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Visualisation of messenger RNA directing peptide synthesis by in situ hybridisation using a novel single-stranded cDNA probe

Potential for the investigation of gene expression and endocrine cell activity

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Summary. The neuropeptide tyrosine precursor (pre-pro-NPY) messenger RNA (mRNA) has been localised in formaldehyde-fixed human phaeochromocytoma tissue using a sensitive in situ hybridisation procedure and a novel single-stranded cDNA probe. The reaction product was revealed by avidin-biotin-peroxidase complex and streptavidin-gold complex with silver enhancement. This technique may be applied for the determination of biosynthetic activity of endocrine and neuronal cell bodies. This is largely due to its rapidity by comparison with conventional autoradiographic procedures, to the permanence of the reaction product and to the sensitivity of the visualisation steps.

Introduction

Regulatory peptide-producing endocrine tumours are routinely diagnosed on the basis of their clinical manifestation and raised plasma hormone levels. Morphological examination, particularly applying immunocytochemistry is used to consolidate the diagnosis and has contributed to the accuracy of identification, particularly in the case of mixed or clinically silent endocrine tumours (Polak and Bloom 1984; Sabate et al. 1984). However, in a large number of cases immunocytochemical localisation of the secretory product often yields poor results, possibly because the normal mechanisms of peptide storage have broken down. For this reason it has become necessary in some cases to demonstrate the presence of secretion-independent markers of the neuroendocrine system, such as neuron-specific enolase (NSE; Schmechel et al. 1979; Tapia et al. 1981) within peptide-producing cells. Furthermore, some endocrine tumours produce abnormal molecular forms of peptides which are not detectable using antisera of limited region specificity (i.e. the mixed population of antibodies recognise single or a small number of epitopes).

In order to be able to determine the amount and rate of each molecular form being synthesised by the tumour cells it is essential to investigate peptide gene expression. It is now possible to study gene expression by identifying particular nucleotide sequences in tissues or their extracts using recombinant DNA probes.

Hybridisation histochemistry is an established technique for the in situ cellular localisation of nucleotide sequences, using labelled (radiolabel or chromogen) probes complementary to the sequence (DNA or RNA) under investigation (Hudson et al. 1981; Singer and Ward 1982; Brigati et al. 1983; Gee and Roberts 1983; Gee et al. 1983; Coghlan et al. 1984). To date only double-stranded DNA probes have been constructed with radiolabelled (^{3}H or ^{32}P) nucleotides for the in situ autoradiographic localisation of mRNA. Double-stranded probes require heating prior to use in order to "melt" the strands which must then be prevented from re-annealing during hybridisation usually by the addition of high concentrations of deionised formamide to the incubation medium. In general the process demands considerable manipulative caution and can often require up to 10 weeks for autoradiographic exposure.

In this paper we present a new in situ hybridisation procedure based on the construction of single-stranded cDNA probes (Fig. 1), which is rapid and sensitive when compared to existing autoradiographic techniques. Briefly, the non-coding strand of pre-pro-NPY cDNA was cloned into a single-stranded bacteriophage vector, m13mp9. A hybridisation primer was used as described in "Material and methods" to incorporate biotinylated UTP into ds-DNA in the vector portion of the single stranded phage. Sites of hybridisation were visualised by the localisation

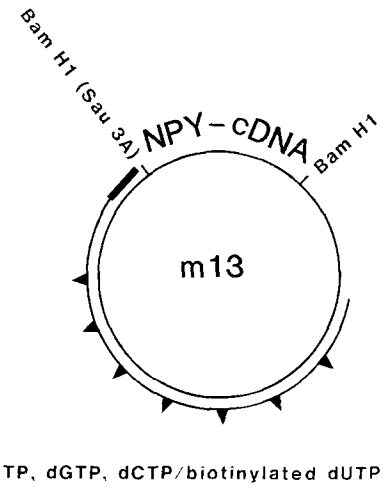

Fig. 1. Schematic representation of the biotinylated DNA probe complementary to NPY mRNA. The biotinylated UTP is incorporated into the double-stranded DNA in the m13 part of the probe while sequences complementary to the NPY mRNA (NPY-cDNA) remain single-stranded

