

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.¹ 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)⁺ 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.

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 multispot 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,

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 immunohistochemistry⁵ with a monoclonal antibody to herpes simplex.

Cryostat sections stored at -70°C were air dried at 22°C , treated with 1% H_2O_2 in methanol (v/v) for 30 minutes at 22°C to block endogenous peroxidase activity, rinsed in ethanol (two \times five minutes), and air dried before *in situ* hybridisation. Paraffin sections were dewaxed in xylene (two \times 10 minutes), rinsed in 99% ethanol (two \times 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: 1 and 10 $\mu\text{g}/\text{ml}$ 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 acetylation²; 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 ³; sequential treatment was with 0.2 M hydrochloric acid, triethanolamine, 2 \times SSC (1 \times SSC = 0.15 mol/l sodium chloride, 0.015 mol/l sodium citrate) at 70°C , 0.05% digitonin (w/v), 5 $\mu\text{g}/\text{ml}$ proteinase K (Sigma, United Kingdom).⁴ After these procedures sections were rinsed in distilled water (two \times five minutes), dehydrated in absolute ethanol, and air dried before *in situ* hybridisation. The method of Angerer and Angerer² 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^{3,4} 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 \times five minutes), rinsed (two \times five minutes) in 99% ethanol, absolute ethanol (two \times 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 .

BIOTINYLATION OF PROBES

Total human placental DNA (TG1) (Chan VT-W, Fleming KA, McGee J'OD, unpublished observations) pHY 2.1 (a Y chromosome probe),⁶ and a β -globin probe⁷ were labelled with biotin-11-dUTP by nick translation using Enzo (New York, United States of America) and Amersham (United Kingdom) nick translation kits.⁸ 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.⁹ Labelled DNA (20 $\mu\text{g}/\text{ml}$) in 1 mmol/l edetic acid, 5 mmol/l Tris-hydrochloric acid (pH 7.3) containing 400 $\mu\text{g}/\text{ml}$ 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 $\mu\text{g}/\text{ml}$) 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.*,¹ with minor modifications. In brief, 10 μl of hybridisation buffer (50% formamide (Sigma), 5% dextran sulphate (BDH, United Kingdom), 2 \times 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 procedure¹ 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 \times 10 minutes) at 22°C in 2 \times SSC and immersed for 15 minutes in

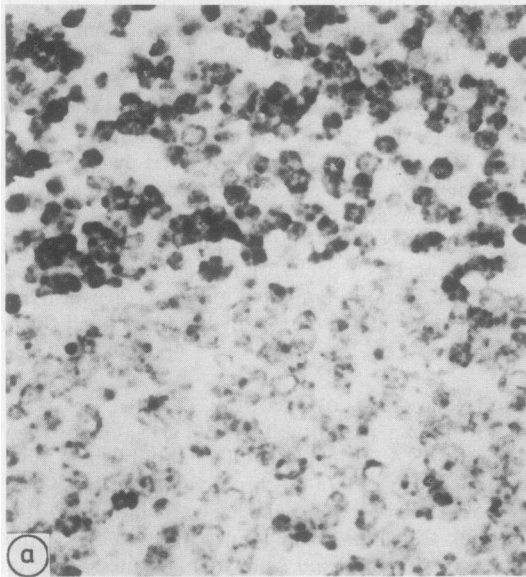


Fig 1(a) All preparations illustrated were weakly counterstained with haematoxylin and eosin. Frozen section of male tonsil fixed in Carnoy's solution and hybridised with biotinylated total human genomic DNA (TG1). As expected, virtually all nuclei in germinal centre and mantle zone are labelled.

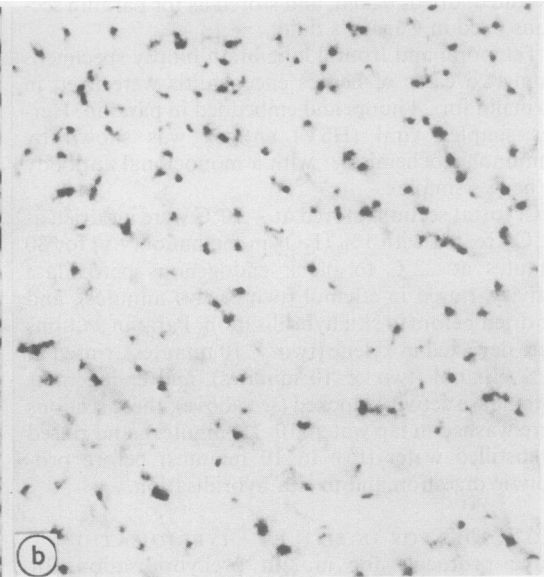


Fig 1(b) Similar area probed with biotinylated pHY 2.1. Almost all nuclei contain Y body stained black.

