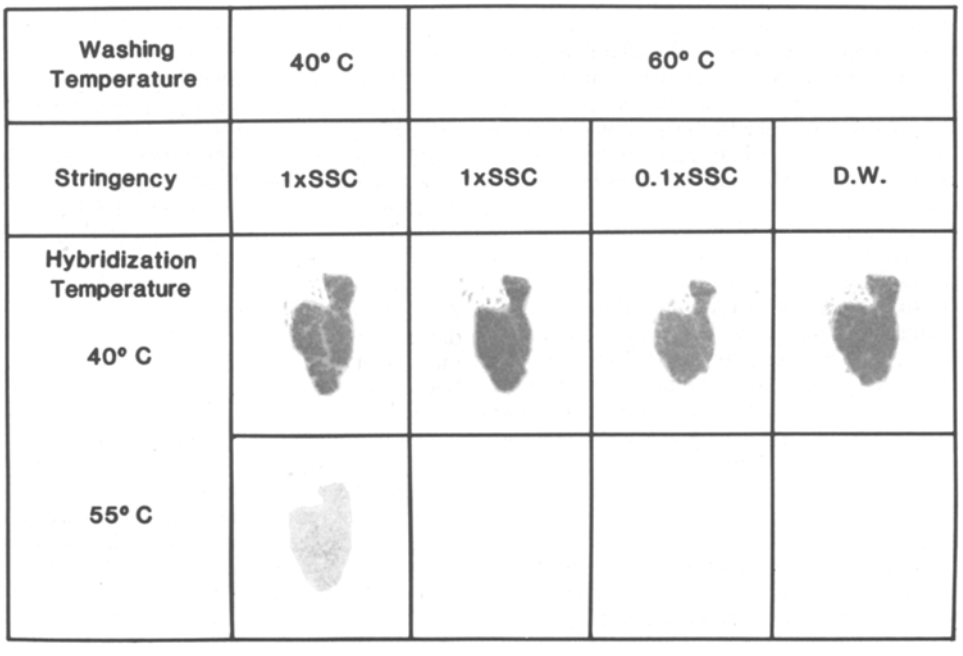

Figure 1. Autoradiographs on X-ray film of 5-μm frozen sections from female mouse salivary glands after hybridization with a ^{32}P-labelled 30mer oligodeoxyribonucleotide probe specific for mouse glandular kallikrein mRNA[87]. For hybridization, salt concentration was 0.6 M and formamide 40%. The temperature for hybridization and the temperature and stringency of post-hybridization washing was varied as illustrated. The remaining procedures were as described previously[82].

Decreasing salt and increasing formamide concentration and hybridization temperature decreases the stability of hybrids and may thus be manipulated to increase the specificity of hybridization. These variables must be adjusted according to the experiment, with regard to the probe length and degree of homology between the probe and target mRNA, and with some consideration of the effects on tissue morphology. To ensure specificity of hybridization with low formamide concentrations temperatures above 60 °C may be necessary. As this can be detrimental to morphology and cause sections to dislodge from slides, for our method of hybridization histochemistry formamide is maintained at 40% and the hybridization temperature varied accordingly[82] whilst maintaining salt concentration at 0.6 M. With formamide and salt constant the appropriate temperature depends on the type and length of probe as well as specificity for the target mRNA (fig. 1), 40 °C is preferred for specific interactions with DNA probes longer than 27 nucleotides, whereas 30 °C or less may be necessary for short probes or for interactions of limited specificity. Higher temperatures may be used as a test of specificity, or for optimal hybridization with SP6 RNA probes[27] as RNA-RNA hybrids are more stable than DNA-RNA.

In a detailed study of hybridization kinetics, Cox et al.[27] have shown that the highest signal to background ratio is obtained where probe concentration is optimal for maximum saturation of target sites. Probe is always in great excess and the optimal concentration for double-stranded probes is higher than for single-stranded due to self-reassociation which leads to a continuous decline in effective probe concentration. Short probes have been shown to give a higher signal than long probes of 500 nucleotides at equivalent concentrations[8,17]. This is suggested to be due to differences in diffusion, only short duplexes being formed in situ[27]. Formation of networks[109] has been shown for probes longer than 700 base pairs to contribute significantly to the signal[68]. Maximum signal to background ratio will not be achieved by an increase in the duration of hybridization where the probe is at a sub-optimal concentration as the hybridization reaction terminates prior to completion, even when probe is in excess[27].

Dextran sulphate has been reported to increase the hybridization signal, presumably by increasing the effective probe DNA concentration[109], and is often included in hybridization mixtures[14,19,49,68,70,108]. 5–10% dextran sulphate has been shown to amplify the hybridization signal with cDNA probes[49,68]. However, for 30mer oligodeoxyribonucleotides at 400 ng/ml we obtained no increase in signal but an increased background.

For prehybridization, Denhart's solution[32] which contains PVP, BSA and Ficoll is a standard buffer component and has background-reducing effects by saturating non-specific binding sites in tissue. Sonicated DNA from salmon or herring sperm is included frequently for the same reasons[19,69,82,99] but has been shown to reduce the hybridization signal in some cDNA probe systems[111]. Slides or sections may be acetylated[52] prior to hybridization to reduce background binding of DNA to charged particles but this has been found in some systems[98] to be ineffective.

Post-hybridization washing

The aim of this procedure is first to remove the coverslip and excess probe, then to reduce background by washing off as much unwanted non-specifically bound probe as possible whilst retaining specific hybrids. For filter hybridization, hybrids may be destabilized by increasing washing stringency (lower salt/higher temperature/longer wash), thus hybrids of a desired specificity may be retained by repeated washes at increasing stringency. For hybridization histochemistry we have, amongst others[68], found extensive washing procedures to be irrelevant (fig. 1). Hybrids formed in situ are extremely stable to low salt/high temperature washes and non-specific interactions which may have occurred during hybridization remain through the most stringent of washes. It is thus essential for minimum background, to select appropriate hybridization conditions and to have probes uncontaminated by vector DNA, very short sequences or unincorporated label. Removal of unbound probe by post-hybridization treatment with RNase for single-stranded RNA probes[27,93,111] has been shown to greatly reduce background and similar treatment with S1 nuclease has been reported for cDNA[44]. We have encountered few problems of excessive background using specific oligodeoxyribonucleotide probes 24mer and longer.

Hybrid detection and quantitation

Methods for locating hybrids are clearly dependent upon the type of probe label. Autoradiography is the most widely used approach, appropriate methods depending on the energy, half-life and specific activity of the isotope. We have found autoradiography with ^{32}P-labelled probes a very convenient method for hybrid detection as short exposure on fast X-ray film[7,9,15,16,22,26,30,54,82,86,114] provides a rapid screening method for numerous samples. Subsequent liquid emulsion autoradiographs, with resolution to single cells in some cases[15,82], can be obtained in a matter of days following the initial X-ray exposure[15,21–26,31,58,86,114].

Tritium has higher resolution but of course requires longer exposure times due to the longer half-life and lower specific activity than ^{32}P. Addition of enhancers to the photographic emulsion tends to offset the gain in resolution sought from the use of tritium in the first place. X-ray film improved for ^{3}H autoradiography is available (L.K.B. Stockholm; Amersham, Buckinghamshire) but it is comparatively slow, thus X-ray exposures are not often used as a screening procedure. Tritium labels were used to develop in situ hybridization procedures[20,38,57,60,61,80] and remain popular due to the high resolution which can be obtained in light microscope autoradiographs[18,27,34,40,46,49,59,69,84,99,108].