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of biotin utilising its ability to bind to avidin in a standard avidin-biotin-peroxidase complex (ABC; Hsu et al. 1981) or by streptavidin-gold conjugate (Bonnard et al. 1984) followed by silver deposition enhancement (Holgate et al. 1983; Springall et al. 1984). Using this approach we have been able to localise the messenger RNA (mRNA) directing the synthesis of neuropeptide tyrosine (Tatemoto 1982) precursor (pre-pro-NPY) within formaldehyde-fixed human phaeochromocytoma. Although the results obtained from this study relate to NPY in phaeochromocytomas the technique could be adapted for investigations on peptide gene expression in normal tissue, particularly to develop an objective index of biosynthetic activity.

Material and methods

Phaeochromocytoma samples were collected at surgery and were snap-frozen or fixed by immersion within 30 min of excision. NPY levels in plasma and wet tissue were measured as described in a previous study (Adrian et al. 1983).

Sample processing

Tissue samples were collected for light microscope hybridisation histochemistry and for immunohistochemistry. The specimens were fixed for 2 h at room temperature in freshly prepared 4% *para*-formaldehyde in 0.1 M phosphate buffer containing 0.1 M sucrose and 20 mM vanadyl sulphate. The samples were then rinsed in fixative-free buffer and snap-frozen in melting arcton.

Synthesis of biotinylated single-stranded hybridisation probes

Total mRNA was extracted and used as a template for ds-cDNA synthesis as previously described (Minth et al. 1982, 1984). ds-cDNA was cloned using standard dG, dC tailing methods and used to transform *E. coli* strain JM83. Recombinants containing sequences corresponding to NPY were isolated as described (Minth et al. 1984). Both strands of the largest clone positive on screening (pNPY3–75) were sequenced using established methods (Sanger et al. 1977; Maxam and Gilbert 1980). Plasmid pNPY3–75 was digested with the restriction enzymes BamHI and Sau3A and a resulting fragment (300 base pairs) was subcloned into the BamHI site of the single-stranded bacteriophage m13mp9 followed by transfection into *E. coli* strain JM103. m13(+) strand DNA was isolated and the orientation of the insert was determined by sequencing (Sanger et al. 1977). The phage producing the m13(+) strand complementary to NPY-mRNA was designated hM13npy.

Incorporation of biotinylated UTP into double-stranded DNA in the m13 part of the probe was carried out as follows: 1 µg of hM13npy DNA in 1 µl of water, 1 µl of buffer (60 mM Tris pH 7.4, 60 mM EDTA, 60 mM NaCl, 60 mM $MgCl_2$, 10 mM dithiothreitol), 1.5 ng of the m13 hybridisation primer (Heidecker et al. 1980) and 5 µl of H_2O was heated to 55° C and allowed to cool to room temperature. This solution was added to 5 µl of dried biotinylated UTP (0.3 mM) along with 1 µl of a mixture containing 500 µM dGTP, dCTP and dATP and 2 units of DNA polymerase I [Klenow fragment]. The reaction mixture (10 µl) was incubated for 2 h at 15° C and was terminated by adding 5 µg of tRNA followed by two ethanol precipitations. DNA synthesis was determined by incorporation of ^{32}P-dATP in the reaction mixture.

Light microscope hybridisation histochemistry and immunohistochemistry

Serial cryostat sections (5–10 µm) were cut and mounted on poly-L-lysine coated glass slides (Huang et al. 1983) such that their common cut surfaces were uppermost. Alternate sections were stained using hybridisation histochemistry or immunohistochemistry as described below.

