RECEPTORS, MEMBRANES AND TRANSPORT MECHANISMS IN MEDICINE

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Preface

The first Heidelberg Conference was held in Melbourne at the Austin Hospital in 1981. Its purpose was to recognise the first fifteen years of the establishment of the University of Melbourne Departments of Medicine at the Austin and Repatriation Hospitals under the Chairmanship of Professor A.E. Doyle. The speakers were all past or present members of the Department or close collaborators. The success of the meeting led to the decision to establish the Conference as a regular forum. Its purpose was to allow present and past members of the Department to meet with close collaborators and distinguished overseas scientists to discuss the growing edge of scientific clinical research. On this occasion the overseas visitor was Dr. Kevin Catt, Chief, Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, Bethesda, MD, U.S.A., a past associate of Austin Doyle's who retains links with the Department. The topic of this conference was broadly the biology of receptors, membranes and transport mechanisms in medicine.

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F.A.O. MENDELSOHN
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HYBRIDIZATION HISTOCHEMISTRY : USE OF COMPLEMENTARY DNA FOR TISSUE LOCALIZATION OF SPECIFIC mRNA POPULATIONS

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Key words : Hybridization Histochemistry, in situ hybridization, Complementary DNA, Autoradiography, Gene Expression, Synthetic genes

INTRODUCTION

Specific hybridization by base pairing between complementary strands of DNA or RNA is not only one of the theoretical foundations of modern molecular biology but also the basis of the rapidly expanding field of recombinant DNA technology. Hybridization using complementary DNA (cDNA) probes is widely used for enrichment of specific mRNA species and for detection of chromosomal DNA components. The products of hybridization can be visualized directly by electron microscopy[1] or indirectly by autoradiography[2]. At the ultrastructural level hybridization has demonstrated visually the presence of intervening sequences within the coding region of the ovalbumin gene[3]. Some of the applications of hybridization have been the development of in situ techniques for the detection of immobilized DNA[4,9], RNA[5] or plasmid DNA from bacterial colonies[6]. These in situ techniques have been extended to the identification of RNA species within individual isolated cells using cDNA probes[7,8]. We report here a novel use of hybridization: the localization in tissue sections of cells which contain specific mRNA populations, using recombinant cDNA probes[12,13].

The basis of this technique is the incubation of a labelled recombinant cDNA probe with a carefully prepared section of tissue. After appropriate washing the tissue is dried and autoradiography is used to identify specific cell populations or tissue regions binding the probe. The principle is thus similar to the widely used immunohistochemical procedures based upon binding of fluorescent, radioactive or peroxidase labelled antibodies.

MATERIALS AND METHODS

The critical elements for optimizing the hybridization reaction have been carefully studied using DNA and RNA bound to nitrocellulose membranes or activated paper supports as the experimental model system[9,10]. Some of the variables which have to be considered are the length and concentration of probe, the degree of nucleotide sequence, homology, time, temperature, and salt concentration as well as a number of options such as the presence of denaturants (eg form -amide) or dextran sulphate[11].

When applying these conditions to the detection of DNA or RNA in fixed sections of tissues it became obvious that the denatured cell -ular debris contributed significantly to the rate of hybridization. The nucleic acid is physically entrapped in the cell debris and thus reduces probe accessibility. 'Background' hybridization results from the probe binding to various cell components and further reduces the signal to noise ratio. Each laboratory has optimized slightly different hybridization conditions and this probably reflects differences in the tissue structure under study as well as the chem -ical method of fixation.

Preparation of Tissue, Preparation of Sections, cDNA probe labelling, Hybridization, Autoradiography and Staining have been described in adequate detail elsewhere[12] and will not be repeated here.

Preparation of Whole Mouse Sections

Adult Swiss male mice were killed by pentobarbitone intraperitoneal injection. The mice were frozen immediately by immersion in hexane/dry ice, and subsequently freeze embedded in a 2% carboxymethylcellulose gel. Midline sagittal sections (30μ - 40μ thick) were cut at -20° using a PMV cryo-microtome (LKB, Sweden). Transparent tape (3M) was stuck to the block prior to cutting the sections. This prevents shattering and the cut section comes off adher -ent to the tape. These sections were immediately fixed at room temperature by immersion in 2.5% glutaraldehyde in 0.1M phosphate pH 7.3, and rinsed at 40°C in hybridization buffer[13]. The sections were then prehybridized at 40°C for 2 hrs, rinsed in ethanol and air dried. Hybridization with P^{32} labelled probes was carried out under glass slides for 3 days at 40°C within a sealed humidified chamber. Following hybridization the sections were washed initially in 2 X SSC, followed by a final wash for 45 min. in 1 X SSC in ethanol and air dried. Autoradiography was carried out by direct exposure to X-ray film (Cronex MRF 32 Medical recording film - Du Pont).