^{125}I has been used extensively as a probe label for chromosome mapping and occasionally for hybridization histochemistry[70,83,94] but its effectiveness for the location of mRNA in tissues is reduced by the high non-specific binding to proteins[52]. ^{35}S autoradiography is being used increasingly for hybrid detection[33,47,51,64,77,115] with the advantages of good resolution, somewhere between ^{3}H and ^{32}P, and shorter exposure times than ^{3}H of comparable specific activity. The difference in β particle energy of ^{3}H and ^{35}S has been exploited for the simultaneous location of two viral genomes within the same cell[48]. High non-specific binding to tissues has been reported with this isotope[64].

A variety of approaches to probe detection which avoid radioactivity have been developed and used mainly for chromosome mapping. The first of these employed labelled antibodies to DNA-RNA hybrids[92,102] or direct detection of fluorochrome-labelled probes[10,11]. The lack of sensitivity of these methods led to the signal enhancement approach using an antibody-sandwich to locate hapten-modified probes[12,19,55,67,72]. Further increases in sensitivity were needed, thus reflection contrast microscopy was developed[66] to amplify immunoprecipitates and avidin-peroxidase conjugates for biotin-labelled probes[6,71,100,107]. These methods have the advantage of rapid detection, but have yet to be proven to have greater sensitivity than optimized autoradiography.

It is tempting to use hybridization histochemistry to measure relative levels of mRNA in different cell populations related to various physiological parameters. Data obtained from extracted mRNA can only provide an average level of gene

expression for any tissue sample but cannot resolve other changes in the cell population where a specific gene is being expressed such as proliferation, differentiation or atrophy, which may occur whilst a constant mRNA level is maintained by transcriptional regulation. Conversely, for the same reasons, in comparisons of hybridization histochemistry from different tissues or animals, relative mRNA levels per cell in any section do not necessarily relate to the total tissue mRNA level. Where possible, a combination of both methods should provide the most informative measure of gene expression.

Methods for quantitation by autoradiography of hybrids in cell cultures[17, 68], tissue sections[8, 24, 26, 27, 111] and chromosome preparations[103] have been described, however potential sources of error should be recognized. These include variations in probe penetration, efficiency of hybridization[27, 103], cell shape[41] or section thickness, loss of mRNA from cell[44] and formation of probe networks with cDNA probes[68], as well as errors in grain-counting of autoradiographs, which vary for different isotopes[1, 90]. We have found quantitation of hybridization histochemistry by competition experiments with ^{32}P-labelled oligodeoxyribonucleotide probes[24, 26] to be a simple, reproducible means for determining relative mRNA levels in tissue sections. Analysis of X-ray films by computer-assisted densitometry may be undertaken to provide colour-coded images which illustrate differences in signal intensity[45].

Applications

One of the major applications of hybridization histochemistry, in conjunction with other cell-specific techniques, lies in the reclassification of cells and tissues on a functional basis. This is particularly applicable to embryogenesis and other areas of cell differentiation where the presence in a cell of specific mRNA may be the first detectable criterion of function. Elegant studies of developing *Drosophila*[49, 64, 72], *Aplysia*[70, 94], sea urchins[27, 108] and nematodes[34] illustrate the value of this technique in developmental biology. Gene expression for some familiar and newly discovered peptide hormones, enzymes, substrates and growth factors has been located at multiple sites in functionally heterogenous cell populations[33, 40, 84, 86, 97] and tissues. This allows further analysis of the hypothesis that 'everything is made everywhere' and recent findings suggest multiple roles for some genes and transcripts which may be differentially processed to heterogenous products which can be cell specific[84, 91, 95]. For these and other applications of hybridization histochemistry, specific oligodeoxyribonucleotide probes can be designed to discriminate between closely homologous mRNA sequences which are encoding multigene families, or which share common sequences[5, 82, 106, 114]. These probes are also invaluable for the location in transgenic animals of expression of introduced gene constructs by sectioning and hybridization of whole animals or isolated tissues. There are clearly many other uses as a research tool and a major role for this technique is becoming increasingly apparent for clinical diagnosis of tumours, infectious diseases and endocrine disorders, or in fact in any situation where a specific recognized mRNA is associated with, or at the root of the problem.

We have selected some examples which show a variety of applications of our method using ^{33}P-labelled probes of different types. Oligodeoxyribonucleotide probes specific for α, β or γ globins, have been used to study globin switching in the ovine foetus by a series of techniques, including colony culture of erythropoietic cells and location of gene expression in haemopoietic tissues[112, 113] (fig. 2, a–c).

Synthetic probes coding for the E2 region of Ross River virus were used for diagnosis in a human skin biopsy of infection with this virus (fig. 3a), innoculated Vero cell cultures acting as hybridization controls (fig. 3b). mRNA for a member of the human growth hormone gene family, choriosomatomammotropin, is demonstrated by a 30mer probe in syncytiotrophoblasts of human placenta (fig. 3c). In human primary medullary thyroid carcinoma, mRNA for calcitonin (fig. 3d) was found to coexist in the primary tumour and lymph node metastasis with mRNA for calcitonin-gene-related peptide (CGRP)[114].