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 anti-biotin 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 anti-rabbit 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 previously¹; 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,¹ 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 K² 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 methods²⁻⁴ 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.^{2,3} TG1, however, was visualised by the method of Blum *et al.*,⁴ but pHY 2.1 was not detected (fig 3); the intensity of the reaction with TG1 was weak by

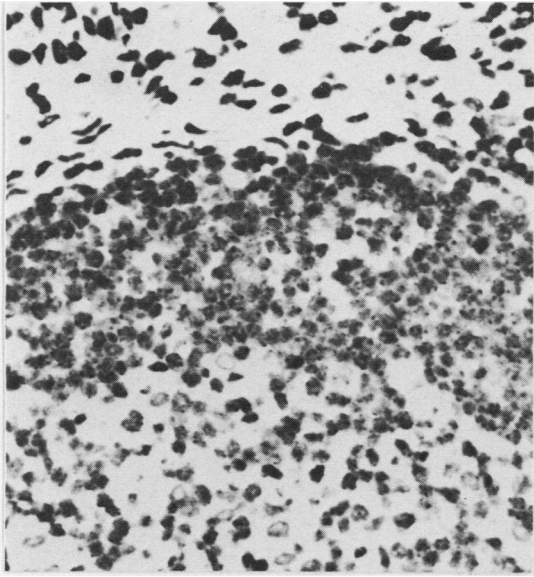


Fig 2 Carnoy fixed paraffin embedded male tonsil probed with TG1 without prehybridisation proteolysis. This result is similar to that seen in fig 1(a).

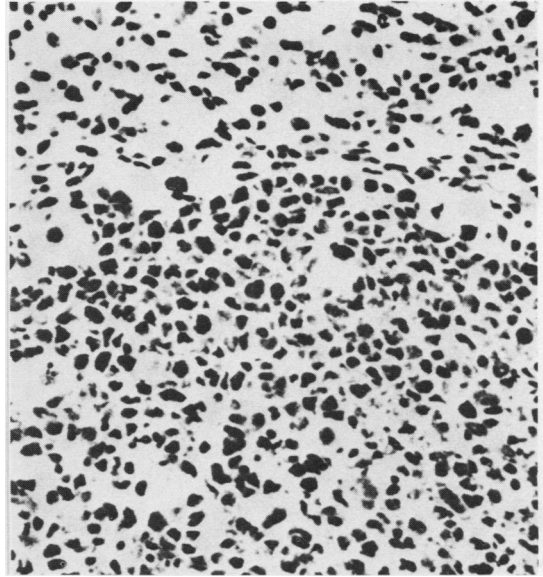


Fig 4 Formalin fixed paraffin embedded male tonsil digested with pepsin (4 mg/ml) in 0.01 M hydrochloric acid and probed with TG1. Staining intensity is better than that shown in fig 1(a).

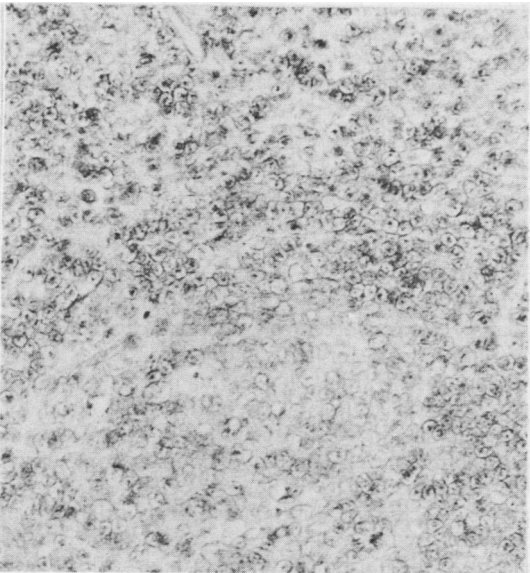


Fig 3 Formalin fixed paraffin embedded male tonsil probed with TG1 after prehybridisation protocol.⁴ By comparison with intensity of figs 1(a) and 2, reaction is greatly diminished.

