Human and viral gene detection in routine paraffin embedded tissue by in situ hybridisation with biotinylated probes: viral localisation in herpes encephalitis

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SUMMARY A simple reproducible protocol for detecting multiple copy human genes and viral DNA in routine formalin fixed paraffin embedded tonsil and brain, by in situ hybridisation with biotinylated probes, is described. The protocol consists of digestion of formalin fixed paraffin sections, with 0.4% pepsin in 0.01 M hydrochloric acid for one hour at 37°C, followed by hybridisation with biotinylated probes. The biotinylated probes used for establishing the conditions for in situ localisation of DNA were total placental DNA (TG1), pHY 2.1 (a Y chromosome probe), and herpes simplex virus I and II. In human male tonsil TG1 labelled all nuclei and pHY 2.1 reacted only with nuclear Y bodies. In herpes encephalitis the virus was detected in some glial cells and neurones.

Biotinylated nucleic acid probes hybridised in situ to Carnoy or methanol and acetic acid fixed cryostat or cytogenetic preparations can be visualised rapidly by immunoperoxidase histochemistry, in which the peroxidase reaction product is amplified by gold and silver precipitation. A protocol that extends this system to routine formalin fixed paraffin embedded material would be of value, because the large stocks of archival material filed in pathology departments would be available for retrospective studies.

Angerer and Angerer2 localised poly (A)4 RNA in glutaraldehyde fixed paraffin embedded sea urchin embryos by in situ hybridisation with radiolabelled probes. Viral DNA was visualised in formalin fixed paraffin embedded tissues by biotin3 and radiolabelled probes.4 These methods, however, are not readily applicable in routine clinical laboratories, because the temperature of fixation must be rigidly controlled2 and the prehybridisation protocols entail several reactions.3 4

In this paper we describe a simple and reproducible protocol for detecting repetitive mammalian genes and viral DNA sequences in human tissues routinely fixed in formalin and embedded in paraffin wax. This was compared with other methods for multiple copy gene detection.

Accepted for publication 8 May 1986

Material and methods

PREPARATION OF TISSUE SECTIONS

Optimal conditions for DNA detection by in situ hybridisation with biotinylated DNA were established on male tonsil. Fresh, postoperative, male tonsillar tissue was divided into five pieces. One piece was frozen immediately in liquid nitrogen and stored at −70°C. 5 µm cryostat sections were mounted on multipost slides (CH Henley, Essex, United Kingdom), precoated with 0.1% aqueous poly-l-lysine hydrobromide, molecular weight = > 300 k (Sigma, United Kingdom), containing 0.1% Tween 20 (v/v) (BDH, United Kingdom). Sections were air dried and fixed sequentially in Carnoy’s fluid (ethanol: chloroform: acetic acid = 6:3:1, v/v) at 22°C for 10 minutes, washed in ethanol at 22°C for 10 minutes, air dried, wrapped, and stored in aluminium foil at −70°C. A second piece was fixed in Carnoy’s fluid at 22°C for four hours, processed to and embedded in paraffin wax, and 5 µm sections cut and mounted as described above; these were dried at 37°C for three hours, baked at 60°C for 24 to 48 hours, and stored at 22°C for two to 24 weeks. The three other pieces of tonsil were fixed in 4% formaldehyde containing 0.15 M sodium chloride, pH 7-4 (formalin) for 24, 48, and 72 hours, respectively, at 22°C. After processing to and embedding in paraffin wax sections were cut at 5 µm,
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mounted, dried, baked, and stored as for paraffin sections fixed in Carnoy's fluid.

Temporal and frontal lobe brain biopsy specimens from two cases of herpes encephalitis were fixed in formalin for 24 hours and embedded in paraffin. Herpes simplex viral (HSV) antigen was shown by immunohistochemistry with a monoclonal antibody to herpes simplex.