Hybridisation histochemistry

Air-dried sections were pre-hybridised in a modified Denhardt's solution (0.02% ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA in 2 × SSC [SSC = 150 mM NaCl + 15 mM Na citrate] containing 10 mM vanadyl sulphate) for 2 h at 40° C. The sections were then briefly rinsed in absolute ethanol and allowed to air dry. This was immediately followed by hybridisation in a medium composed of 1 vol NPY-mRNA cDNA probe, 2 vol deionised formamide and 1 vol 20 × SSC. Approximately 30 µl of the hybridisation solution was applied per section. Each section was covered with a coverslip and incubated in a sealed humidity chamber for 60–96 h at 40° C. At the end of the incubation period the sections were rinsed in 2 × SSC (4–5 changes), followed by prolonged washing (2–4 h, 15 min changes) in PBS (pH 7.2).

Sites of mRNA-cDNA hybridisation were developed in two different ways: 1. using freshly prepared avidin-biotin-peroxidase complex according to the standard procedure (Hsu et al. 1981); 2. using streptavidin-linked colloidal gold (Bonnard et al. 1984; 5 nm diameter). The gold particles were then made visible at the light microscopical level by silver enhancement as previously described (Danscher and Nörgaard 1983; Holgate et al. 1983; Springall et al. 1984).

Immunohistochemistry

Sites of NPY storage were visualised on serial cryostat sections using the avidin-biotin-peroxidase procedure. Details of the antisera used are given elsewhere (Adrian et al. 1983; Gu et al. 1983; Varndell et al. 1984).

Controls

Standard controls for the immunocytochemical methods were applied, including pre-absorption with NPY, homologous and non-homologous fragments as previously described (Allen et al. 1983; Varndell et al. 1984).

Controls for the hybridisation procedures included incubation with probe-less biotinylated vector and non-homologous cDNA probes (including ds-cDNA probes to rat insulin and rat somatostatin). In addition, small volumes of NPY-mRNA cDNA probe were allowed to hybridise overnight at 4° C with an excess of cold NPY-mRNA in an attempt to "pre-absorb" the cDNA probe. Sections were then incubated with pre-absorbed cDNA probe. The complete hybridisation reaction was performed on cryostat sections of formaldehyde-fixed perfused rat brain. Rat brain contains significant levels of immunoreactive NPY (Allen et al. 1983) however, the full nucleotide sequence of rat pre-pro-NPY mRNA is not known at present.

Some phaeochromocytoma tissue sections were also treated with ribonuclease (4–6 h, 37° C) prior to hybridisation. Excess ribonuclease was washed off the slides with large volumes of sterile saline before pre-hybridisation.

Results

In a previous study (Adrian et al. 1983), the amount of NPY present in the tissue and in the circulation from patients with phaeochromocytomas was established. The plasma NPY concentration averaged 460 pmol/l in patients with a phaeochromocytoma compared with 55 pmol/l in healthy subjects.

NPY-mRNA extracted from the samples of phaeochromocytomas used in this study composed approximately 0.05% of total RNA (1,096 ± 32.4 µg/g tissue; mean ± SD) and about 5% of poly A^+ RNA.

Using a novel biotinylated double-stranded DNA vector with a single-stranded 300 base BamH1-Sau3A insert of pNPY3–75 as probe we have succeeded in localising pre-pro-NPY mRNA in cells within human phaeochromocyto-

