

Technical reports

Detection of immunoglobulin light chain mRNA by in situ hybridisation using biotinylated tyramine signal amplification

G King, G Chambers, G I Murray

Abstract

A highly sensitive method for the light microscopic *in situ* hybridisation of immunoglobulin light chain mRNA in formalin fixed, paraffin wax embedded sections is reported. This method is based on signal amplification using horseradish peroxidase catalysed deposition of biotinylated tyramine at the sites of hybridisation. κ and λ light chain immunoglobulin mRNA in situ hybridisation was performed with fluorescein isothiocyanate conjugated oligonucleotide probe cocktails. The hybridisation signal was detected using a biotinylated tyramine signal amplification procedure with streptavidin-biotin-horseradish peroxidase complex as the final layer. Peroxidase was demonstrated using 3,3'-diaminobenzidine. The biotinylated tyramine signal amplification method resulted in the sensitive detection of immunoglobulin light chain mRNA, with the whole procedure being completed in one day. Moreover, the use of peroxidase as the final reporter molecule also allowed haemamatoxylin to be used as counterstain, thereby permitting the evaluation of cellular morphology.

Keywords: biotinylated tyramine; catalysed signal deposition; in situ hybridisation; light chain immunoglobulin mRNA; lymphoma

The detection of immunoglobulin light chain restriction in B cell lymphomas is an integral part of their histopathological examination.¹ Current practice involves the detection of light chain immunoglobulin by immunohistochemistry; however, interpretation of immunoglobulin light chain immunohistochemistry can be difficult because of the large amount of immunoglobulin present in serum, which results in background immunostaining. In principle, the detection of immunoglobulin light chain mRNA should overcome such problems. Although there have been some reports of in situ hybridisation in the detection of immunoglobulin light chain mRNA, most of those studies have concentrated on the detection of immunoglobulin light chain mRNA in plasma cells,^{2,3} which have abundant

mRNA. It is more difficult to detect the lower amounts of light chain mRNA found in other types of B lymphoid cells with conventional methods, and long incubations in substrate solution are often required to identify the hybridisation signal. The prolonged incubation, if performed overnight, can be difficult to monitor and might also result in high background staining.

To identify small amounts of mRNA more quickly by in situ hybridisation methods improved sensitivity of detection is required. The recent development of reporter catalysed methods using biotinylated tyramine potentially provides the necessary sensitivity.⁴ There have been several reports of DNA-DNA in situ hybridisation using this detection procedure,⁵⁻⁷ and the application of biotinylated tyramine signal amplification has been shown to detect both human⁸ and viral⁹ single copy genes. However, there have been few reports of this technique being applied to the detection of mRNA by in situ hybridisation.⁸ We have shown recently that the application of biotinylated tyramine signal amplification in immunohistochemistry provides enhanced sensitivity,¹⁰ and in this report we describe the application of the biotinylated tyramine signal amplification method as a sensitive system for the detection of immunoglobulin light chain mRNA by in situ hybridisation.

Materials and methods

TISSUE

The tissue samples used in our study were of normal tonsil submitted to the Department of Pathology, University of Aberdeen for diagnostic purposes. All specimens were divided into slices no thicker than 3 mm and fixed in 10% neutral buffered formalin at room temperature for 24 hours. The tissue blocks were then routinely processed to paraffin wax. Sections of 4 μ m thickness were cut, mounted on to 3-aminopropyltriethoxysilane (Sigma, Poole, Dorset, UK) coated slides and dried at 60°C for 30 minutes.

PROBES

Oligonucleotide probes for κ and λ immunoglobulin light chain mRNA were obtained from Dako (High Wycombe, Buckinghamshire, UK). These probes are supplied in hybridisation

Department of Pathology, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, Scotland, UK
G King
G Chambers
G I Murray

Correspondence to:
Dr Murray.

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buffer as a mixture consisting of either 13 oligonucleotides (κ) or 14 oligonucleotides (λ), and were supplied labelled with fluorescein isothiocyanate (FITC).

