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Improvement of Non-radioactive In Situ Hybridization in Human Airway Tissues: Use of PCR-generated Templates for Synthesis of Probes and an Antibody Sandwich Technique for Detection of Hybridization

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SUMMARY We describe the use of non-traditional methods of probe synthesis and quantification and detection of hybridization that appreciably improved non-radioactive in situ hybridization (ISH) in human airway tissue. To avoid the problems of bacterial cloning, plasmid digestion, and probe hydrolysis, we synthesised complementary RNA probes (riboprobes) for ISH from PCR-generated DNA. DNA template was produced by nested PCR incorporation of T7 and SP6 RNA polymerase promoters. We then compared the efficiency of in vitro transcription from PCR-generated template with traditional plasmid template by quantifying the relative probe fluorescence in denaturing gels. Transcription with SP6 or T7 polymerase in either orientation produced TNF riboprobes from a single PCR-generated template more efficiently than from plasmid, providing there were no primer hairpin loops. Fluorescence quantification enabled equal amounts of probe label to be used in ISH, eliminating signals from the sense probe and demonstrating that probes transcribed from PCR templates were as sensitive as hydrolyzed probe transcribed from plasmid. Detection of ISH by a conventional anti-hapten, alkaline phosphatase-based technique was found to cause tissue damage due to extended substrate incubation at high pH. We therefore developed a four-layer, avidin–biotin–peroxidase technique that afforded greater sensitivity, allowing brief substrate incubation and resulting in structural preservation of tissue.

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KEY WORDS

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AIRWAY ENDOBRONCHIAL BIOPSIES (EBBx) and bronchoalveolar lavage (BAL) obtained at bronchoscopy have proved very valuable in enabling researchers to gain insights into the pathophysiology of asthma (Walters et al. 1996). We are interested in analyzing cytokine gene expression in EBBx and BAL samples in longitudinal studies investigating the effects of various medications in the treatment of asthma, using the complementary techniques of in situ hybridization (ISH), competitive RT-PCR, and immunohistochemistry (IHC). ISH is a technique that allows the detection

of specific nucleic acid sequences within the intact tissue and cellular architecture. It has the advantage of identifying the gene products from de novo synthesis rather than protein uptake or receptor-bound protein, which may result in false-positive immunostaining results. Conversely, because proteins may be rapidly secreted or destroyed, this may lead to false-negative immunostaining results.

Complementary RNA probes or riboprobes are the probes of choice for ISH (Cox et al. 1984) because they are sensitive and specific and there is no competition between hybridization and probe re-annealing, as with double-stranded probes (Melton et al. 1984). They can be efficiently transcribed from cDNA clones containing T7, SP6, or T3 RNA polymerase promoters (Green et al. 1983), but this depends on the avail-

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ability of the cDNA of interest and cloning it into a suitable vector. Clones must also be linearized by enzyme restriction at opposite ends of the cDNA to allow transcription of sense and antisense probes from the appropriate promoter. We have found this to be problematic because the choice of restriction sites is limited and complete linearization was not consistently achievable, leading to inclusion of undesirable vector sequences in the probe. Furthermore, hydrolysis of the probe is often required to reduce it to a size that is capable of penetrating into tissue and hybridizing with the target (Angerer and Angerer 1992; Wilkinson 1998). Notably, some plasmid-generated probes have also been observed to hybridize nonspecifically due to the presence of sequences similar to human 28S rRNA in the plasmid multiple cloning site (Witkiewicz et al. 1993; Baumgart et al. 1997). We wished to avoid these problems by using an alternative means of riboprobe production.

One technique that circumvents these problems is the generation of DNA template for *in vitro* transcription using simple PCR technology (Higuchi et al. 1988; Weier and Rosette 1988). Young et al. (1991) first produced a DNA fragment with both SP6 and T7 RNA polymerase promoters, enabling production of sense and antisense transcripts from a single template. This technique obviated the need for cloning, screening, and large-scale plasmid culture and the difficulties associated with plasmid restriction digestion. Probe hydrolysis to facilitate ISH is also not required because the PCR-generated template can be designed to yield probe of optimal length. The distinct advantages of this technique are that the probe produced has a user-defined length and sequence without extraneous vector sequences.