Cryostat sections stored at −70°C were air dried at 22°C, treated with 1% H₂O₂ in methanol (v/v) for 30 minutes at 22°C to block endogenous peroxidase activity, rinsed in ethanol (two × five minutes), and air dried before in situ hybridisation. Paraffin sections were dewaxed in xylene (two × 10 minutes), rinsed in 99% ethanol (two × 10 minutes), and endogenous peroxidase activity blocked (see above); these sections were washed in tap water (10–15 minutes), and rinsed in distilled water (five to 10 minutes) before proteolytic digestion and in situ hybridisation.

PROTOCOLS FOR IN SITU PRE-HYBRIDISATION

Three protocols for in situ prehybridisation for paraffin sections were used for comparison with the method developed in this laboratory. Sections were treated with: I and 10 fg/ml proteinase K (Sigma, United Kingdom) in 100 mmol/l Tris-hydrochloric acid, 50 mmol/l edetic acid buffer (pH 8.0) at 37°C for 30 minutes, with or without acetylation; sequential treatment of sections with 0.02 M hydrochloric acid, 0.01% Triton X-100 (v/v), and 2.0 mg/ml “pronase” (Protease, type VII, Sigma, United Kingdom) in 50 mmol/l Tris-hydrochloric acid (pH 7.4) for five minutes at 22°C; sequential treatment was with 0.2 M hydrochloric acid, triethanolamine, 2 × SSC (1 × SSC = 0.15 mol/l sodium chloride, 0.015 mol/l sodium citrate) at 70°C, 0.05% digitonin (w/v), 5 fg/ml proteinase K (Sigma, United Kingdom). After these procedures sections were rinsed in distilled water (two × five minutes), dehydrated in absolute ethanol, and air dried before in situ hybridisation.

The method of Angerer and Angerer was applied to sections of male tonsil fixed in Carnoy's fluid for four hours and embedded in paraffin and to sections of paraffin embedded male tonsil that had been fixed in formalin for 24 hours. The prehybridisation in situ efficiency of the two other protocols was tested only on sections of male tonsil fixed in formalin for 24 hours and embedded in paraffin.

The method developed in this laboratory for formalin fixed paraffin embedded tissue was as follows: sections from male tonsil fixed in formalin for 24, 48, and 72 hours and embedded in paraffin were used to establish the optimal conditions for pepsin-hydrochloric acid unmasking of DNA. Pepsin (3200–3800 units/μg protein; Sigma, United Kingdom) was used at concentrations ranging from 0.01 to 16.0 mg/ml dissolved in hydrochloric acid; the hydrochloric acid molarity tested ranged from 0.01 to 0.2 M. Digestion of sections was performed at 37°C for one hour. Thereafter, sections were washed in distilled water (three × five minutes), rinsed (two × five minutes) in 99% ethanol, absolute ethanol (two × five minutes), and air dried before in situ hybridisation. Undigested and sections treated with hydrochloric acid only were also studied.

Having established optimal conditions for unmasking DNA prehybridisation, in situ treatment of brain sections consisted only of digestion with pepsin (4 mg/ml) in 0.01 M hydrochloric acid for one hour at 37°C.

BIOTINYLLATION OF PROBES

Total human placental DNA (TG1) (Chan VT-W, Fleming KA, McGee J'O'D, unpublished observations) pH 2–I (a Y chromosome probe), and a β-globin probe were labelled with biotin-11-dUTP by nick translation using Enzo (New York, United States of America) and Amersham (United Kingdom) nick translation kits. Labelled probes were purified by ethanol precipitation. The degree of dUTP biotin substitution (for thymidyl residues) for each probe varied from 30–40%. The average size of each biotinylated probe ranged from 150–250 bases determined by Southern blot analysis on glyoxal gels.

Labelled DNA (20 fg/ml) in 1 mmol/l edetic acid, 5 mmol/l Tris-hydrochloric acid (pH 7.3) containing 400 μg/ml of sheared herring sperm DNA (type XIV, Sigma, United Kingdom) was stored at −70°C. A biotinylated HSV probe containing HSV I and II fragments (12 μg/ml) was obtained from Enzo (New York, United States of America) and stored at 4°C.