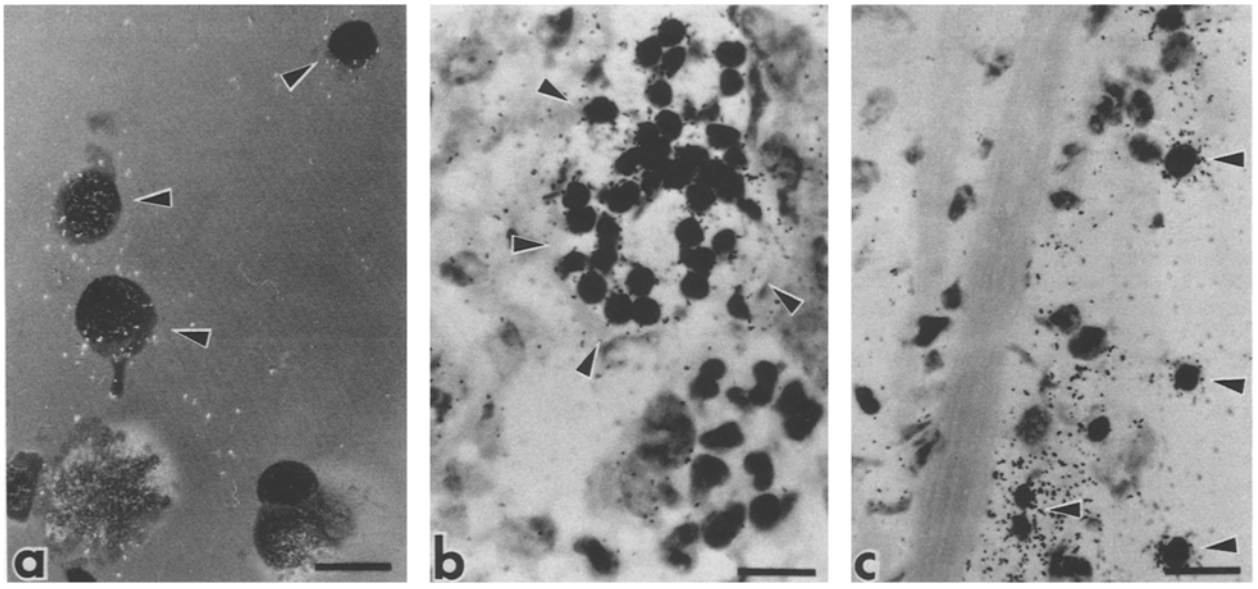

Figure 2. Autoradiograph after hybridization with ^{32}P-labelled 30mer oligodeoxyribonucleotide probes designed to discriminate mRNA's for ovine β and γ globin[65]. *a* mRNA for β globin shown in erythropoietic cell (arrowed) from a 3-day methylcellulose culture of ovine 129-day foetal bone marrow. Cytocentrifuged preparation, stained with Wright's and photographed by polarized epi-illumination. Bar = 13 μm. *b* A 5-μm frozen section showing mRNA for γ globin in cells of a haemopoietic focus (arrowed) within liver parenchyma of an ovine 124-day foetus. Stain: H & E. Bar = 30 μm. *c* A 5-μm frozen section of ovine 99-day foetal bone marrow showing mRNA for γ globin in erythropoietic cells (arrowed). Stain. H & E. Bar = 35 μm.

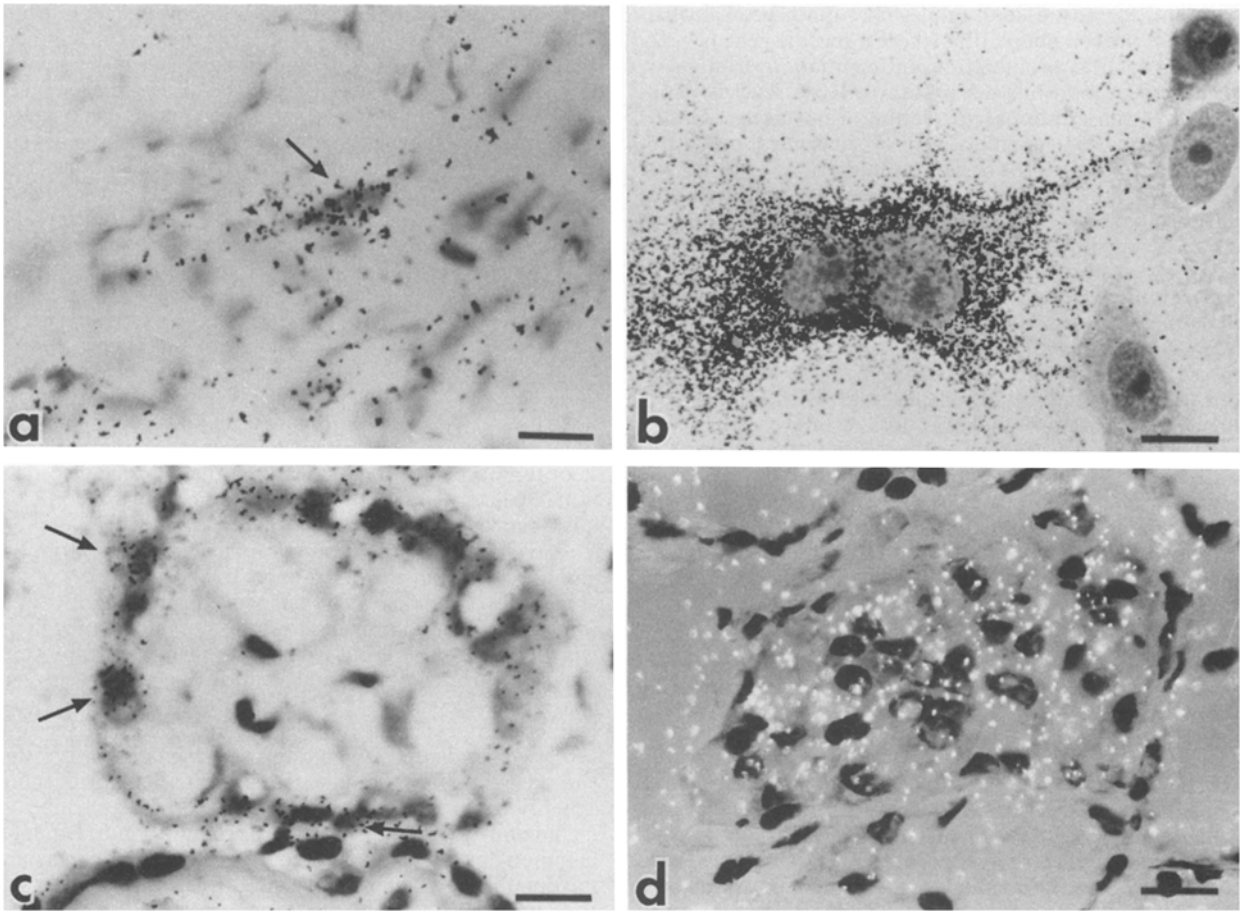

Figure 3. Autoradiographs after hybridization with ^{32}P-labelled 30mer (*a, b, c*) or 40 mer (*d*) oligodeoxyribonucleotide probes. *a* A 10-μm frozen section of human skin biopsy taken 4 days after onset of a rash. mRNA coding for Ross River virus (RRV) envelope protein is demonstrated in an infected cell (arrowed) by a probe corresponding to the E2 region of the RRV genome[29]. Bar = 35 μm. *b* Vero cells from a culture infected with RRV and hybridized with the RRV E2 probe used in 3(a). Bar = 10 μm. *c* A 5-μm frozen section of human placenta showing mRNA for chorioso-matomammotropin[96] in syncytiotrophoblasts (some arrowed) of foetal cotyledons. Bar = 30 μm. *d* mRNA for calcitonin in neoplastic C-cells of a human primary medullary thyroid carcinoma, demonstrated in a 5-μm frozen section with a 40mer oligodeoxyribonucleotide probe complementary to the calcitonin coding region. Photomicrograph by polarized epi-illumination. Bar = 25 μm.

mRNA for the enzyme renin is demonstrated with a cDNA probe[81] in the afferent arteriole of the mouse kidney (fig. 4a) and its substrate angiotensinogen with a 36mer probe, including the angiotensin coding region, in neurones of the arcuate nucleus of rat brain (fig. 4b). Attempts by ourselves and others to locate renin mRNA in the brain[33] have been unsuccessful and the mechanism of a brain renin-angiotensin system is still being investigated. Genes of the kallikrein family of serine protease enzymes have been shown to be expressed in a diversity of tissues although the major site of expression of most of the functional genes of this multigene family is in granular convoluted tubule cells of the submandibular gland[106]. Kallikrein mRNA is demonstrated by an SP6 RNA probe containing the mouse kallikrein insert pMK-1[87] in striated ducts of the murine parotid gland (fig. 4c), where products of the renal kallikrein gene, a kininogenase, may have a role in local regulation of blood flow.