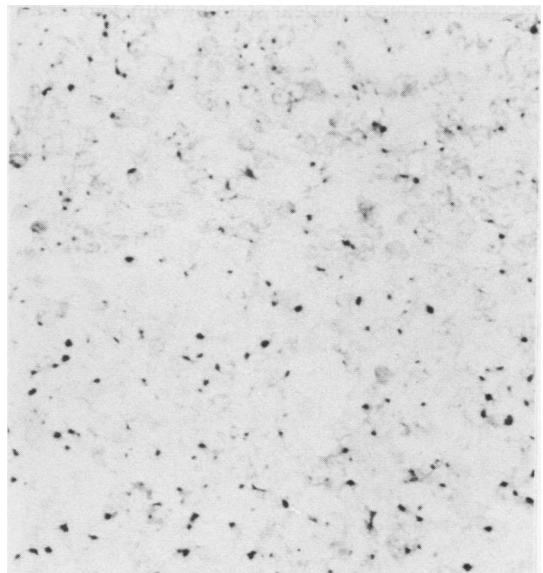


Fig 5 As for fig 4, but probed with biotinylated pHY 2-1. Y bodies are evident as block spots in most cells. Intensity of reaction product is diminished by comparison with that of fig 1(b).

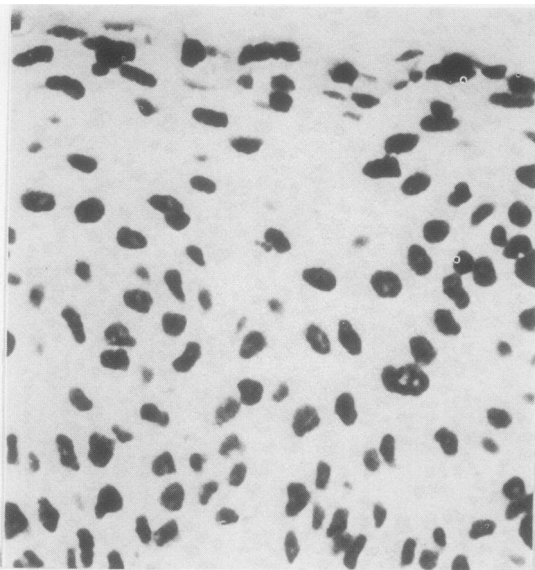


Fig 6 As for fig 4, except that tonsillar epithelium is illustrated. TG1 labels all nuclei in pepsin-hydrochloric acid digested section.

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

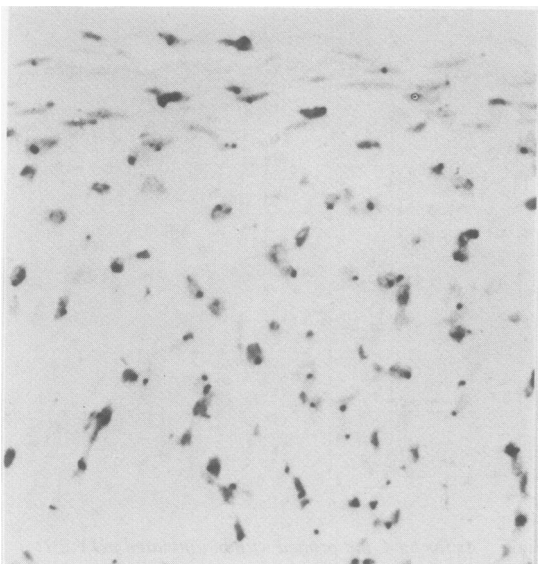


Fig 7 As for fig 6, except that tissue was probed with biotinylated pHY 2.1. Y bodies are evident.



Fig 8 Brain from herpes encephalitis fixed in formalin for 24 hours, pepsin-hydrochloric acid digested, and probed with HSV I 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.

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.