IN SITU HYBRIDISATION

The procedure used was essentially that of Burns et al., with minor modifications. In brief, 10 μl of hybridisation buffer (50% formamide (Sigma), 5% dextran sulphate (BDH, United Kingdom), 2 × SSC (pH 7.4) containing 20 ng of biotinylated DNA, 400 ng of sheared herring sperm DNA, 0.1 mmol/l edetic acid, 0.5 mmol/l Tris-hydrochloric acid (pH 7.3) was added to multispot wells and covered with glass coverslips 14 mm in diameter. The modifications to the original procedure comprised a 10-fold increase in probe concentration, a reduction in dextran sulphate concentration, and the addition of carrier DNA to the hybridisation mix. Tissue sections and probes were denatured simultaneously in a hot air oven for 10 minutes at 73°C in sealed Terasaki plates containing about 1 ml of deionised water. The sections were hybridised for 16 hours at 42°C in a hot air incubator. Slides were washed (two × 10 minutes) at 22°C in 2 × SSC and immersed for 15 minutes in...
5% bovine serum albumin (v/v), 0.1% Triton X-100 (v/v) 0.1 M phosphate buffered 0.15 M sodium chloride, pH 7.4, (PBT). The biotinylated DNA was detected by indirect immunocytochemistry. Sections were treated with rabbit antibiotin IgG (Enzo, United States of America), diluted 1/200 in PBT, in a moist chamber at 37°C for one hour, washed in PBT for 15 minutes at 22°C, and incubated at 37°C for one hour in peroxidase labelled swine antirabbit IgG (Dako-patts, Denmark), diluted 1/50 in PBT. Sections were washed twice in 0.1 M phosphate buffered 0.15 M sodium chloride (PBS) containing 0.1% Tween 20 for 15 minutes, rinsed in PBS, and reacted for four minutes with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB, Polysciences, New York) in PBS containing 0.012% (v/v) hydrogen peroxide, and washed in distilled water. Sections were sequentially incubated and washed at 22°C in 2.5 mmol/l aqueous sodium chloroaurate (BDH), pH 2.3, for five minutes, water for five minutes, and 0.1 mol/l aqueous sodium sulphide (BDH), pH 7.5, for five minutes and washed in water for five minutes. Silver was precipitated on DAB complexes by incubating the slides at 22°C for one to five minutes in silver reagent, as described previously; all of the reagents for silver amplification were obtained from BDH (United Kingdom).

After gold and silver amplification the slides were washed in 1.0% (v/v) aqueous acetic acid (two × 10 minutes), washed in water for 30 minutes at 22°C, and counterstained by haematoxylin and eosin, dehydrated, cleared in xylol, and mounted in DPX.

Results

In cryostat sections of male tonsil fixed in Carnoy’s fluid biotinylated TG1 and pHY 2-1 were readily visualised. The reaction product for TG1 was spread across each nucleus whereas, as previously shown, pHY 2-1 was confined to nuclear Y bodies (figs 1a and b). Both probes were also visualised in paraffin sections fixed in Carnoy’s fluid. With pHY 2-1, however, the sections required pretreatment with proteinase K to obtain a result similar in intensity to that seen in cryostat sections; TG1 detection did not require proteolysis before in situ hybridisation (fig 2).

Three published methods for visualising nucleic acids in aldehyde fixed paraffin sections by in situ hybridisation were tested. TG1 and pHY 2-1 were not detectable in sections pretreated by two of these methods. TG1, however, was visualised by the method of Blum et al., but pHY 2-1 was not detected (fig 3); the intensity of the reaction with TG1 was weak by
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Fig 2 Carnoy fixed paraffin embedded male tonsil probed with TG1 without prehybridisation proteolysis. This result is similar to that seen in fig 1(a).

Fig 3 Formalin fixed paraffin embedded male tonsil probed with TG1 after prehybridisation protocol. By comparison with intensity of figs 1(a) and 2, reaction is greatly diminished.

Fig 4 Formalin fixed paraffin embedded male tonsil digested with pepsin (4 mg/ml) in 0.01 M hydrochloric acid and probed with TG1. Staining intensity is better than that shown in fig 1(a).