In this study we wished to determine whether we could synthesize sensitive non-radioactive riboprobes for detection of TNF mRNA as efficiently from PCR-generated DNA as from plasmid DNA and so avoid the problems associated with plasmid clones. We therefore compared the transcription efficiency and ISH sensitivity of fluorescein-labeled riboprobes produced from "nested" PCR-generated template with plasmid-generated probes. In addition, we encountered problems with the non-radioactive detection of hybrids by conventional anti-hapten antibody (Ab) coupled to alkaline phosphatase (AP) (Herrington et al. 1991; McNicol and Farquharson 1997; Wilkinson 1998; Kadkol et al. 1999). Although anti-hapten AP detection has been the method of choice in many studies (Baumgart et al. 1997; Lazarov et al. 1998; Kitazawa et al. 1999; Hauptmann 2001), we found that the extended incubations at high pH required for the enzymic modification of substrate caused degradation of delicate airway biopsy tissue with concomitant loss of signal resolution. In an effort to preserve tissue morphology,

we have attempted to increase the sensitivity of detection and thus reduce the time required for color development by expanding the detection protocol to a four-layer Ab sandwich technique.

Materials and Methods

Collection of Human Airway Tissues

This study was performed as part of a large study investigating the effects of doubling inhaled corticosteroid medication in asthma vs the addition of a long-acting β_2 -agonist (Li et al. 1999). The study was approved by the Alfred Hospital Ethics Committee and all subjects gave informed written consent. EBBx were obtained at fiberoptic bronchoscopy (Rennard et al. 1998; Robinson et al. 1998) from the segmental subcarinae, fixed in 10% neutral buffered formalin (NBF) for 2 hr, then processed for embedding in paraffin. Nasal polyps were obtained at routine polypectomy from the Alfred Hospital Ear, Nose and Throat clinical service, resected into 0.5-cm² pieces, fixed in NBF for 4 hr, then processed into paraffin.

Synthesis of DNA Template by PCR

THP-1 monocyte cells (ATCC TIB 202; Tsuchiya et al. 1980) at 1.5×10^6 cells/ml were stimulated with 10 μ g/ml lipopolysaccharide (*E. coli*, serotype 026:B6; Sigma, Sydney, Australia) for 4 hr at 37C in 5% CO₂. Cells were harvested, washed, and total RNA extracted with RNazol B solution (Tel-Test; Friendswood, TX) according to the manufacturer's instructions. Primers (Table 1; GeneWorks, Adelaide, South Australia) were designed using the human TNF mRNA sequence (GenBank accession number M10988; Wang et al. 1985) and Oligo Primer Analysis Software, version 5.0 (National Biosciences; Plymouth, MN). cDNA synthesis and PCR were carried out using the GeneAmp RNA PCR Kit (Applied Biosystems; Melbourne, Australia) in a PTC-200 DNA Engine (MJ Research; Watertown, MA). cDNA was synthesized according to the manufacturer's protocol with 0.32 μ M external TNF antisense primer and 1 μ g THP-1 total RNA in a volume of 20 μ l. cDNA synthesis was commenced at 13C, increased to 15C for 1 min, then increased in increments of 0.1C/2 sec to 42C and held at 42C for 1 hr. The enzymes were then denatured at 85C for 5 min and the reactions cooled to 4C.

PCR was then carried out using a nested approach to ensure specificity. The sense external primer was designed to span an intron-exon boundary to discriminate between amplification products from cDNA and contaminating genomic DNA. PCR was first conducted in 50- μ l reactions using 0.2 μ M external primers, 1.5 mM MgCl₂, 1 \times PCR Buffer II, 200 μ M each deoxyribonucleotide triphosphate, 2 μ l TNF-specific cDNA, and 0.02 U/ μ l AmpliTaq Gold DNA polymerase (Applied Biosystems). The thermal profile was as follows: 94C initial denaturation for 8 min, followed by 94C for 1 min, 58C for 1 min, and 72C for 1 min for 35 cycles, then 72C for 7 min, followed by a 4C hold. The product of 325 base pairs (bp) was then diluted 1:100 and 2 μ l re-amplified in 100- μ l reactions using a nested set of primers. These primers (nested set 1 or 2) were designed to hybridize to sequences internal to the outer set of primers and include SP6 or T7