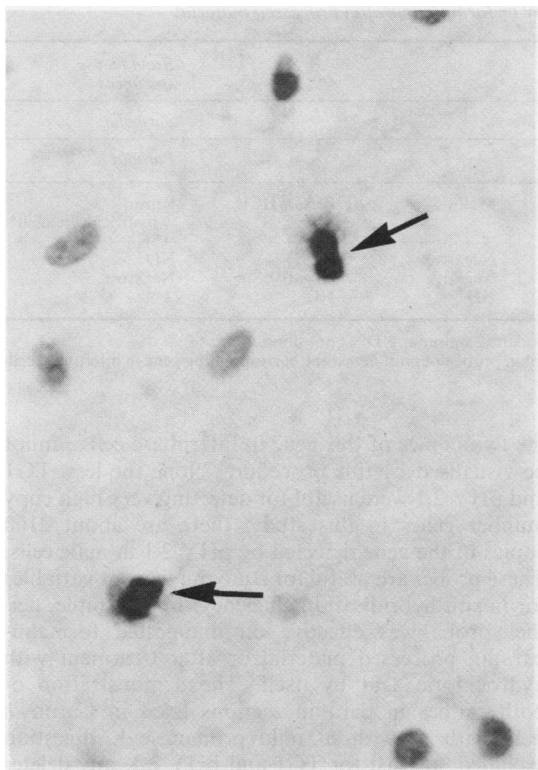


Fig 9 Similar field to that shown in fig 9. HSV antigen is shown in nuclei and cytoplasm of two glial cells (arrows) by indirect immunoperoxidase histochemistry with monoclonal antibody to HSV.

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^{3,4} were used without modification, except that "pronase" in our experiments differed in origin

from that previously described.³ 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,^{10,11} or alternatively, with immunocytochemistry, if biotin or N-acetoxy-2-acetylaminofluorene (AAF) labelled.^{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 group³ has been used for detecting mammalian or viral nucleic

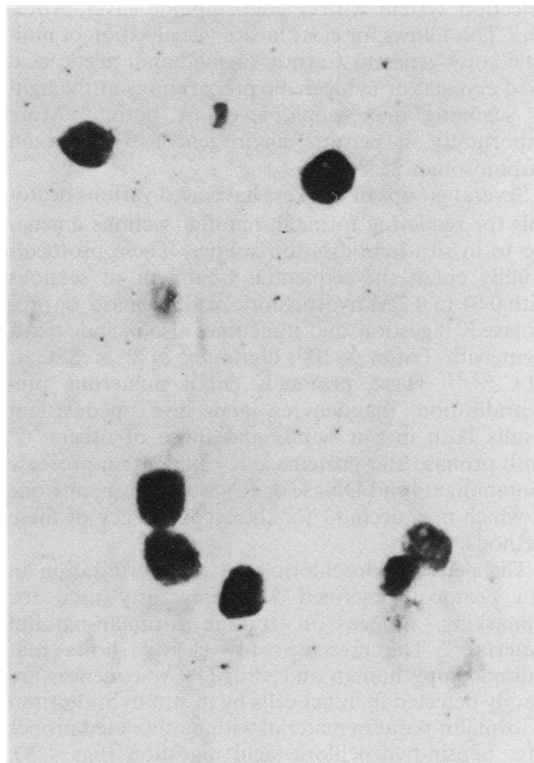


Fig 10 Field similar to that shown in fig 8, except that this was probed with TG1. Compared with those in fig 8 all nuclei are labelled indicating specificity of reaction in fig 8.

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

Tissue	Male tonsil			Brain biopsy specimens		
	Carnoy's fluid		Formalin	Formalin		
Fixative	Cryostat	Paraffin	Paraffin	Paraffin		
Sections	Cryostat	Paraffin	Paraffin	Paraffin		
Probe:			Method ³	Method ⁴	Pepsin/HCl*	Pepsin-hydrochloric acid*
TG1	++++	++++	Negative	++ to +++	++++	++++
pHY 2-1	++++	+++	Negative	Negative	++++	ND
β -globin†	Negative	Negative	Negative	Negative	Negative	Negative
HSV I + II	ND	ND	ND	ND	ND	++++

++++ = very intense staining; +++ = intense staining; ++ = moderate staining; ND = not done.