Hybridization histochemistry – in situ tissue hybridization using cDNA, RNA or synthetic oligodeoxynucleotide probes with various labelled compounds is beyond the countdown stage as an emergency technique and is in the early stage of lift-off. Recent contributions from many laboratories have accelerated the transition through generation one, two and three. The technique is user-friendly apart from the autoradiographic end point which is not too difficult. The technique offers valuable, unique information which is not obtainable by application of other procedures. The specific cellular address of gene expression as in the developing embryo during narrow time windows offers very significant advances and insight into differentiation and development. The ability to detect viral, oncogene, growth factor, or hormonal gene expression in carcinomas offers the possibility of a new taxonomy of tumours with the likelihood of an appropriately structured chemotherapy or direction of the most relevant specific cytotoxins; furthermore, the profile of gene expression in secondary metastases will provide vital information about the primary tumour and appropriate treatment. Identification of viruses by this technique will have eventually a permanent niche in this area of diagnosis. Location of gene expression in specific cells is a crucial piece of information in many on-going studies of hormones, enzymes and proteins. This information profile includes transgenic animals, chimeric embryos and immortal cell lines, where knowledge of promoter regions and their interaction with enhancers and other transactive agents will be the bases of the 'new pharmacology'.

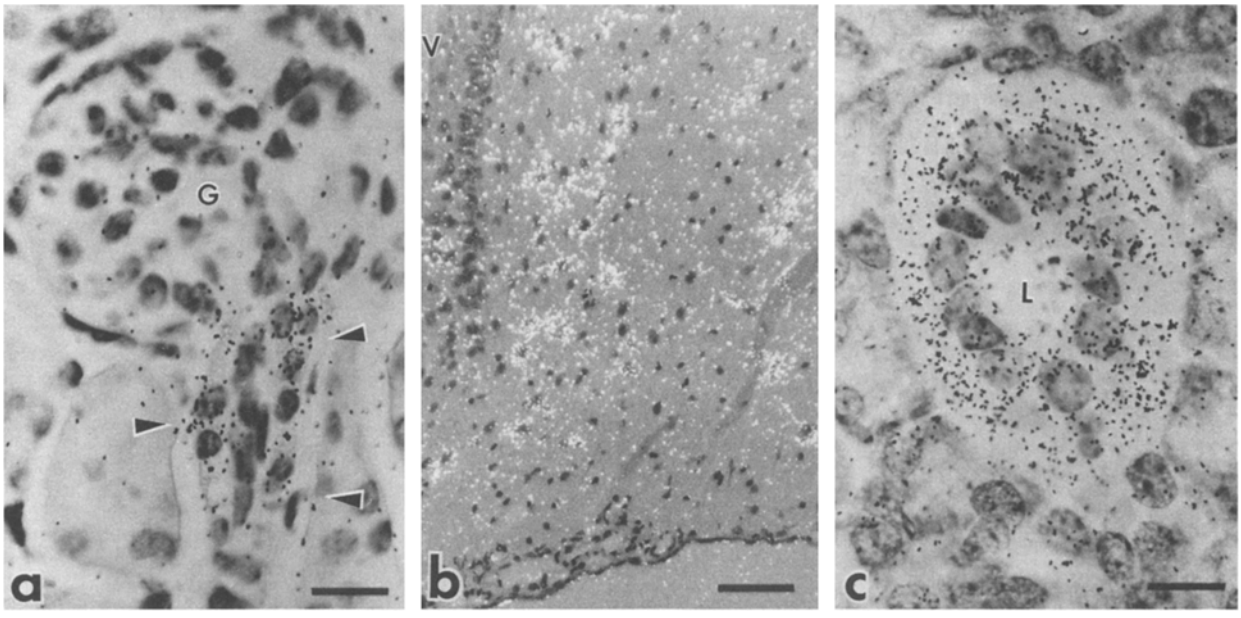

Figure 4. *a* mRNA for renin demonstrated in cells of the afferent arteriole (arrowed) of a renal glomerulus (G) by a ^{32}P-labelled m. renin cDNA probe of 1400 base pairs[81]. Autoradiograph of a 5-μm paraffin section of freeze-dried mouse kidney. Stain: H & E. Bar = 25 μm. *b* Coronal 8-μm frozen section of rat brain after hybridization with a ^{32}P-labelled 36mer oligodeoxyribonucleotide complementary to the angiotensin coding region of rat angiotensinogen mRNA[79]. mRNA-DNA hybrids are located by autoradiography in neurones of the arcuate nucleus, adjacent to the third ventricle (V). Photomicrograph by transmitted light dark-field illumination. Stain: cresyl violet. Bar = 300 μm. *c* Autoradiograph of a 4-μm paraffin section of freeze-dried mouse salivary glands after hybridization with a ^{32}P-labelled RNA probe generated in the SP6 vector system[75] from the 550 base pair mouse kallikrein cDNA pMK-1[87]. Kallikrein-RNA is demonstrated in striated duct cells of the parotid gland. (L) lumen of duct. Stain: H & E. Bar = 20 μm.

Acknowledgments. We are grateful to Professor T. J. Martin and Dr R. J. Fraser from the University of Melbourne Department of Medicine at the Repatriation Hospital and Royal Melbourne Hospital respectively, for their significant contributions to the clinical studies. – This work was supported by grants-in-aid from the National Health and Medical Research Council of Australia, the Myer Family Trusts, the Ian Potter Foundation, and the Howard Florey Biomedical Foundation, and by Grant HD11908 from the National Institutes of Health. – The oligodeoxyribonucleotide probes used in this study were supplied by Syngene Ltd, 225 Queensberry Street, Melbourne, 3000, Australia.