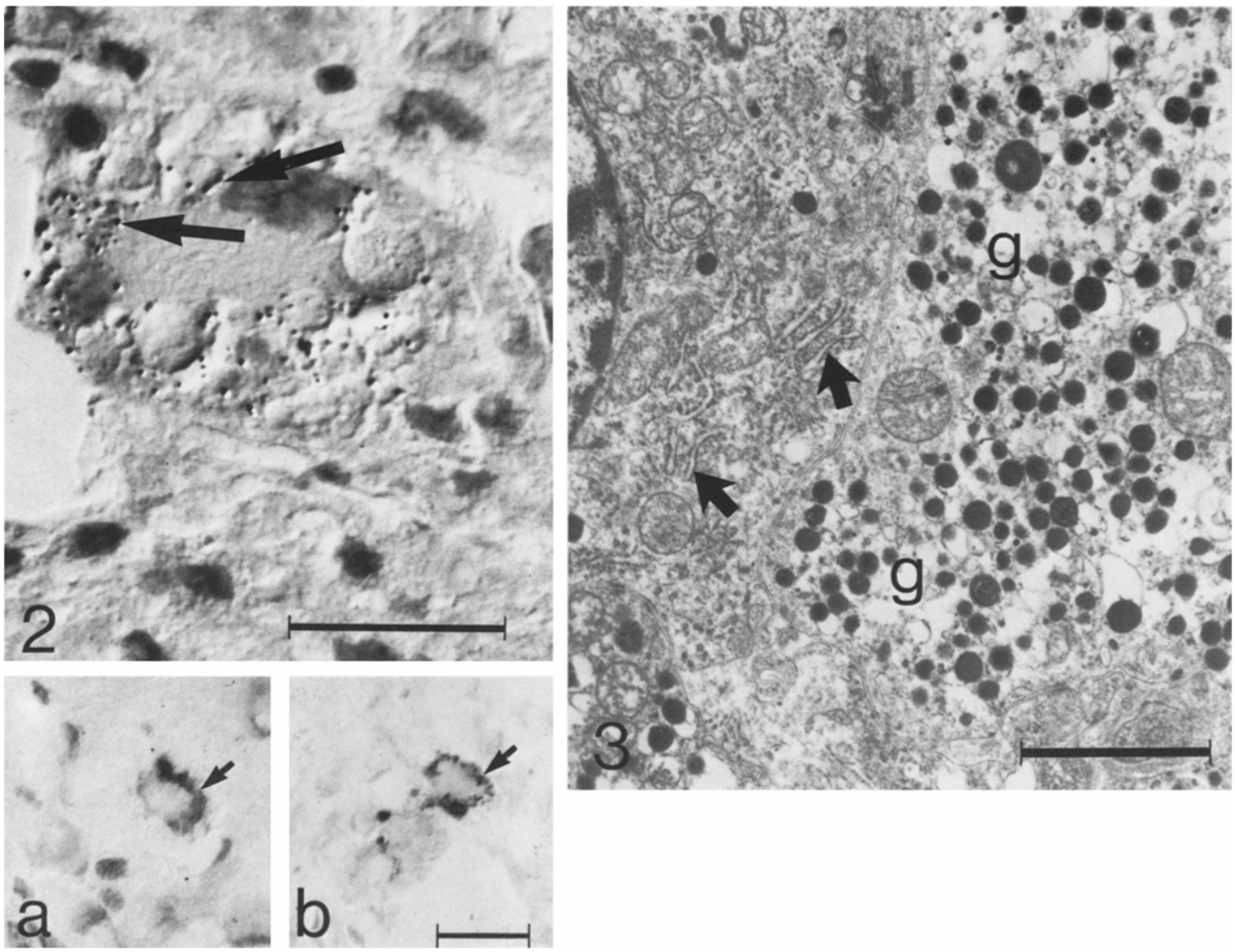

Fig. 2. NPY-containing human phaeochromocytoma tumour cells exhibiting NPY mRNA-biotinylated cDNA hybridisation sites (*arrowed*) visualised using the avidin-biotin technique. The highly concentrated, punctate, appearance of the hybridisation sites is evident in the perinuclear region. Presumably this reflects the localised distribution of messenger molecules in the endoplasmic reticulum. Scale bar = 10 μm. **a** NPY immunoreactivity in phaeochromocyte. **b** NPY mRNA-cDNA hybridisation sites in serial sections of same phaeochromocyte. Scale bar = 25 μm

Fig. 3. Electron micrograph of human phaeochromocytoma exhibiting the typical, highly granulated, appearance of the majority of the tumour cells (*g*). Localised arrays of rough endoplasmic reticulum are visible (*arrowed*) in an adjacent cell which is poorly granulated. The poorly granulated cells compose a minor tumour cell population. Glutaraldehyde-fixed, osmicated, Araldite-embedded surgical specimen; silver-grey ultrathin section counterstained with uranyl acetate and lead citrate. Scale bar = 1 μm

mas (Fig. 2). Serial sections of the same cells have been shown to contain immunoreactive NPY. It is clear that some cells exhibit NPY immunoreactivity but no apparent NPY mRNA, whereas other cells contain hybridisable components although immunoreactive NPY could not be demonstrated on serial sections. Discrete, punctate, NPY-mRNA-cDNA hybridisation sites were observed which correlates well with the poorly ordered, disaggregated arrays of endoplasmic reticulum observed at the electron microscopical level (Fig. 3).