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

acid sequences in cytogenic, cryostat, or formalin fixed paraffin embedded material.¹⁵⁻²¹ The initial detection systems for biotinylated probes, however, did not give the sensitivity of radiolabelled probes¹² on filters, but an appreciable increase in sensitivity has been reported.⁹ This problem has also been overcome for *in situ* hybridisation in cells and tissue sections by amplifying the end product of the biotin detection system with a gold-sulphide silver protocol.¹ This allows for clear *in situ* visualisation of multiple copy genes in Carnoy or methanol acetic acid fixed cryostat or cytogenetic preparations at the light or scanning microscopic level, or both.¹⁹ More importantly, it permits single gene assignment on chromosomes.^{22 23}

Several groups of workers have used various protocols for rendering formalin-paraffin sections amenable to *in situ* hybridisation studies. These protocols usually entail the sequential treatment of sections with 0.01 to 0.2 M hydrochloric acid, pronase, or proteinase K digestion, and sometimes also include treatment with Triton X-100, digitonin, or $2 \times$ SSC at 70°C.^{3 4 22} These protocols entail numerous prehybridisation manoeuvres and give inconsistent results both in our hands and those of others.^{3 24} Both pronase and proteinase K can contain protease contaminants and DNase or RNase, or both, any one of which may account for the inconsistency of these methods.

The pepsin-hydrochloric acid prehybridisation *in situ* protocol described was previously used for unmasking antigens in routine formalin-paraffin material.²⁶ The present study clearly shows that multiple copy human and viral DNA sequences are readily detected in intact cells by *in situ* hybridisation of formalin-paraffin material with biotinylated probes after pepsin-hydrochloric acid digestion (figs 4-9). The exact level of sensitivity—that is, gene copy number detectable—remains to be determined. From the experiments with β -globin, however, it is evident that

the two copies of this gene in interphase cells cannot be visualised by this procedure. None the less, TG1 and pHY 2-1 were useful for detecting very high copy number genes in this study; there are about 2100 copies of the gene detected by pHY 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 excluded, at least for TG1 and pHY 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 This, however, does not explain the present results, because only selected cells hybridise with the HSV probe, and this has a distribution similar to HSV antigen, as shown here and elsewhere.⁵

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 histones²⁷ and may facilitate denaturation of DNA. Pepsin does not remove histones

but digests nuclear tryptophan rich acidic proteins present on chromosomes.²⁸ 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 modification²⁹ of their original method³ for viral localisation in paraffin sections.