1 Ada, G. L., Humphrey, J. H., Askonas, B. A., McDevitt, H. O., and Nossal, G. J. V., Correlation of grain counts with radioactivity (^{125}I and tritium) in autoradiography. Exp. Cell Res. *41* (1966) 557–572.
2 Adams, S. P., Kavka, K. S., Wykes, E. J., Holder, S. B., and Galluppi, G. R., Hindered dialkylamino nucleoside phosphite reagents in the synthesis of two DNA 51mers. J. Am. chem. Soc. *105* (1983) 661–663.
3 Adams, P. S., Noonan, D., and Jeffery, W. R., A model for the organization of the poly (A) protein complex in messenger ribonucleoprotein. FEBS Lett. *114* (1980) 115–118.
4 Agrawal, S., Christodoulu, C., and Gait, M. J., Efficient methods for attaching non-radioactive labels to the 5'ends of synthetic oligodeoxyribonucleotides. Nucl. Acids Res. *14* (1986) 6226–6245.
5 Amara, S. G., Arriza, J. L., Leff, S. E., Swanson, L. W., Evans, R. M., and Rosenfeld, M. G., Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene-related peptide. Science *229* (1985) 1094–1097.
6 Ambros, P. F., Matzke, A. J. M., and Matzke, M. A., Localization of agrobacterium rhizogenes T-DNA in plant chromosomes by in-situ hybridization. EMBO *5* (1986) 2073–2077.
7 Anderson, M. A., Cornish, E. C., Mau, S.-L., Williams, E. G., Hoggart, R., Atkinson, A., Bonig, I., Grego, B., Simpson, R., Roche, P. J., Haley, J. D., Penschow, J. D., Niall, H. D., Tregear, G. W., Coghlan, J. P., Crawford, R. J., and Clarke, A. E., Cloning of cDNA for a stylar glycoprotein associated with expression of self-incompatibility in *Nicotiniana alata*. Nature *321* (1986) 38–44.
8 Angerer, L. M., and Angerer, R. C., Detection of poly (A)$^+$ RNA in sea urchin eggs and embryos by quantitative in-situ hybridization. Nucl. Acids Res. *9* (1981) 2819–2840.
9 Arentzen, R., Baldino, F. Jr, Davis, L. G., Higgins, G. A., Lin, Y., Manning, W., and Wolfson, B., In-situ hybridization of putative somatostatin mRNA within hypothalamus of the rat using synthetic oligonucleotide probes. J. Cell Biochem. *27* (1985) 415–422.
10 Bauman, J. G. J., Wiegant, J., Borst, P., and von Duijn, P., A new method of fluorescence microscopical localisation of specific DNA sequences by in-situ hybridization of fluorochrome labelled RNA. Exp. Cell Res. *128* (1980) 485–490.
11 Bauman, J. G. J., Wiegant, J., and van Duijn, P., Cytochemical hybridization with fluorochrome-labelled RNA II. Applications. J. Histochem. Cytochem. *29* (1981) 238–246.
12 Bauman, J. G. J., Wiegant, J., and van Duijn, P., Cytochemical hybridization with fluorochrome-labelled RNA III. Increased sensitivity by the use of anti-fluorescein antibodies. Histochemistry *73* (1981) 181–185.
13 Beaucage, S. L., and Caruthers, M. H., Deoxynucleoside phosphoramidites – A new class of key intermediates for deoxypolynucleotide synthesis. Tetrahedron Lett. *22* (1981) 1859–1862.
14 Blum, H. E., Stowring, L., Figus, A., Mongomery, C. K., Haase, A., and Vyas, G. N., Detection of hepatitis B virus DNA in hepatocytes, bile duct epithelium, and vascular elements by in-situ hybridization. Proc. natl Acad. Sci. USA *80* (1983) 6685–6688.
15 Bloch, B., LeGuellec, D., and de Keyzer, Y., Detection of the messenger RNAs coding for the opioid peptide precursors in pituitary and adrenal by in situ hybridization: study in several mammalian species. Neurosci. Lett. *53* (1985) 141–148.
16 Bloch, B., Milner, R. J., Baird, A., Gubler, E., Reymond, C., Bohlen, P., le Guellec, D., and Bloom, F. E., Detection of the messenger RNA coding for preproenkephalin A in bovine adrenal in situ hybridization. Reg. Peptides *8* (1984) 345–354.
17 Brahic, M., and Haase, A. T., Detection of viral sequences of low reiteration frequency by in-situ hybridization. Proc. natl Acad. Sci. USA *75* (1978) 6125–6129.
18 Brahic, M., Haase, A. T., and Cash, E., Simultaneous in-situ detection of viral RNA and proteins. Proc. natl Acad. Sci. USA *81* (1984) 5445–5448.
19 Brigati, D. J., Myerson, D., Leary, J. J., Spalholz, B., Travis, S. Z., Fong, C. K. Y., Hsiung, G. D., and Ward, D. C., Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labelled hybridization probes. Virology *126* (1983) 32–50.

20 Buongiorno-Nardelli, S., and Amaldi, F., Autoradiographic detection of mulecular hybrids between RNA and DNA in tissue sections. Nature *225* (1970) 946–948.

21 Coghlan, J.P., Aldred, P., Butkus, A., Crawford, R.J., Darby, I.A., Fernley, R.T., Haralambidis, J., Hudson, P.J., Mitri, R., Niall, H.D., Penschow, J.D., Roche, R.J., Scanlon, D.B., Tregear, G.W., Richards, R., van Leeuwen, B.L., Rall, L., Scott, J., and Bell, G., Hybridization histochemistry, in: Endocrinology, pp. 18–24. Eds R. Labrie and L. Proulx. Excerpta Medica, Amsterdam 1984.

22 Coghlan, J.P., Penschow, J.D., Tregear, G.W., and Niall, H.D., Hybridization histochemistry: Use of complementary DNA for tissue localization of specific mRNA populations, in: Receptors, Membranes and Transport Mechanisms in Medicine, pp. 1–11. Eds A. Doyle and F. Medelson. Excerpta Medica, Amsterdam 1984.

23 Coghlan, J.P., Penschow, J.D., Hudson, P.J., and Niall, H.D., Hybridization histochemistry: Use of recombinant DNA for tissue localizations of specific mRNA populations. Clin. exp. Hypert. *A6* (1984) 63–78.

24 Coghlan, J.P., Aldred, P., Haralambidis, J., Niall, H.D., Penschow, J.D., and Tregear, G.W., Hybridization histochemistry. Analyt. Biochem. *149* (1985) 1–28.

25 Coghlan, J.P., Aldred, P., Butkus, A., Crawford, R.J., Darby, I.A., Haralambidis, J., Penschow, J.D., Roche, P.J., Troiani, C., and Tregear, G.W., Hybridization histochemistry – locating gene expression, in: Neuroendocrine Molecular Biology, pp. 33–39. Eds G. Fink, A.J. Harmar and K.W. McKerns. Plenum Publ. Corp., 1986.

26 Coghlan, J.P., Penschow, J.D., Fraser, J.R., Aldred, P., Haralambidis, J., and Tregear, G.W., Location of gene expression in mammalian cells, in: In-Situ Hybridization. Eds K. Valentino, J. Eberwine and J.D. Barchas. Cambridge Press 1987, in press.

27 Cox, K.H., DeLeon, D.V., Angerer, L.M., and Angerer, R.C., Detection of mRNA's in sea urchin embryos by in-situ hybridization using asymmetric RNA probes. Devl Biol. *101* (1984) 485–502.

28 Craig, R.K., Hall, L., Edbrooke, M.R., Allison, J., and MacIntyre, I., Partial nucleotide sequence of human calcitonin precursor mRNA identifies flanking cryptic peptides. Nature *295* (1982) 345–347.

29 Dalgarno, L., Rice, C.M., and Strauss, J.H., Ross River Virus 26S RNA: complete nucleotide sequence and deduced sequence of the encoded structural proteins. Virology *129* (1983) 170–187.

30 Darby, I.A., Aldred, G.P., Coghlan, J.P., Fernley, R.T., Penschow, J.D., and Ryan, G.B., Use of synthetic oligonucleotide and recombinant DNA probes to study renin gene expression. Clin. exp. Pharm. Physiol. *12* (1985) 199–203.

31 Darby, I.A., Aldred, P., Crawford, R.J., Fernley, R.T., Niall, H.D., Penschow, J.D., Ryan, G.B., and Coghlan, J.P., Renin gene expression in vessels of the ovine renal cortex. J. Hypert. *3* 1985) 9–11.

32 Denhart, D.T., A membrane-filter technique for the detection of complementary DNA. Biochem. biophys. Res. Commun. *23* (1966) 641–646.

33 Deschepper, C.F., Mellon, S.H., Cumin, F., Baxter, J.D., and Ganong, W.F., Analysis by immunocytochemistry and in-situ hybridization of renin and its mRNA in kidney, testis, adrenal and pituitary of rat. Proc. natl. Acad. Sci. USA *83* (1986) 7552–7556.

34 Edwards, M.K., and Wood, W.B., Location of specific messenger RNA's in *Caenorhabditis elegans* by cytological hybridization. Devl Biol. *97* (1983) 375–390.

35 Favre, A., Morel, C., and Scherrer, K., The secondary structure and poly (A) content of globin messenger RNA as a pure RNA and in polyribosome-derived ribonucleoprotein complexes. Eur. J. Biochem. *57* (1975) 147–157.