Table 1 Primer sets used to generate DNA template by PCR for *in vitro* transcription of TNF riboprobes^a

Primer pair	Sense primer	Antisense primer	Product size (bp)	Probe size (b)
TNF external primers	CAG AGG GAA GAG TTC CCC AG	CCT TGG TCT GGT AGG AGA CG	325	—
Nested set 1	T7 GTG TAA TAC GAC TCA CTA TAG—TCT TCT CGA ACC CCG AGT GAC AA	SP6 TGG ATT TAG GTG ACA CTA TAG [A]GG AGA CCG CGA TGC GGC TGA TG	292	250
Nested set 2	SP6 CGA TTT AGG TGA CAC TAT AGA—GCC CAG GCA GTC AGA TCA TCT TCT C	T7 AAT TAA TAC GAC TCA CTA TAG GG—CGG CTG ATG GTG TGG GTG AG	298	254

^aExternal primers were used for the initial amplification, then nested PCR was carried out with the internal primers, nested set 1 or 2, which incorporated SP6 and T7 RNA polymerase promoters. Promoter sequences are underlined with 5' leader sequences and 3' sequences determined from pGEM plasmids (Promega). TNF primer sequences are separated from the promoter sequences by a dash and brackets indicate a shared base between the promoter and the primer sequences. The respective PCR products and probes are indicated in base pairs and bases.

RNA polymerase promoters at the 5'-end. Conditions for nested PCR were as for external PCR, except that annealing and extension steps were combined into one step of 74C for 2 min for 40 cycles. The products were 292 and 298 bp from nested set 1 and 2, respectively, so designed to yield probes of 250 and 254 bases (b) respectively, which are optimal for ISH (Angerer and Angerer 1992). Specificity of the PCR-generated templates was confirmed by DNA sequencing (Baker Institute of Medical Research; Melbourne Australia).

In Vitro Transcription

Nested PCR product was purified away from primers and nucleotides using the Qiaquick PCR Purification Kit (Qiagen; Melbourne, Australia) and concentrated by vacuum desiccation. Transcription of fluorescein-labeled riboprobes was carried out using the RNA color kit (Amersham; Sydney, Australia).

Control Riboprobes

To assess the efficiency of transcription from PCR-generated template, plasmid-generated probes were produced for comparison. Human TNF cDNA of 800 bp (Genentech, San Francisco, CA; Pennica et al. 1984) was subcloned from pSP64 into pGEM-3z (Promega; Sydney, Australia) to allow synthesis of sense and antisense riboprobes. A subclone of 246 bp (EcoRI/AccI digest) was also made. A 280-bp chicken lysozyme control clone was also provided in the RNA color kit.

Fluorometric Analysis of Riboprobes in Agarose Gels

Probes were resolved on 4.5% denaturing formaldehyde agarose gels (DNA Grade Agarose; Progen Industries, Brisbane, Australia) according to Sambrook et al. (1989), with some modifications. One- μ l probe samples were denatured for 15 min at 65C with 1 μ l 10 \times MOPS buffer, 3.5 μ l 37% formaldehyde, and 10 μ l deionized formamide in a total volume of 20 μ l. Samples were then snap-cooled and 1 μ l RNA dye added before loading into the gel. Probes were analyzed by a FluorImager 575 (Molecular Dynamics; Sunnyvale, CA) using laser-based fluorometric scanning. Analysis of the relative band intensities was carried out using ImageQuaNT software (Molecular Dynamics) by selecting the band area, then calculating the volume by integration relative to background. Data were expressed as relative probe intensities.