Synthesis of DNA probes

Oligodeoxyribonucleotide probes were prepared by the solid-phase phosphoramidite procedure[14] on an Applied Biosystems Inc. Model 380 A DNA Synthesizer. Syntheses were commenced with 20 mg of the appropriate nucleotide coupled by the 3' - hydroxyl to a Fractosil - 200 silica support at a loading of 50 μmole/gm. (total 1 μmole nucleotide). The standard phosphite chemistry program was used with 15. fdd excess of protected diisoprophyl phosphoramidites. Following chain assembly and removal of the phosphate methyl ester protecting groups with thiophenol, the oligodeoxyribonucleotide was cleaved from the silica support in aqueous ammonia solution. Additional concentrated ammonia solution was added and the oligodeoxyribonucleotide solution treated for a further 16 hr at 55oC to remove the base protecting groups. After removal of the ammonia solution by rotary evaporation the residue was made up to a final volume of 3 ml in freshly purified water. Aliquots (200 μl) of

this aqueous solution were purified by ion exchange HPLC on a Whatman Partisil 10 - SAX column using a phosphate-formamide gradient system or by gel electrophoresis on 18% acrylamide gels. The sequence of the purified probes was confirmed by the Maxam and Gilbert procedure[15]. The synthetic oligodeoxyribonucleotide probes were 5' - end labelled with [α - ^{32}P] ATP using T4 polynucleo -tide kinase to a specific activity of 6 x 10^6 cpm/pmole.

RESULTS

We now have considerable experience with several cDNA probes of differing length (base pairs/b.p) which we have used to study gene expression. These include probes of 800 b.p. including the whole of the coding region for growth hormone; two probes for the pro opio -melano-cortin system, one only 150 b.p. at the endorphin coding - end; the whole of the coding region for rat and human relaxin; rat calcitonin of 945 b.p. containing the entire coding region; human somatostatin of 600 b.p. including the whole coding region; mouse kallikrein of 500 b.p. including the C terminal coding region; renin of 500 b.p. including the 3' end and others.

For this presentation we have chosen to detail two probes, to illustrate some new aspects of Hybridization Histochemistry. The probes selected are mouse kallikrein, 500 b.p. including the C terminal coding region (MK1), a synthetic 30 mer probe of mouse kallikrein, and a synthetic 39 mer probe for arginine vasopressin (AVP). We will apply these probes to demonstrate firstly, that we have solved the problems of applying the technique to sections of whole mouse; and secondly, that synthetic oligodeoxyribonucleotide probes only 30 mer in length are effective.

Figure 1 shows an autoradiograph of a whole section of a male mouse hybridized with a synthetic 30 mer probe for kallikrein. This autoradiograph can be compared with a photo of the whole block taken immediately before the section was cut, to identify tissues where kallikrein family mRNA sequences have been detected.

Whole mouse section hybridization demonstrates clearly the presence of kallikrein mRNA within the submandibular glands, an unidentified central location, kidney and possible labelling of some sublingual glands and the gut as well as the vertebrae and bone. Hybridization amongst the salivary glands occurred mainly in the submandibular/submaxillary with lower levels of labelling in the sublingual. The intensity of labelling of the male submandibular gland was much greater than the female. In the whole mouse sections kallikrein gene expression was also detected in the cortex of the kidney.