36 Forster, A.C., McInnes, J.L., Skingle, D.C., and Symons, R.H., Non-radioactive hybridization probes prepared by the chemical labelling of DNA and RNA with a novel reagent, photobiotin. Nucl. Acids Res. *13* (1985) 746–761.

37 Fuller, P.J., Clements, J.A., Whitfield, P.L., and Funder, J.W., Kallikrein gene expression in the rat anterior pituitary. Molec. cell. Endocr. *39* (1985) 99–105.

38 Gall, J., and Pardue, M., Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proc. natl. Acad. Sci. USA *63* (1969) 378–383.

39 Galloway, D.A., Fenoglio, C.M., Shevchuk, M., and McDougall, J.K., Detection of *Herpes simplex* RNA in human sensory ganglia. Virology *95* (1979) 265–268.

40 Gee, C.E., Chen, C.-L.C., and Roberts, J.L., Identification of proopiomelanocortin neurones in rat hypothalamus by in-situ cDNA-mRNA hybridization. Nature *306* (1983) 374–376.

41 Gee, C.E., and Roberts, J.L., In-situ hybridization histochemistry: a technique for the study of gene expression in single cells. DNA *2* (1983) 155–161.

42 Gerhard, D.S., Kawasaki, E.S., Carter Bancroft, F., and Szabo, P., Localization of a unique gene by direct hybridization in-situ. Proc. natl. Acad. Sci. USA *78* (1981) 3755–3759.

43 Gillespie, D., and Spiegelman, S., A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J. molec. Biol. *12* (1965) 829–842.

44 Godard, C.M., and Jones, K.W., Improved method for detection of cellular transcripts by in-situ hybridization. Detection of poly (A) sequences in individual cells. Histochemistry *65* (1980) 291–300.

45 Goochee, C., Rasband, W., and Sokoloff, L., Computerized densitometry and colour coding of [^{14}C] deoxyglocuse autoradiographs. Ann. Neurol. *7* (1980) 359–370.

46 Gowans, E.J., Burrell, C.J., Jilbert, A.R., and Marmion, B.P., Detection of hepatitis B virus sequences in infected hepatocytes by in-situ cytohybridization. J. med. Virol. *8* (1981) 67–78.

47 Haase, A.T., Gantz, G., Eble, B., Walker, D., Stowring, L., Ventura, P., Blum, H., Wietgrefe, S., Zupancic, M., Tourtellotte, W., Gibbs, C.J. Jr, Norrby, E., and Rozenblatt, S., Natural history of restricted synthesis and expression of measles virus genes in subacute sclerosing panencephalitis. Proc. natl. Acad. Sci. USA *82* (1985) 3020–3024.

48 Haase, A.T., Walker, D., Stowring, L., Ventura, P., Geballe, A., Blum, H., Brahic, M., Goldberg, R., and O'Brien, K., Detection of two viral genomes in single cells by double label hybridization in-situ and colour microradioautography. Science *227* (1985) 189–191.

49 Hafen, E., Levine, M., Garber, R.L., and Gehring, W.J., An improved in-situ hybridization method for the detection of cellular RNA's in *Drosophila* tissue sections and its application for localising transcripts of the homeotic Antennapedia gene complex. EMBO *2* (1983) 617–623.

50 Hall, B.D., and Spiegelman, S., Sequence complementarity of T-2 DNA and T-2 specific RNA. Proc. natl. Acad. Sci. USA *47* (1961) 137–163.

51 Harper, M.E., Marselle, L.M., Gallo, R.C., and Wong-Staal, F., Detection of lymphocytes expressing human T-lymphotropic virus type III in lymph nodes and peripheral blood from infected individuals by in-situ hybridization. Proc. natl. Acad. Sci. USA *83* (1986) 772–776.

52 Hayashi, S., Gillam, I.C., Delaney, A.D., and Tener, G.M., Acetylation of chromosome squashes of *Drosophila melanogaster* decreases the background in autoradiographs from hybridization with ^{125}I-labelled RNA. J. Histochem. Cytochem. *36* (1978) 677–679.

53 Hopman, A.H.N., Wiegant, J., Tesser, G.I., and Van Duijn, P., A nonradioactive in-situ hybridization method based on mercurated nucleic acid probes and sulfhydryl-hapten ligands. Nucl. Acids Res. *14* (1986) 6471–6488.

54 Hudson, P., Penschow, J., Shine, J., Ryan, G., Niall, H., and Coghlan, J., Hybridization histochemistry: Use of recombinant DNA as a 'homing probe' for tissue localisation of specific mRNA populations. Endocrinology *108* (1981) 353-356.

55 Hutchinson, N.J., Langer-Sofer, P.R., Ward, D.C., and Hamkalo, B.A., In situ hybridization at the electron microscope level: hybrid detection by autoradiography and colloidal gold. J. Cell Biol. *95* (1982) 609–618.

56 Jablonski, E., Moomaw, E.W., Tullis, R.H., and Ruth, J.L., Preparation of oligodeoxynucleotide-alkaline phosphatase conjugates and their use as hybridization probes. Nucl. Acids Res. *14* (1986) 6114–6128.

57 Jacobs, J., Todd, K., Birnstiel, M.L., and Bird, A., Molecular hybridization of ^{3}H-labelled ribosomal RNA with DNA in ultrathin sections prepared for electron microscopy. Biochim. biophys. Acta *228* (1971) 761–766.

58 Jacobs, J.W., Simpson, E., Penschow, J., Hudson, P., Coghlan, J., and Niall, H., Characterisation and localisation of calcitonin messenger ribonucleic acid in rat thyroid. Endocrinology *113* (1983) 1616–1622.

59 Jamrich, M., Mahon, K.A., Gavis, E.R., and Gall, J.G., Histone RNA in amphibian oocytes visualized by in-situ hybridization to methacrylate-embedded tissue sections. EMBO *3* (1984) 1939–1943.

60 John, H.A., Birnstiel, M.L., and Jones, K.W., RNA-DNA hybrids at the cytological level. Nature *223* (1969) 582–587.

61 Jones, K.W., Bishop, J.O., and Brito-da-Cuhna, A., A complex formation between Poly-r-(U) and various chromosomal loci in *Rhyncosciara*. Chromosoma *43* (1973) 375–390.

62 Karnovsky, M.J., A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol. *27* (1965) 137A–140A.

63 Koenig, S., Gendelman, H.E., Orenstein, J.M., Dal Canto, M.C., Pezeshkpour, G.H., Yungbluth, M., Janotta, F., Askamit, A., Martin, M.A., and Fauci, A.S., Detection of AIDS virus in macrophages