In Situ Hybridization

ISH for TNF mRNA was carried out according to the method of Zhou et al. (1994), with some modifications. Briefly, 3- μ m sections of airway tissue were collected on duplicate silanized slides (Applied Biosystems), dried overnight and adhered at 60C for 30 min, then dewaxed and rehydrated to Tris-buffered saline (TBS). Deproteinization was carried out with 0.2 M HCl for 20 min, followed by digestion with 0.025% w/v protease type VIII (Subtilisin carlsberg; Sigma) in TBS at 37C. EBBx were treated for 45 sec and nasal polyps treated for 3 min. These times were determined empirically and allowed the probe to penetrate into the tissue to give the best signal-to-noise ratio with the best concomitant morphology. Sections were then rinsed in PBS, postfixed for 15 min with 4% paraformaldehyde-PBS, and washed twice more with PBS. Slides were then acetylated for 10 min in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0). Slides were rinsed in TBS between each treatment and all procedures were carried out at room temperature unless otherwise specified. Sections were then dehydrated through graded ethanols and air-dried. Prehybridization was carried out at 47C for 1.5 hr in a buffer consisting of 20% deionized formamide, 4 \times SSC, 10 \times Denhardt's solution, 0.5 mg/ml denatured fish sperm DNA (Roche Molecular Biochemicals; Melbourne, Australia), 0.25 mg/ml yeast tRNA (Roche Molecular Biochemicals), and 10% dextran sulfate.

Sections were then washed three times in TBS, dehydrated, and air-dried as previously and probed with fluorescein-labeled TNF antisense, sense, and chicken lysozyme control riboprobes diluted in ISH buffer (RNA color kit). Further controls included omission of probe and abolition of signal by RNase A (Roche Molecular Biochemicals) treatment of tissues. The concentration of antisense probe was determined by simple comparison with chicken lysozyme control probe in a dot-blot dilution series, then used at approximately 0.5 μ g/ml. Corresponding amounts of control probes were determined by comparative fluorescence quantitation in denaturing gels, as described above. Sections were sealed with riboprobe solution using AmpliCover discs, clips, and an assembly tool (Applied Biosystems), denatured at 75C for 3 min, and hybridized at 47C for 15 hr in the GeneAmp In Situ PCR System 1000 (Applied Biosystems).

After hybridization, slides were rinsed briefly in 2 \times SSC, then subjected to stringency washes consisting of two washes in 1 \times SSC-0.1% SDS for 5 min at RT, followed by two

washes in $0.1 \times \text{SSC}$ –0.1% SDS for 10 min at 47°C. Finally, slides were rinsed in Hi-TBS (0.1 M Tris, pH 7.5, and 0.4 M NaCl) before detection of hybridization.

Detection of Hybrids Formed In Situ

Sections were blocked for 1 hr in 0.5% w/v blocking agent (RNA color kit) in Hi-TBS to prevent nonspecific Ab binding, washed briefly in Hi-TBS, followed by single-Ab AP detection or four-layer, avidin–biotin complex (ABC)–peroxidase detection on the consecutive section. For both detection techniques, the primary Ab consisted of a sheep anti-fluorescein-AP conjugate (RNA color kit). This was applied at 1:1000 dilution in 0.5% bovine serum albumin (BSA) in Hi-TBS for 1 hr. Slides were washed in Hi-TBS between each treatment for both techniques and all procedures were carried out at RT.

Single-Ab AP Detection

Sections were then flooded with AP substrate-chromogen, nitroblue tetrazolium chloride-5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP; RNA color kit) and incubated in the dark. After 4.5 hr, when adequate staining intensity was achieved, sections were washed in running water, counterstained with Mayer's hematoxylin, and mounted with permanent aqueous mountant [Permanent Mounting Medium (Aqueous); ScyTek, Logan, UT].

Four-layer ABC–Peroxidase Detection

The secondary Ab consisted of a monoclonal mouse anti-AP Ab (Zymed; South San Francisco, CA), which was applied to sections at 1:200 in 0.5% BSA–Hi-TBS for 1 hr. Tertiary biotinylated horse anti-mouse Ab (Vectastain Elite ABC kit; Vector, Burlingame, CA) then followed at 1:100 in 0.5% BSA–5% normal human serum–Hi-TBS for 30 min. Sections were then dehydrated through graded ethanols and endogenous peroxidase blocked by immersion in 0.12% hydrogen peroxide in methanol for 10 min. Sections were rehydrated through reversed graded ethanols and the quaternary avidin–biotin–horseradish peroxidase layer (Vectastain kit) applied for 30 min. Sections were flooded with metal-enhanced diaminobenzidine chromogen-substrate (Immunopure; Pierce, Rockford, IL) for ~10 min, as standardized against staining in positive control nasal polyp tissue. Sections were washed in running water, counterstained with Harris hematoxylin, dehydrated, and cleared and coverslipped with permanent xylene-based mountant (Biomount-X; Bio-Service, Melbourne, Australia).