IN SITU HYBRIDISATION

Sections were dewaxed, washed in ethanol, and endogenous peroxidase was blocked, using a solution of 0.3% (vol/vol) hydrogen peroxide in methanol. Unmasking of target mRNA was performed by proteolytic enzyme digestion using proteinase K (Boehringer Mannheim, Lewes, East Sussex, UK) at a concentration of 10 $\mu\text{g/ml}$, 15 $\mu\text{g/ml}$, or 20 $\mu\text{g/ml}$ for up to 30 minutes at 37°C. Sections were then washed in diethyl pyrocarbonate (DEPC; Sigma) treated water, rinsed in 95% ethanol, and air dried. The sections were then hybridised with the probe for 120 minutes at 37°C in hybridisation buffer using a Hybaid Omnislide thermal cycler (Hybaid Ltd, Teddington, Greater London, UK). After hybridisation, the sections were washed in Tris buffered saline (TBS) containing 0.05% Triton X-100 for three periods of three minutes. Detection of the hybridised probes was then performed.

A single step method for the detection of hybrids was performed with a rabbit anti-FITC antibody conjugated with alkaline phosphatase (1/200; Novocastra, Newcastle-upon-Tyne, UK), which was applied for 30 minutes. After removing unbound antibody by washing with TBS, alkaline phosphatase was demonstrated using bromochloroindolyl phosphate (BCIP) and nitroblue tetrazolium (NBT). Sections were incubated for up to 16 hours at room temperature. The reaction was stopped by washing in cold tap water, sections were optionally counterstained with haematoxylin, and then mounted in glycerin jelly. Omission of the κ or λ oligonucleotide probes and replacement of the oligonucleotide probes by FITC conjugated random oligonucleotides (Dako) acted as negative controls.

BIOTINYLATED TYRAMINE METHOD

When the biotinylated tyramine signal amplification method was used, endogenous biotin was blocked following the proteinase K digestion step using a biotin blocking kit (Vector Laboratories, Peterborough, Cambridgeshire, UK). This involved sequential 15 minute applications of avidin D and biotin blocking solution with a rinse in RNase free water between applications, after which the sections were rinsed in 95% ethanol, air dried, and the probe applied.

After hybridisation and post hybridisation washes, as described above, biotinylated rabbit anti-FITC (1/1000; Molecular Probes Europe, Leiden, The Netherlands) was applied for 30 minutes. The sections were then washed in TNT buffer (0.1 M Tris/HCl, pH 7.6, 0.15 M NaCl, 0.05% Tween 20) for three periods of five minutes. The streptavidin–biotin–horseradish peroxidase complex (sABC/HRP; Dako), used at the dilution recommended by the manufacturer and diluted with TNT buffer, was then applied for 30 minutes. After three successive five minute washes with TNT, biotinylated