in brain tissue from AIDS patients with encephalopathy. Science *223* (1986) 1089–1093.
64 Kornberg, T., Sidén, I., O'Farrell, P., and Simon, M., The engrailed locus of *Drosophila:* in-situ localization of transcripts reveal compartment-specific expression. Cell *40* (1985) 45–53.
65 Kretschner, P.J., Coon, H.C., Davis, A., Harrison, M., and Nienhuis, A.W., Haemoglobin switching in sheep. J. biol. Chem. *256* (1981) 1975–1982.
66 Landegent, J.E., Jansen in de Wal, N., Ploem, J.S., and van der Ploeg, M., Sensitive detection of hybridocytochemical results by means of reflection-contrast microscopy. J. Histochem. Cytochem. *33* (1985) 1241–1246.
67 Langer-Sofer, P.R., Levine, M., and Ward, D.C., Immunological method for mapping genes on Drosophila polytene chromosomes. Proc. natl. Acad. Sci. USA *79* (1982) 4381–4385.
68 Lawrence, J.B., and Singer, R.H., Quantitative analysis of in-situ hybridization methods for the detection of actin gene expression. Nucl. Acids Res. *13* (1985) 1777–1799.
69 Lawrence, J.B., and Singer, R.H., Intracellular localization of messenger RNA's for cytoskeletal proteins. Cell *45* (1986) 407–415.
70 McAllister, L.B., Scheller, R.H., Kandel, E.R., and Axel, R., In-situ hybridization to study the origin and fate of identified neurones. Science *222* (1983) 800–808.
71 McDougall, J.K., Myerson, D., and Beckmann, A., Detection of viral DNA and RNA by in-situ hybridization. J. Histochem. Cytochem. *34* (1986) 33–38.
72 McGinnis, W., Levine, M.S., Hafen, E., Kuroiwa, A., and Gehring, W.J., A conserved DNA sequence in homoeotic genes of the *Drosophila* Antennapedia and bithorax complexes. Nature *308* (1984) 428–433.
73 McWilliams, D., and Boime, I., Cytological localisation of placental lactogen messenger ribonucleic acid in syncytiotrophoblast layers of human placenta. Endocrinology *107* (1980) 761–765.
74 Maxam, A.M., and Gilbert, W., Sequencing end-labelled DNA with base-specific chemical cleavages. Meth. Enzymol. *65* (1980) 499–560.
75 Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K., and Green, M.R., Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP-6 promoter. Nucl. Acids Res. *12* (1984) 7035–7056.
76 Mendelsohn, F.A.O., Quirion, R., Saavedra, J.M., Aguilera, G., and Catt, K.J., Autoradiographic localization of angiotensin II receptors in rat brain. Proc. natl. Acad. Sci. USA *81* (1984) 1575–1579.
77 Moench, T.R., Gendelman, H.E., Clements, J.E., Narayan, O., and Griffin, D.E., Efficiency of in-situ hybridization as a function of probe size and fixation technique. J. virol. Meth. *11* (1985) 119–130.
78 Muragagi, A., and Wallace, R.B., Biotin-labelled oligonucleotides: enzymatic synthesis and use as hybridization probes. DNA *3* (1984) 269–277.
79 Ohkubo, H., Kageyama, R., Ujihara, M., Hirose, T., Inayama, S., and Nakanishi, S., Cloning and sequence analysis of cDNA for rat angiotensinogen. Proc. natl. Acad. Sci. USA *80* (1983) 2196–2200.
80 Orth, G., Jeantur, P., and Croissant, O., Evidence for and localisation of vegetative viral DNA replication by autoradiographic detection of RNA-DNA hybrids in sections of tumours induced by Shope papilloma virus. Proc. natl. Acad. Sci. USA *68* (1971) 1876–1880.
81 Panthier, J.J., Foote, S., Chambrand, B., Strosberg, A.D., Corvol, P., and Rougeon, F., Complete amino acid sequence and maturation of the mouse submaxillary gland renin precursor. Nature *298* (1982) 90–92.
82 Penschow, J.D., Haralambidis, J., Aldred, P., Tregear, G.W., and Coghlan, J.P., Location of gene expression in CNS using hybridization histochemistry. Meth. Enzymol. *124* (1986) 534–548.
83 Pfeifer-Ohlsson, S., Goustin, A.S., Rydnert, J., Bjersing, L., Wahlström, T., Stehelin, D., and Ohlsson, R., Spatial and temporal pattern of cellular myc oncogene expression in developing human placenta: implications for embryonic cell proliferation. Cell *38* (1984) 585–596.
84 Pintar, J.E., Schachter, B.S., Herman, A.B., Durgerian, S., and Krieger, D.T., Characterization and localization of proopiomelanocortin messenger RNA in the adult rat testis. Science *225* (1984) 632–634.
85 Pochet, R., Brocas, H., Vassart, G., Toubeau, G., Seo, H., Refetoff, S., Dumont, J.E., and Pasteels, J.L., Radioisotopic localisation of prolactin mRNA on histological sections by in-situ hybridization. Brain Res. *211* (1981) 433–438.
86 Rall, L.B., Scott, J., Bell, G.I., Crawford, R.J., Penschow, J.D., Niall, M.D., and Coghlan, J.P., Mouse prepro-epidermal growth factor synthesis by the kidney and other tissues. Nature *313* (1985) 228–231.
87 Richards, R.I., Catanzaro, D.F., Mason, A.J., Morris, B.J., Baxter, J.D., and Shine, J., Mouse glandular kallikrein genes. J. biol. Chem. *257* (1982) 2758–2761.
88 Rigby, P.W.J., Dieckmann, M., Rhodes, C., and Berg, P., Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. molec Biol. *113* (1977) 237–241.
89 Robins, D.M., Ripley, S., Henderson, A.S., and Axel, R., Transforming DNA integrates into the host chromosome. Cell *23* (1981) 29–39.
90 Rogers, A.W., Techniques of autoradiography, pp.201–284. Elsevier, North Holland, Amsterdam 1979.
91 Rosenfeld, M.G., Mermod, J.-J., Amara, S.G., Swanson, L.W., Sawchenko, P.E., Rivier, J., Vale, W.W., and Evans, R.M., Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. Nature *304* (1983) 129–135.
92 Rudkin, G.T., and Stollar, B.D., High resolution detection of DNA-RNA hybrids in-situ by indirect immunofluorescence. Nature *265* (1977) 472–473.
93 Schalling, M., Hökfelt, T., Wallace, B., Goldstein, M., Filer, D., Yamin, C., and Schlesinger, D.H., Tyrosine-3-hydroxylase in rat brain and adrenal medulla: hybridization histochemistry and immunohistochemistry combined with retrograde tracing. Proc. natl. Acad. Sci. USA *83* (1986) 6208–6212.
94 Scheller, R.H., Kaldany, R.-R., Kreiner, T., Mahon, A.C., Nambu, J.R., Schaefer, M., and Taussig, R., Neuropeptides: mediators of behaviour in *Aplysia*. Science *225* (1984) 1300–1308.
95 Scott, J., Patterson, S., Rall, L., Bell, G.I., Crawford, R., Penschow, J.D., Niall, H.D., and Coghlan, J., The structure and biosynthesis of epidermal growth factor precursor. J. Cell Sci. Suppl. *3* (1985) 19–28.
96 Seeburg, P., The human growth hormone gene family: nucleotide sequences show recent divergence and predict a new polypeptide hormone. DNA *1* (1982) 239–249.
97 Shivers, B.D., Harlan, R.E., Hejtmancik, J.F., Conn, P.M., and Pfaff, D.W., Localization of cells containing LHRH-like mRNA in rat forebrain using in-situ hybridization. Endocrinology *118* (1986) 883–885.
98 Shivers, B.D., Harlan, R.E., Pfaff, D.W., and Schachter, B.S., Combination of immunocytochemistry and in-situ hybridization in the same tissue section of rat pituitary. J. Histochem. Cytochem. *34* (1986) 39–43.
99 Shivers, B.D., Schachter, B.S., and Pfaff, D.W., In-situ hybridization for the study of gene expression in the brain. Meth. Enzymol. *124* (1986) 497–510.
100 Singer, R.H., and Ward, D.C., Actin gene expression visualized in chicken muscle tissue culture by using in-situ hybridzation with a biotinated nucleotide analog. Proc. natl. Acad. Sci. USA *79* (1982) 7331–7335.
101 Smith, F.M., Fung, S., Hunkapiller, M.W., Hunkapiller T.J., and Hood, L.E., The synthesis of oligonucleotides containing an aliphatic amino group at the S'terminus: synthesis of fluorescent DNA primers for use in DNA sequence analysis. Nucl. Acids Res. *13* (1985) 2399–2502.
102 Stuart, W.D., and Porter, D.L., An improved in-situ hybridization method. Exp. Cell Res. *113* (1978) 219–222.
103 Szabo, P., Elder, E., Steffensen, D.M., and Uhlenbeck, O.C., Quantitative in-situ hybridization of ribosomal RNA species to polytene chromosomes of *Drosophila melanogaster*. J. molec. Biol. *115* (1977) 539–563.
104 Taylor, J.M., Illmensee, R., and Summers, J., Efficient transcription of RNA into DNA by avian sarcoma virus polymerase. Biochim. biophys. Acta *442* (1976) 324–330.
105 Tu, C-P.D., and Cohen, S.N., 3'end-labelling of DNA with [α-^{32}P] cordycepin-5-triphosphate. Gene *10* (1980) 177–181.
106 van Leeuwen, B.H., Evans, B.A., Tregear, G.W., and Richards, R.I., Mouse glandular kallikrein genes. J. biol. Chem. *261* (1986) 5529–5535.
107 Varndell, I.M., Polak, J.M., Sikri, K.L., Minth, C.D., Bloom, S.R., and Dixon, J.E., Visualisation of messenger RNA directing peptide synthesis by in-situ hybridization using a novel single-stranded cDNA probe. Histochemistry *81* (1984) 597–601.
108 Venezky, D.L., Angerer, L.M., and Angerer, R.C., Accumulation of histone repeat transcripts in the sea urchin egg pronucleus. Cell *24* (1981) 385–391.
109 Wahl, G.M., Stern, M., and Stark, G.R., Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization using dextran sulphate. Proc. natl. Acad. Sci. USA *76* (1979) 3683–3687.