The controls applied for all methods were upheld.

The complete procedure from fixation to final result takes 3–4 days.

Discussion

One significant limitation of immunohisto(cyto)chemistry in the study of regulatory peptides is the fact that only a static representation of the immunoreactive component(s) of interest, stored at the time of cell death, is obtainable. Correlation of light and electron immunocytochemical results with biochemical analysis may give some indication of cell activity by comparison of immunoreactive cell number and intensity, secretory granule number and maturity (if assessment can be made), mitochondrial, Golgi and endoplasmic reticulum volume with extractable or circulating peptide levels. Furthermore, the use of a panel of region-specific antisera to fragments of the peptide precursor may also give some indication of the dynamic state of the cell. However, the results thus obtained are still circumstantial when interpreted to reflect the biosynthetic activity of the cell.

We present here a technique for the investigation of peptide gene expression which is based on the in situ hybridisation of tissue-bound mRNA to a novel biotinylated single-stranded cDNA probe. The procedure has advantages

over established autoradiographic techniques, particularly in that less time is required to obtain a result. The single-stranded probe was generated from the $m13^+$ strand utilising a primer which initiates 5′ to the NPY-DNA sequence and a limiting concentration of nucleotides. Under these conditions the biotinylated UTP will be incorporated into double-stranded DNA in the m13 part of the probes while sequences complementary to the NPY-mRNA will remain single-stranded (Fig. 1). The present results support these predictions and indicate that double-stranded DNA of approximately 1000–3000 base pairs was obtained. Probes prepared by this method have several advantages over nick-translated probes prepared using biotinyl UTP:
1. the probes prepared are single-stranded and will not re-anneal; 2. the biotinylated nucleotide is not incorporated into the DNA sequence which must base pair to the mRNA; and 3. hybridisation conditions do not require elevated temperatures or dissociation of double-stranded DNA prior to use.

Hybridisation sites were identified using two different methods for the localisation of biotin. Both the avidin-biotin-peroxidase complex and streptavidin-gold – silver enhancement procedures are rapid and easy to use and provide a permanent chromogenic result.

In order to evaluate this new procedure we selected pre-pro-NPY gene expression in human phaeochromocytomas. These tumours are known to produce considerable quantities of NPY (Adrian et al. 1983), a 36 amino acid peptide (Tatemoto 1982) which is widely distributed throughout the central (Allen et al. 1983), and peripheral (Lundberg et al. 1982; Adrian et al. 1983; Varndell et al. 1984) nervous systems often in association with noradrenalin-containing elements. In accord with the majority of, if not all, regulatory peptides, NPY is enzymatically cleaved post-translationally from a larger (precursor) molecular form (pre-pro-NPY). We have been able to localise pre-pro-NPY mRNA in tumour cells of human phaeochromocytomas using a single-stranded cDNA probe. Adjacent cryostat sections immunostained for NPY revealed that not all the cells contained both NPY and its messenger RNA. The variable results may be explained by differential state and rates of synthesis. Obviously, other factors such as NPY cDNA cross-reaction with other, similar to but non-NPY, mRNA sequences cannot be absolutely discounted nor, indeed, non-specific adherence to other structures even with the controls applied. At present work is progressing to perform hybridisation histochemistry at the electron microscopical level which would serve to resolve the latter interpretative problem.

In conclusion, we present here a novel probe and improved procedure for in situ hybridisation on formaldehyde-fixed tissue sections. The rapidity of use, when compared to autoradiographic procedures and sensitivity of the technique are real advantages in the morphological study of gene expression and peptide biosynthesis.

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