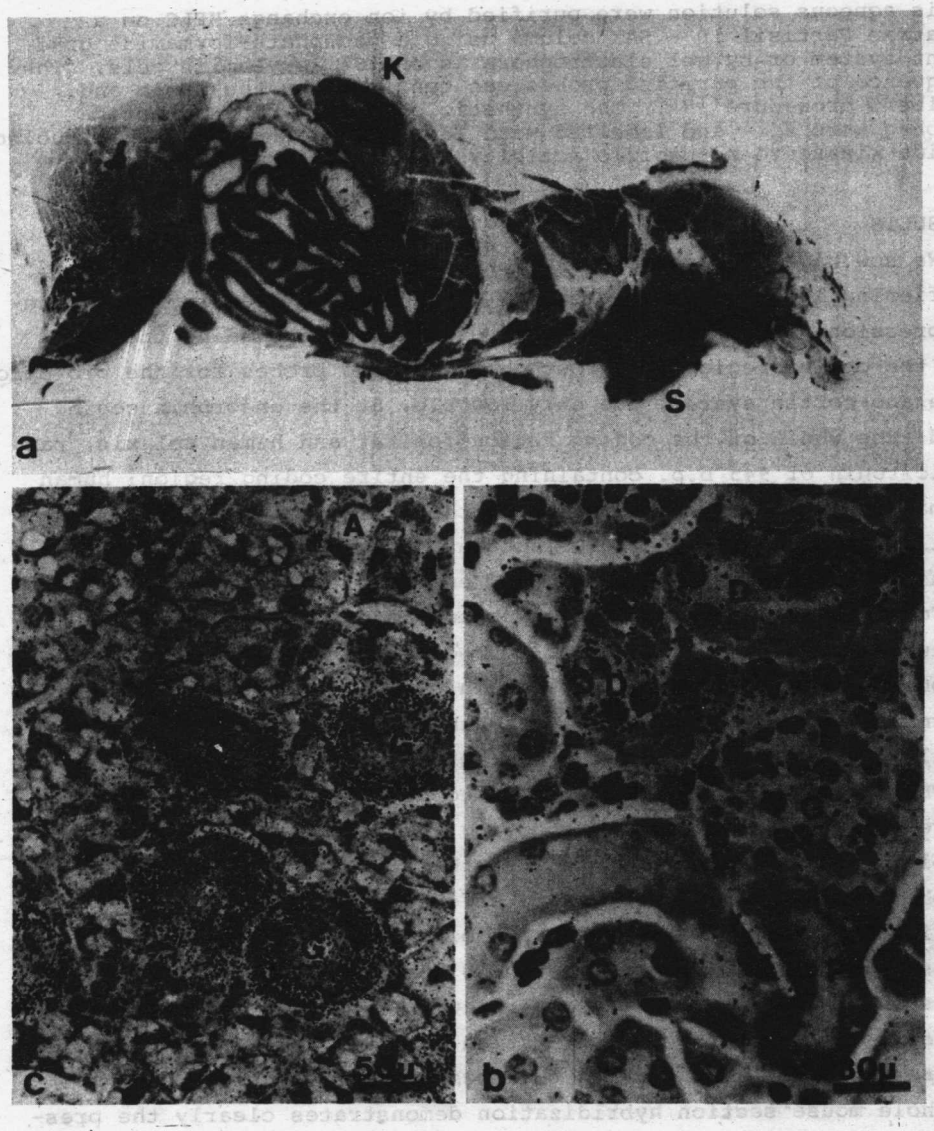

Fig. 1 Autoradiographs prepared from male Swiss mice after hybridization with a ^{32}P-labelled synthetic oligodeoxyribunucleotide 30 mer probe corresponding to amino acid sequence 111-120 of the mouse kallikrein gene PMK1[24].
a) Autoradiograph on X-ray film of a 30μ sagittal section of a whole adult mouse. K - Kidney, S - Submaxillary gland.
b and c are liquid emulsion autoradiographs of 5μ sections of isolated tissues.
b) Kidney cortex. D - Distal tubules, G - Glomerulus, P - Proximal tubules.
c) Submandibular gland. G - Granular convoluted tubules, A - Acinar tissue.

Male and female mouse kidney were similar in both distribution and levels of kallikrein mRNA. The kidney appears to be likely a major site of kallikrein production as the intensity of labelling of the kidney is about the same as in the male salivary gland. The precise anatomical nature of the intensely labelled region above the salivary gland has not been resolved. The low level labelling of the gut could only be regarded as suggestive at this time, but the kallikrein family of enzymes and hormones have a wide anatomical distribution.

The presence of kallikrein mRNA within the kidney and submaxillary gland was investigated further using liquid emulsion autoradiography and 5 μ sections of isolated tissue to improve the autoradiographic resolution (Fig. 1a & b). Using the synthetic oligodeoxyribonucleotide probe, hybridization of the submaxillary gland demonstrates that the major site of kallikrein mRNA is within the cells of the granular convoluted tubules, with less intense labelling in female confirming earlier results with the "natural" cDNA probe[12] not reproduced here.

The higher resolution analysis of kallikrein mRNA location in the kidney supports the results of the whole mouse sections. Figure 1b shows a high magnification picture of the outer kidney cortex where the kallikrein gene is clearly being expressed in the convoluted distal tubule.