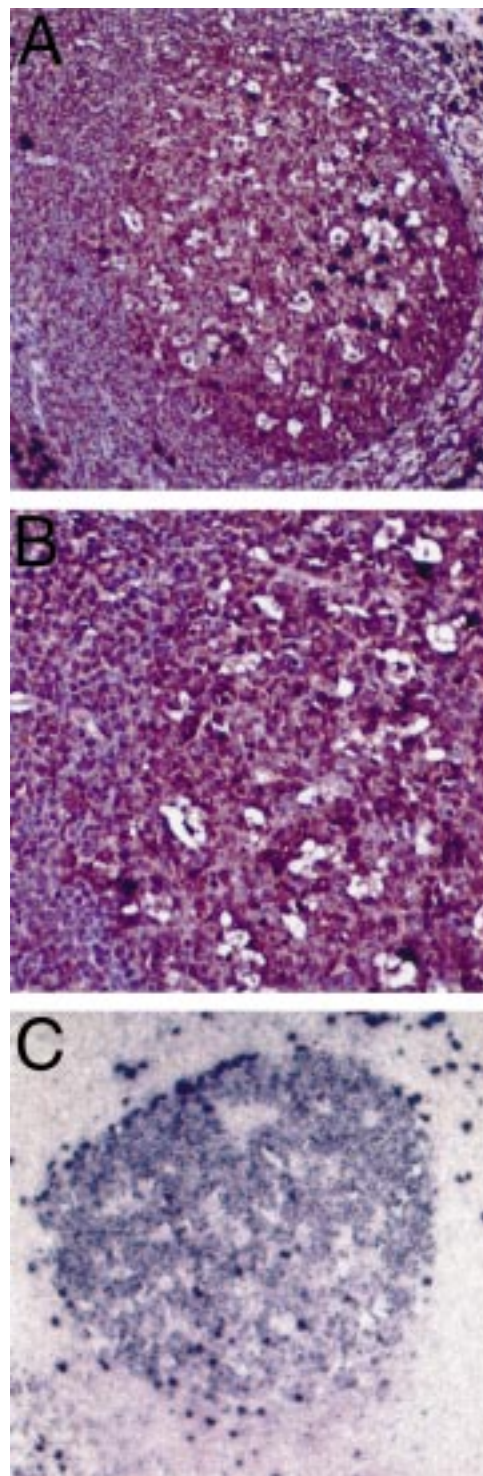


Figure 1 Detection of κ immunoglobulin light chain mRNA using (A and B) biotinylated tyramine detection method and (C) one step alkaline phosphatase conjugated antibody. In (A) and (B), horseradish peroxidase was the reporter molecule and the section was counterstained with haematoxylin. Follicle centre cells stain strongly (A) and mantle zone lymphocytes stain less intensely (B). In (C) alkaline phosphatase was the reporter molecule with BCIP/NBT as the enzyme substrate and the section was not counterstained with haematoxylin.

tyramine (NEN Life Science Products, Hounslow, Greater London, UK) was applied for 10 minutes. A working solution of biotinylated tyramine was prepared by diluting the stock solution 1/160 in 0.05 M Tris/HCl, pH 7.6, and

adding H₂O₂ at a concentration of 1/1000 (vol/vol) as described previously.¹⁰ Three successive five minute washes with TNT were followed by a 30 minute incubation in sABC/HRP. Sections were then incubated for 10 minutes at room temperature in 3,3'-diaminobenzidine to visualise the HRP. The reaction product was intensified by incubation with 0.5% copper sulphate. The sections were then washed in cold tap water, counterstained with haematoxylin, dehydrated in alcohol, cleared in xylene, and mounted in DPX.

In one experiment, HRP was demonstrated following the initial application of sABC/HRP and before incubation in the biotinylated tyramine, while in another experiment two or three cycles of application of sABC/HRP and biotinylated tyramine were performed before demonstration of HRP.

Results

The different detection methods were assessed using normal tonsil cells to determine which cell types showed positive staining, the intensity of staining, and the presence or absence of any background staining. A hybridisation signal in plasma cells was obtained with the single step method after 60 minutes, whereas a signal in follicle centre cells and mantle zone cells was obtained with the single step detection method only after overnight incubation in substrate solution. By contrast, a hybridisation signal in follicle centre cells and mantle zone cells was obtained with the biotinylated tyramine method after 10–15 minutes incubation in substrate solution (fig 1). This also produced very strong staining of plasma cells. Repeated application of biotinylated tyramine and sABC/HRP resulted in a further increase in intensity of the hybridisation signal. No background staining was seen with any of the methods investigated. Only plasma cell staining was seen when peroxidase was visualised without previous tyramine signal amplification. Cellular morphology was much easier to evaluate using the biotinylated tyramine method in which the nuclei were counterstained with haematoxylin and the sections mounted in a synthetic mounting medium.

Discussion

Plasma cells contain abundant light chain immunoglobulin mRNA and a variety of conventional in situ hybridisation procedures, often using alkaline phosphatase as the reporter molecule, have been described for the consistent, reliable, and rapid detection of mRNA in these cells.^{2,3} These methods have also been used to detect the lower amounts of immunoglobulin light chain mRNA present in follicle centre cells and mantle zone lymphocytes but this has required a prolonged incubation in substrate solution. Moreover, because alkaline phosphatase has usually been the reporter molecule, with BCIP and NBT as the enzyme substrate, this has prevented the use of haematoxylin counterstaining. In our study, we have developed a method for the detection of light chain immunoglobulin mRNA using biotinylated tyramine signal amplification and we

have taken advantage of the enhanced sensitivity of this method to use HRP as the reporter molecule.

Amplification procedures based on the deposition of biotinylated tyramine are the most sensitive detection technique currently available for both in situ hybridisation⁸ and immunohistochemistry,¹⁰ and they can achieve sufficient sensitivity to detect single copy genes.⁸ The enhanced sensitivity of the biotinylated tyramine amplification method is a result of the HRP catalysed deposition of biotin at sites of peroxidase activity.⁴ The HRP catalyses the formation of reactive biotinylated tyramine, which binds to protein molecules at the sites of HRP activity.⁴ The phenol moiety of the biotinylated tyramine reacts with peroxidase in the presence of hydrogen peroxide and forms an electrophilic reactive group with a short half life, which binds covalently to proteins at electron rich amino acid residues, such as tyrosine and tryptophan, present on the antibody complex or in adjacent tissue. This results in the accumulation of a further layer of biotin at the reaction site, which can be targeted with enzyme or fluorescent labelled streptavidin. This provides both a highly sensitive and specific method for signal amplification. Because endogenous peroxidase has been blocked previously and HRP is only present at sites of hybridisation, biotin is deposited only at the sites of hybridisation.