Fig 5 As for fig 4, but probed with biotinylated pH Y 2-1. Y bodies are evident as block spots in most cells. Intensity of reaction product is diminished by comparison with that of fig 1(b).
comparison with that obtained in paraffin sections fixed in Carnoy's fluid (figs 2 and 3).

The pepsin-hydrochloric acid prehybridisation in situ schedule applied to sections of male tonsil formalin fixed for 24, 48, or 72 hours and paraffin embedded produced nuclear staining with TG1 at all concentrations of pepsin-hydrochloric acid tested. The strongest reaction for both TG1 and pHY 2.1 was obtained with 4 mg/ml pepsin in 0.2 M hydrochloric acid, but unlike TG1 preparations, there was considerable loss of stroma with concomitant loss of histological detail with pHY 2.1 (not shown). A compromise between acceptable histological detail and signal:noise ratio for TG1 and pHY 2.1 was found with a concentration of 4 mg/ml pepsin in 0.01 M hydrochloric acid (figs 4–7). The staining result for pHY 2.1 was mildly diminished in intensity in sections of tonsil fixed in formalin for 72 hours by comparison with that fixed for 24–48 hours (not shown). No signal was detected with biotinylated TG1 or pHY 2.1 in formalin fixed paraffin sections not treated with pepsin-hydrochloric acid, or with hydrochloric acid only.

After treatment with pepsin-hydrochloric acid biotinylated HSV probe bound to nuclei of some glial cells and neurones in the two cases of herpes encephalitis fixed in formalin for 24, 48, or 72 hours, pepsin-hydrochloric acid digested, and probed with HSV 1 and II. Viral inclusions are evident in neurone (large arrow) and in glial cells (small arrow). Cell indicated by two arrowheads contains endogenous pigment, which is present in unprobed sections.

Fig 6 As for fig 4, except that tonsillar epithelium is illustrated. TG1 labels all nuclei in pepsin-hydrochloric acid digested section.

Fig 7 As for fig 6, except that tissue was probed with biotinylated pHY 2.1. Y bodies are evident.

Fig 8 Brain from herpes encephalitis fixed in formalin for 24 hours, pepsin-hydrochloric acid digested, and probed with HSV 1 and II. Viral inclusions are evident in neurone (large arrow) and in glial cells (small arrow). Cell indicated by two arrowheads contains endogenous pigment, which is present in unprobed sections.
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from that previously described. Variations in stringency washes after hybridisation were not investigated in these experiments.

**Discussion**

In situ hybridisation brings together single stranded DNA (denatured DNA) or mRNA with a labelled complementary nucleic acid sequence (probe) to form an intracellular hybrid. The probe, and hence the sequence of interest, is shown by autoradiography, if radiolabelled,\textsuperscript{10,11} or alternatively, with immunocytochemistry, if biotin or N-acetoxy-2-acetyl aminofluorene (AAF) labelled.\textsuperscript{3,12-14} Because of the intrinsic disadvantages of autoradiography, time consumption, radioactivity, background noise, poor microscopic resolution, and incompatibility with many staining methods, alternative means of labelling and rapid detection of nucleic acid probes are necessary if in situ hybridisation is to become available for routine diagnostic use. Of the alternative non-radiolabelled in situ hybridisation systems available, the biotin system introduced by Ward's group\textsuperscript{3} has been used for detecting mammalian or viral nucleic

litis (fig 8). This reaction was not abolished by ribonuclease before in situ hybridisation, and no signal was seen when the tissue DNA was not denatured. These data indicate that the nuclear reaction is due to viral DNA rather than viral mRNA. HSV antigen was present in similar cells (fig 9). In contrast, TG1 reacted with all glial and neuronal nuclei (fig 10).

No reaction was obtained with biotinylated β-globin (as a control) before or after any of the prehybridisation in situ protocols tested on interphase cells. This probe, as expected, labelled the short arm of chromosome 11 in metaphase spreads (personal observation). Since the β-globin probe contained as much biotin as TG1 and pHY 2-1 this indicates the specificity of the reactions observed with TG1 and pHY 2-1.