Fig. 2 shows a coronal section of (a) sheep brain and (b) rat brain, using a synthetic oligodeoxyribonucleotide probe for arginine vasopressin. These section are of the anterior hypothalamus and show the 3rd ventricle. As would have been anticipated from other studies the magnocellular neurones label, including the supraoptic and paraventricular nuclei and the nucleus circularis in the rat.

DISCUSSION

The general implication of the results presented here are more important than the particular, which in any case, have been touched upon elsewhere[12].

In recent times the very considerable advances in molecular biology have led to a proliferation of putative peptide hormones which for one reason or another have been fished from the genome. Numerous oncogens and growth factors have been recognized also. Successful gene transfer experiments have made the knowledge of the anatomical sites at which these genes are switched on a compelling issue.

In any of these instances the search for the "switched on" site on an organ to organ basis is tedious. The whole mouse section has a great deal to offer for this type of enquiry. Indeed the approach is obviously very useful for organs which are morphologically complex and functionally heterogenous, eg. brain from larger animals. Indeed for some applications this would be the approach of preference. The method is not absolutely definitive and depends on whether the physiological conditions were such that the gene would be

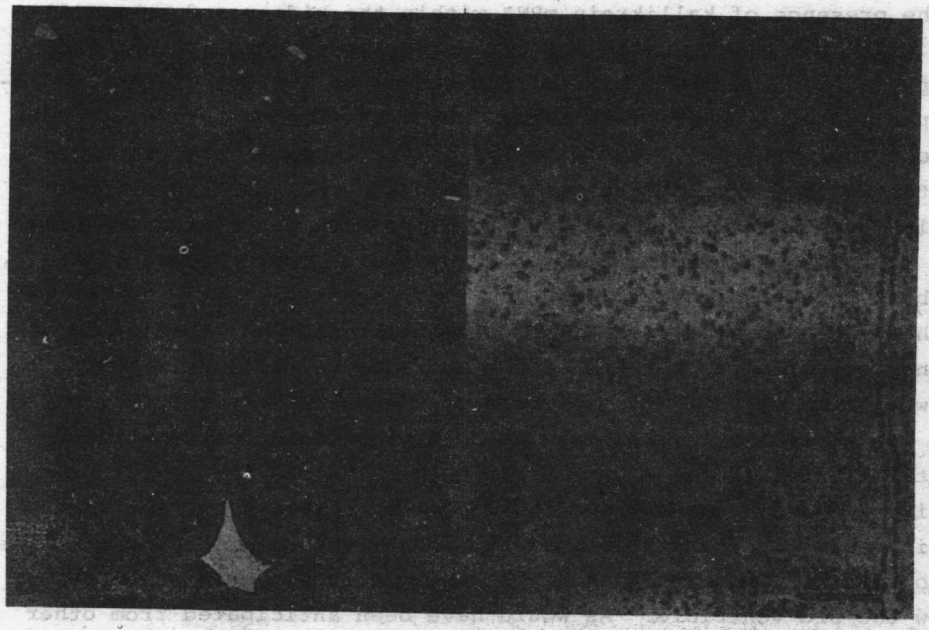

Fig. 2. Liquid emulsion autoradiographs of 10μ sections of brain after hybridization with a ^{32}P-labelled synthesizer oligodeoxyribonucleotide 30- mer probe coding for the amino sequence (-1 to +12) of the bovine vasopressin-neurophysin II precursor(26). (a) Sheep hypothalamus with the 3rd ventricle in centre field. The magnocellular paraventricular nucleus is delineated by the extent of the labelled areas adjacent to the top, centre and bottom of the 3rd ventricle. (b) Rat hypothalamus with the upper two-thirds of the 3rd ventricle to the right of the field. Some labelled magnocellular neurones of the paraventricular nucleus are evident adjacent to the top of the 3rd ventricle and the nucleus circularis (NC) is clearly delineated.

switched on; on the spatial arrangement of the block and the distance of the section from the midline - not infrequent histological problems. Whole mouse/rat sections of this type have been widely used for various reasons, but not successfully handled after cutting, to the extent reported here without the awkward problem of the

sections detaching from the tape.