The detection of immunoglobulin mRNA by means of the signal amplification method was more rapid and could be completed in one day even though it involved several additional antibody incubations compared with the single step method. The biotinylated tyramine method produced an equally intense signal and appeared to be of at least equivalent sensitivity. The repeated application of biotinylated tyramine and sABC/HRP increased the speed of visualisation of the hybridisation signal. A further advantage of this particular signal amplification technique was that it allowed HRP to be used as the reporter molecule and thus enabled the slides to be counterstained with haematoxylin and mounted with a synthetic and permanent mounting medium. This ensured easy evaluation of cellular morphology and provided sections of similar appearance to those produced by immunohistochemical procedures used routinely in diagnostic histopathology.

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Origin of spurious multiple bands in the amplification of microsatellite sequences

D Bovo, M Rugge, Y-H Shiao

Abstract

Multiple band artifacts are seen commonly in the analyses of short repetitive sequences, also known as microsatellites, using the polymerase chain reaction (PCR). In this study, the conditions of PCR were examined for five microsatellite loci (D2S119, D2S123, D5S409, D11S904, and interferon α) in an attempt to eliminate this artifact. In addition, and a possible mechanism for the formation of the multiple band artifact in non-denaturing polyacrylamide gel electrophoresis was also explored. The intensity of multiple bands increased when the numbers of PCR cycles were increased. The multiple bands were abolished simply by reducing PCR cycle numbers and were reproduced from single specific PCR products undergoing alternate denaturation and reassociation without primer extension. This finding suggests that formation of multiple bands in non-denaturing gel electrophoresis is a result of improper annealing of PCR fragments, rather than being the result of polymerase slippage and 3' non-template extension, as has been reported previously.

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Keywords: microsatellite; polymerase chain reaction artifact, multiple bands

Microsatellites are short, tandemly repeated DNA sequences that are present throughout mammalian genomes. The tandem repeats of (dC-dA) n are particularly abundant.¹ Alterations in the numbers of repeats at each site, known as microsatellite instability, have been implicated in various human diseases, including neoplasms.² Screening for microsatellite instability in different stages of tumorigenesis could contribute to the understanding of the progression of neoplasms. However, reported

polymerase chain reaction (PCR) screening methods often encounter the artifact of multiple bands.³⁻⁴ Artifactual fragments shorter than specific PCR products are detected commonly in denaturing gel electrophoresis and larger fragments are observed frequently in non-denaturing conditions. The presence of these extra non-specific bands makes interpretation of microsatellite genotypes difficult.

Polymerase slippage and 3' non-template extension during PCR amplification have been suggested as possible mechanisms for this artifact.³⁻⁵ However, elimination of the multiple bands by increased stringency of denaturing gel electrophoresis has been reported in the analysis of single stranded products, suggesting that secondary DNA structures might also contribute to formation of the artifact.⁶

The mechanism of the formation of PCR fragments larger than specific products in non-denaturing gel electrophoresis has not been investigated. In our study, PCR conditions were examined to eliminate the multiple band artifact.

Methods

Primers for the amplification of D2S123, D5S409, D11S904, and interferon α (IFN- α) were obtained from the Website: "http://www.gdb.org". For the amplification of D2S119, an upper primer (5'-CCAGTTTGGAAGCATTTTCA-3') and a lower primer (5'-CCCTCAATTTCTTTGGATT-3') were used. An aliquot of 20 μ l genomic DNA from formalin fixed, paraffin wax embedded gastric tissues was used in a final 50 μ l PCR mixture containing 10 mM Tris base (pH 8.3), 50 mM KCl 1.5 mM MgCl₂, 125 μ M deoxyribonucleoside triphosphates, 0.2 μ M primers, and 1.25 U Ampli-Taq polymerase (Cetus Perkin Elmer, Norwalk, Connecticut, USA). PCR was carried out in the Perkin-Elmer 480 automated thermal cycler (Cetus Perkin Elmer). Thermal cycling was performed as follows: denaturation

Department of Pathology, University of Padova, I-35121, Padova, Italy
D Bovo

Department of Pathology, University of Padova, I-35121, Padova, Italy
M Rugge

Laboratory of Comparative Carcinogenesis, Building 538, Room 205, National Cancer Institute, Frederick Cancer Research and Development Center, National Institutes of Health, Frederick, MD 21702, USA
Y-H Shiao