110 Warembourg, M., Tranchant, O., Perret, C., Desplan, C., and Thomasset, M., In-situ detection of vitamin D-induced calcium binding protein (9-kDa CaBP) messenger RNA in rat duodenum. J. Histochem. Cytochem. *34* (1986) 277–280.
111 Wilcox, J. N., Gee, C. E., and Roberts, J. L., In-situ cDNA:mRNA hybridization: development of a technique to measure mRNA levels in individual cells. Meth. Enzymol. *124* (1986) 510–533.
112 Wintour, E. M., The role of the adrenal in control of foetal-developmental gene-switches in haemoglobin. Proc. XXXth Congress I.U.P.S. (1986) 121 (abstr.).
113 Wintour, E. M., Smith, M. B., Bell, R. J., McDougall, J. G., and Cauchi, M. N., The role of fetal adrenal hormones in the switch from fetal to adult globin synthesis in sheep. J. Endocr. *104* (1985) 165–170.
114 Zajac, J. D., Penschow, J. D., Mason, T., Tregear, G. W., Coghlan, J. P., and Martin, T. J., Identification of calcitonin and calcitonin gene-related peptide messenger ribonucleic acid in medullary thyroid carcinomas by hybridization histochemistry. J. clin. Endocr. Metab. *62* (1986) 1037–1043.
115 Zawatsky, R., De Maeyer, E., and De Maeyer-Guignard, J., Identification of individual interferon-producing cells by in-situ hybridization. Proc. natl. Acad. Sci. USA *82* (1985) 1136–1140.

0014-4754/87/070741-10$1.50 + 0.20/0

Regulatory peptide receptors: visualization by autoradiography

by J. M. Palacios and M. M. Dietl

Preclinical Research, SANDOZ LTD, CH 4002–Basel (Switzerland)

Summary. The receptors for regulatory peptides have been extensively characterized using radioligand binding techniques. By combining these binding techniques with autoradiography it is possible to visualize at the light and electron microscopic levels the anatomical and cellular localization of these receptors. In this review we discuss the procedures used to label peptide receptors for autoradiography and the peculiarities of peptides as ligands. The utilization of autoradiography in mapping peptide receptors in brain and peripheral tissues, some of the new insights revealed by these studies particularly the problem of 'mismatch' between endogenous peptides and receptors, the existence of multiple receptors for a given peptide family and the use of peptide receptor autoradiography in human tissues are also reviewed.
Key words. Receptors; multiplicity; radioligand binding; quantitative autoradiography; light- and electron microscopy; peptidases; endogenous ligands; brain; gut.

Introduction

The physiological and pharmacological effects of the regulatory peptides, like those of other hormones and neurotransmitters, are mediated by the interaction of these substances with specific recognition sites named 'receptors'. In parallel with the steadily growing number of peptides being discovered, multiple receptors for these peptides have been characterized[7,50,54,55,116,117]. Pharmacological and biochemical techniques have provided evidence for the existence of multiple receptors for the different peptide families. Historically the characterization of the opioid receptor opened the path for the isolation and characterization of the opiate peptides[12,51,114,118]. The use of the so-called high-affinity radioligand binding techniques was instrumental in beginning the search for endogenous ligands. By using radiolabeled molecules at high specific activity like, for example, the same peptides, it is possible to study the binding of these ligands to membrane preparations. These techniques allow the characterization of sites with high affinity (in the low nanomolar range) and low capacity (fmol/mg of protein) and presenting selectivity and specificity for a given peptide and its analogues with similar physiological effects. In recent years this relatively simple approach has allowed the detailed study of the structure-activity relationship and the biochemical mechanism involved in peptide action and progress is being made with solubilization and purification and towards the ultimate goal of the elucidation of the molecular characteristics of these receptors[115,123].

One main limitation of these biochemical techniques is, however, the lack of sufficient anatomical resolution to answer questions such as: where are these sites localized at the microscopic level? Are there specific cell populations in a given tissue or organ enriched in a particular peptide receptor? Finally; which subcellular structures bear these receptor sites?

Techniques with high anatomical resolution are required to answer these questions. Because of the wide use of radioligand binding assays in the study of peptides and other receptors it is not surprising that *autoradiography* has been one of the techniques most widely used in the localization of peptide receptors at the microscopic level. In particular the in vitro autoradiographic procedure originally developed by Young and Kuhar[134] is specially well suited for the study of peptide receptors. Organ and tissue barriers encountered in in vivo labeling are overcome, as is the problem of ligand metabolism, a very important problem when dealing with peptide ligand both in vivo and in vitro[59,60,89]. In this paper we will review the use of this technique in the visualization of peptide receptors both in the central nervous system and peripheral tissues at the light and electron microscopic levels of resolution as well as other alternatives for the visualization of peptide receptors.

Methodology

The procedures for the labeling of peptide receptors for autoradiography are essentially identical with those used for the labeling of other hormone, drug or neurotransmitter receptors[59,60,89]. Some of the particularities encountered in peptide receptor labeling are related to the use, in most cases, of radiolabeled peptides as ligands[123]. These ligands present, in general 1) a high susceptibility to degradation by peptidases present on the tissue, and 2) a tendency to bind in a non-specific way to tissue, glass and gelatine in the histological