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References

- Burns J, Chan VT-W, Jonasson JA, Fleming KA, Taylor S, McGee JO'D. Sensitive system for visualising biotinylated DNA probes hybridised in situ: rapid sex determination of intact cells. *J Clin Pathol* 1985;**38**:1085-92.
- Angerer LM, Angerer RC. Detection of poly A⁺ RNA in sea urchin eggs and embryos by quantitative in situ hybridisation. *Nucleic Acids Res* 1981;**9**:2819-40.
- Brigati DJ, Myerson D, Leary JJ, *et al*. Detection of viral genomes in cultured cells and paraffin embedded tissue sections using biotin-labelled hybridisation probes. *Virology* 1983;**126**:32-50.
- Blum HE, Haase AT, Vyas GN. Molecular pathogenesis of hepatitis B virus infection: simultaneous detection of viral DNA and antigens in paraffin embedded liver sections. *Lancet* 1984;**ii**:771-5.
- Esiri MM. Herpes simplex encephalitis. *J Neurol Sci* 1982;**54**:209-26.
- Cooke HJ, Schmidtke J, Gosden JR. Characterisation of a human Y chromosome repeated sequence and related sequences in higher primates. *Chromosoma* 1982;**87**:491-502.
- Kaufman RE, Kretschmer PJ, Adams JW, Coon HC, Anderson WF, Nienhuis AW. Cloning and characterisation of DNA sequences surrounding the human gamma-, delta- and beta-globin genes. *Proc Natl Acad Sci USA* 1980;**77**:4229-33.
- Ferguson DJP, Harrison D, Burns J, Jonasson JA, McGee JO'D. Chromosomal localisation of genes by scanning electron microscopy using in situ hybridisation with biotinylated probes: Y chromosome repetitive sequences. *Histochem J* 1986;**18**:266-70.
- Chan VT-W, Fleming KA, McGee JO'D. Detection of sub-picogram quantities of specific DNA sequences on blot hybridisation with biotinylated probes. *Nucleic Acids Res* 1985;**13**:8083-91.
- Coghlan JP, Aldred P, Haralambidis J, Niall HD, Penschow JD, Tregear GW. Review. Hybridisation histochemistry. *Analyt Biochem* 1985;**149**:1-28.
- Pardue ML. In: Hames BD, Higgins SJ, eds. *In situ hybridisation, nucleic acid hybridisation. A practical approach*. Vol 1. Oxford: IRL Press, 1985:179-202.
- Langer PR, Waldrop AA, Ward DC. Enzymatic synthesis of biotin-labelled polynucleotides: novel nucleic acid affinity probes. *Proc Natl Acad Sci USA* 1981;**78**:6633-7.
- Landegent JE, Jansen in de Wal N, van Ommen G-JB, *et al*. Chromosomal localisation of a unique gene by non-autoradiographic in situ hybridisation. *Nature* 1985;**317**:175-7.
- Van der Ploeg M, van Duijn P, Bauman JGJ, Landegent JE, Raap AK. Hybridocytochemistry as a tool for the investigation of chromatin organisation. *Basic Appl Histochem* 1985;**29**:181-9.
- Royston ME, Augenlicht LH. Biotinylated probe containing a long terminal repeat hybridised to a mouse colon tumour and normal tissue. *Science* 1983;**222**:1339-41.
- Albertson DG. Mapping muscle protein genes by in situ hybridisation using biotin-labelled probes. *EMBO J* 1985;**4**:2493-8.
- Beckmann AM, Myerson D, Daling JR, Kiviat NB, Fenoglio CM, McDougall JK. Detection and localisation of human papillomavirus DNA in human genital condylomas by in situ hybridisation with biotinylated probes. *J Med Virol* 1985;**16**:265-73.
- Forghani B, Dupuis KW, Schmidt NJ. Rapid detection of herpes simplex virus DNA in human brain tissue by in situ hybridisation. *J Clin Microbiol* 1985;**22**:656-8.
- Lau Y-F. Detection of Y-specific repeat sequences in normal and variant human chromosomes using in situ hybridisation with biotinylated probes. *Cytogenet Cell Genet* 1985;**39**:184-7.
- Negro F, Berninger M, Chiaberge E, *et al*. Detection of HBV-DNA by in situ hybridisation using a biotin-labelled probe. *J Med Virol* 1985;**15**:373-82.
- Myerson D, Hackman RC, Nelson JA, Ward DC, McDougall JK. Widespread presence of histologically occult cytomegalovirus. *Hum Pathol* 1984;**15**:430-9.
- Nicholls RD, Jonasson JA, McGee JO'D, *et al*. High resolution gene mapping of the human α -globin locus *J Med Genet* (in press).
- McGee JO'D, Jonasson J, Burns J, Chan VT-W. β -globin and c-Ha-ras-1 genes are located on p15.5 and p14/15 of chromosome II, respectively, in normal subjects. *J Pathol* 1986;**148**:63A.
- McAllister HA, Rock DL. Comparative usefulness of tissue fixatives for in situ viral nucleic acid hybridisation. *J Histochem Cytochem* 1985;**33**:378-83.
- Burns J. The unlabelled antibody peroxidase-anti-peroxidase method (PAP). In: Bullock GR, Petrusz P, eds. *Techniques in immunocytochemistry*. Vol 1. London: Academic Press, 1982:91-105.
- Jones TR, Parks CL, Spector DJ, Hyman RW. Hybridisation of herpes simplex virus DNA and human ribosomal DNA and RNA. *Virology* 1985;**144**:384-97.
- Gall JG, Pardue ML. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc Natl Acad Sci USA* 1969;**63**:378-83.
- Pearse AG. *Histochemistry, theoretical and applied*. Vol 1. 4th ed. London: Churchill Livingstone, 1980:289-91.
- Unger ER, Budgeon LR, Myerson D, Brigati DJ. Viral diagnosis by in situ hybridisation. Description of a rapid simplified colorimetric method. *Am J Surg Pathol* 1986;**10**:1-8.

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