Hybridization histochemistry since its introduction in 1980[16] has been applied in several studies. A short history of the genera background and a chronological perspective has been published[12]. There were a number of studies which used a polynucleotide or partially purified rather than recombinant cDNA probes. This is an important distinction because recombinant probes, with their absolute homogeneity guaranteed through the cloning procedure used to produce them, provide a degree of specificity of labelling which cannot be matched by any partially purified probe enriched for a particular molecular species. cDNA has been successfully used by ourselves[16,12,13,17,18] and by Gee et al[19] to demonstrate pro-opiomelanocortin neurones in the peri-arcuate region of the rat hypothalamus, and ingeniously by McAllister et al[20] to study the origin and fate of identified neurones in the marine mollusc Aphysia. Ward and his group[21,22] have pioneered the use of non-radio active labelling.

The wide application of hybridization histochemistry is limited only by the availability of suitable probes. In the absence of commercially available cDNA probes produced by recombinant technology the use of "natural" cDNA requires access at least to the skill and resources of a fully set up biotechnology laboratory. Major advances have been made recently in the production of synthetic oligonucleotide (DNA) (14) and the use of second generation program able DNA synthesizers (23). The illustrations in this paper are ample evidence that synthetic probes do work satisfactory. These two probes were 30 mer for kallikrein and 39 mer for AVP. Probes of this length are well within the capacity of current technology, and are long enough for adequate specificity. In general, based on statistical considerations a sequence of only 20 nucleotides is needed for a probe to be unique for a specific gene. Resolution between the member genes of a closely related family eg. the kallikrein, AVP - oxytocin etc. is a more difficult issue and the influence of frame shifts and a small number of mismatches are currently the subject of further formal investigation in our laboratory.

However, the fact that small synthetic probes will work in the technique of hybridization histochemistry opens up vast possibilities. While immunohistochemistry and hybridization histochemistry provide complementary but different information - the contents of warehouses vis a vis state of the plant in a factory. Making a

synthetic 20-30 mer oligodeoxyribonucleotide in a day is akin to manufacturing a highly specific antibody. Even with ^{32}P labelling suitable probes for hybridization histochemistry can be prepared in any laboratory from stocks of DNA which can easily be prepared in massive amounts relative to each usage. Thus as the fall out from the molecular biology explosion continues synthetic probes will be available to every basic scientist and for every pathologist for diagnosis. These will include peptides (hormones) proteins, enzymes, viruses and oncogenes.

In regard to the particular example chosen here the autoradiograph of the whole mouse section Fig. 1, speaks for itself. This particular probe was selected from a family of genes which are all possible sereine proteases (24). It shows the presence of kallikrein mRNA within the submandibular gland and other glands in the mouth, including a conspicuous unidentified site, kidney and possibly some labelling of the gut. The labelling of the vertebrae and bone is thought to be non-specific and represents binding of the polyphosphate probe to bone. This was not unexpected but could mask specific labelling. Similar labelling has occurred with several unrelated probes. Fig. 1c shows a highpower emulsion autoradiograph of the submandibular gland where the location of the gene expression of kallikrein is in the granulated duct cells. These granulated duct cells are the only site where cells are morphologically equipped to secrete enzymes or hormones. At higher magnification Fig.1b shows a section through mouse kidney where the kallikrein gene being expressed is clearly located in the convoluted distal tubule. Fig. 2a shows the labelling of hypothalamic nuclei as would be expected. These areas labelled have been recognized previously by immunohistochemistry (25).

SUMMARY

This paper illustrates further application of the technique of hybridization histochemistry to whole sections of heterogenous tissue to identify sites where specific genes are being transcribed. The scanning of tissue in the whole mouse sections, especially for the potential discovery of the site of synthesis of new peptides with biological functions represents an exciting advance.

The horizons of the technique are advanced at once from finite to infinite with the finding here that synthetic oligodeoxyribonucleotides of relatively short length are as satisfactory as long

lengths of cDNA at least in the several situations we have now tried; two of which are reported here. At the same time the general applicability of the method is enhanced enormously by unshackling it from heavy dependence on the availability of biotechnology skills at a high level.

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This paper is a small part only of an extensive program on the development and application of hybridization histochemistry. Many people have generously provided cDNA probes and valuable advice. The kallikrein studies in particular form part of a large ongoing program concerning the kallikrein gene family with Dr. Rob Richards and his group at the Department of Biological Sciences, Australian National University. We were assisted in the probe synthesis by James Haralambidis, Denis Scanlon, Janice Turton and Bruce Thorley, and with probe labelling by Dr. Peter Hudson, Dr. Ross Fernley, Peter Aldred and Peter Roche.

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