The table summarises a semiquantitative assessment of in situ hybridisation efficiency with the probes, tissues, fixatives, and prehybridisation in situ schedules. The published prehybridisation schedules\textsuperscript{3,4} were used without modification, except that "pronase" in our experiments differed in origin

![Fig 9](image1.png)

**Fig 9** Similar field to that shown in fig 9. HSV antigen is shown in nuclei and cytoplasm of two glial cells (arrows) by indirect immunoperoxidase histochemistry with monoclonal antibody to HSV.

![Fig 10](image2.png)

**Fig 10** Field similar to that shown in fig 8, except that this was probed with TG1. Compared with those in fig 8 all nuclei are labelled indicating specificity of reaction in fig 8.
Table

Comparison of prehybridisation in situ protocols on Carnoy or formalin paraffin processed material

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Male tonsil</th>
<th>Brain biopsy specimens</th>
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<tr>
<td></td>
<td>Carnoy's fluid</td>
<td>Formalin</td>
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<tr>
<td>Fixative</td>
<td>Paraffin</td>
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<tr>
<td>Sections</td>
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<td></td>
<td>Method(^1)</td>
<td>Method(^4)</td>
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<tr>
<td>Probe:</td>
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<tr>
<td>TG1</td>
<td>++ + +</td>
<td>Negative</td>
</tr>
<tr>
<td>pH2 2:1</td>
<td>+ + + +</td>
<td>Negative</td>
</tr>
<tr>
<td>(\beta)-globin(^†)</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>HSV I + II</td>
<td>ND</td>
<td>ND</td>
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\(+ + + + = very intense staining; + + + = intense staining; + + = moderate staining; ND = not done.\)

\(^\ast\)Pepsin (4 mg/ml) in 0.01 M hydrochloric acid for one hour at 37°C; \(^\dagger\)\(\beta\)-globin probe does not detect the corresponding gene in interphase cells but does detect \(\beta\)-globin on chromosome 11 in metaphase spreads.\(^24\)

The two copies of this gene in interphase cells cannot be visualised by this procedure. None of the less, TG1 and pH2 2:1 were useful for detecting very high copy number genes in this study; there are about 2000 copies of the gene detected by pH2 2:1 in male cells.

These probes are useful for studying reagent variables for in situ hybridisation efficiency. For example, neither probe was effective on undigested formalin-paraffin processed material or after treatment with hydrochloric acid by itself. The demonstration of both probes in paraffin sections fixed in Carnoy's fluid with or without mild proteinase K digestion was excluded, at least for TG1 and pH2 2:1, any deleterious effect of dehydration, and clearing and embedding reagents on the accessibility to hybridisation of these biotinylated probes.

The finding that HSV DNA was detectable after treatment with pepsin-hydrochloric acid of brain biopsy specimens will facilitate the investigation of the role of viruses in neurological disease and other diseases in which viruses have been implicated, such as cervical cancer. Sequences similar to those of HSV have been reported in mammalian cells.\(^26\)

The choice of tonsil and brain tissue sections as model tissues for study was useful from a purely technical point of view. Both tissues contain very little supportive connective tissue stroma, which may facilitate retention of sections to slides during enzyme digestion, denaturing, and hybridisation treatments. No loss of sections was noted, however, when using the recommended pepsin-hydrochloric acid protocol detailed in this study.

The rationale behind treatment with pepsin-hydrochloric acid is hypothetical. Treatment of cells with acid removes histones\(^27\) and may facilitate denaturation of DNA. Pepsin does not remove histones.
but digests nuclear tryptophan rich acidic proteins present on chromosomes. The combination of these two independent activities of hydrochloric acid and pepsin may explain their combined efficiency in rendering DNA in routine formalin-paraffin sections available for in situ hybridisation.

**Addendum**

Brigati *et al.* have since published a modification of their original method for viral localisation in paraffin sections.

Miss S Taylor gave technical assistance. Miss L Watts typed the manuscript. The work was supported in part by grants from the Cancer Research Campaign (JO'DMG), and DMR was supported by an ICI funded Royal College of Pathologists Studentship.

**References**


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