Correspondence to:
Dr Shiao.
email: Shiao@ncifcrf.gov

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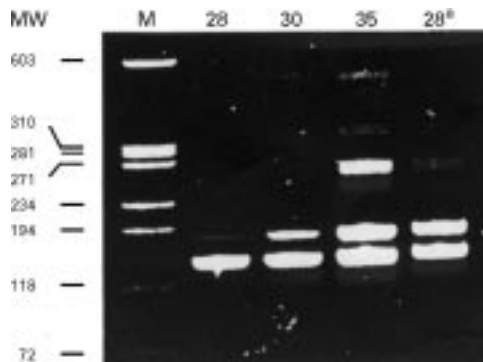


Figure 1 PCR products (144 bp, homozygosity) from 28, 30, or 35 cycles of amplification. M, ϕ x174/HaeIII molecular weight marker; 28, 28 cycle PCR products followed by removal of primers and then seven additional PCR cycles. MW, molecular weight.

at 94°C for five minutes in the first cycle followed by 30 seconds annealing at 60°C, one minute extension at 72°C, and 30 seconds at 94°C for a total of 25–35 cycles of PCR. The extension step of the final cycle was increased to five minutes to ensure complete extension. The PCR products were resolved by a 10% non-denaturing Tris borate EDTA (TBE) polyacrylamide gel, 8 × 10 cm² (Novex, San Diego, California, USA) and were visualised by ethidium bromide staining. The gel was then photographed with the IS-1000 imaging system (Alpha Innotech Corp, San Leandro, California, USA).

Results and Discussion

The PCR products obtained from 28, 30, and 35 cycle amplification of D5S409 dinucleotide microsatellite sequences from the same DNA template are shown in fig 1. The intensity of non-specific multiple bands increased when the numbers of PCR cycles were increased. Our finding confirms a previous report that the numbers of amplification cycles need to be optimised for each microsatellite investigated.⁷ In a separate experiment, 5% or 10% dimethylsulphoxide were included in the PCR and the annealing temperature was raised in an attempt to reduce the non-specific products. However, none of these changes were able to abolish the multiple band artifact effectively. Failure to abolish the multiple bands by increasing PCR stringency in our study suggests that the artifact does not originate from non-specific amplification. The artifact is also not a result of 3' non-template extension, because the sizes of the multiple bands are much higher than a one base overhang. We hypothesise that the formation of multiple bands is a result of the secondary structure of the PCR fragments.

A proportion of the PCR products from the 28 cycle amplification was purified by a spin column (Microcon 30; Amicon Inc, Beverly, Massachusetts, USA). After purification and drying, the PCR products were resuspended with a PCR reaction mixture lacking primers and DNA polymerase, and returned to the same thermocycle programme for an addi-

tional seven cycles. The products were then electrophoresed side by side with 28, 30, and 35 cycle PCR products (fig 1). The additional seven cycles, without primer extension, transformed 28 cycle PCR products free of multiple bands into a multiple band containing sample (28*) and the pattern was comparable with the 30 and 35 cycle samples. Similar findings were also obtained consistently for other microsatellite loci.

Our findings support the hypothesis that the artifact of multiple bands in the amplification of microsatellites is a secondary phenomenon resulting from improper reannealing. Detection of the artifact after a high number of PCR cycles and in samples given thermal cycling without polymerisation suggests that multiple bands might result from unextended PCR fragments that undergo denaturation and reassociation only after PCR efficiency reaches the amplification plateau. It has been shown that reassociation of repetitive sequences is much faster than that for non-repetitive sequences.⁸ Under such circumstances, misalignment of the double stranded DNA by one and/or more units of repeat might occur during reannealing, because regions containing repetitive sequences reassociate far more quickly than the flanking non-repetitive sequences. The random slippage induced misalignment might appear as high molecular weight products in non-denaturing gel electrophoresis because of overhanging sequences at the 5' and 3' ends of the PCR fragments. In addition to misalignment of PCR fragments, other secondary structures, such as single stranded, hairpin, and mismatched DNA, are also possible.

In our study, we have shown that secondary structures contribute to the formation of multiple bands in the amplification of microsatellites by extensive PCR cycling. Elimination of this artifact in non-denaturing gel electrophoresis, by minimising the generation of unextended PCR fragments when amplification efficiency reaches a plateau, provides a less ambiguous picture for correct microsatellite genotyping.

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