Results

PCR-generated DNA Provided an Efficient Template for In Vitro Transcription

Fluorescein-labeled riboprobes produced from PCR product were electrophoresed and compared with riboprobes produced from two human TNF clones and probes transcribed from an unrelated clone (Figure 1). Relative probe fluorescence showed that transcription from the PCR template with SP6 RNA polymerase was more efficient than from plasmid DNA, producing the

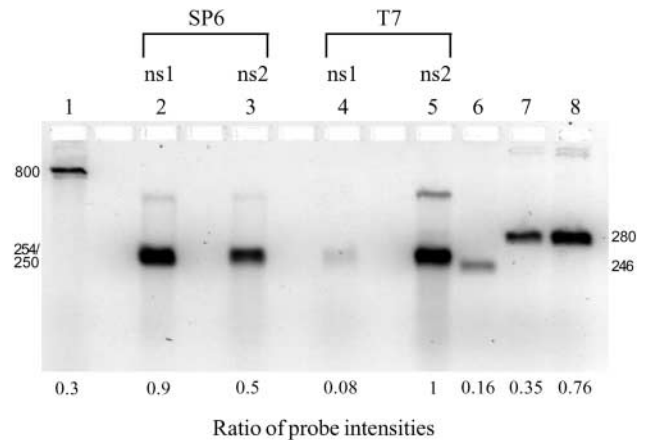


Figure 1 Comparison of riboprobes transcribed from PCR-generated template with probes transcribed from plasmid clones. Fluorescein-labeled riboprobes were resolved on a 4.5% denaturing formaldehyde agarose gel and visualized by laser-based fluorescent scanning in the FluorImager 575. Lane 1, an 800-b antisense probe transcribed from a human TNF subclone. PCR product from nested set 1 (ns1) was transcribed with SP6 RNA polymerase (Lane 2) and T7 RNA polymerase (Lane 4), yielding antisense and sense probes, respectively. PCR product from nested set 2 (ns2) was transcribed with SP6 polymerase (Lane 3) and T7 polymerase (Lane 5), yielding sense and antisense probes, respectively. Lane 6, a 246-b antisense probe transcribed from a second TNF subclone. Lanes 7 and 8, 280-b riboprobes transcribed from a chicken lysozyme clone with SP6 and T7 polymerases, respectively. Indicated are the relative probe intensities as measured by volume integration for each band. Probe intensities were measured relative to the antisense probe in Lane 5, which was the most intense and therefore assigned the value "1." Band sizes are indicated in bases.

highest signal from a shorter probe (Figure 1, Lane 2). Transcription with T7 polymerase from the PCR template, however, was not as efficient (Figure 1, Lane 4), the signal being weaker than that from a similar-sized probe generated from plasmid (Figure 1, Lane 6).

PCR-generated DNA Provided an Efficient Template for In Vitro Transcription in the Absence of Primer Hairpin Loops

Reanalysis of the T7 primer with our primer designing software revealed a hairpin loop in the TNF sequence with T_m of 64°C, which was observed to be suitable for PCR but which prevented efficient transcription at 37°C. Thus,



We then assessed the effect of minimal base substitution to disrupt the hairpin loop and found that substitution of all bases except the 5' C or 3' G in the hairpin stem completely eliminated the loop. Substitution of these terminal bases leaving a three-base stem, TCG/CGA, only reduced stability of the loop to a T_m of 42°C. The

presence of a stable hairpin loop was therefore considered likely. Primers were therefore redesigned so as to avoid hairpin formation, with the SP6 promoter on the sense primer and the T7 promoter on the antisense primer. DNA template was made as before with the new nested primers and probes transcribed and analyzed (Figure 1, Lanes 3 and 5). Relative probe intensities demonstrated comparable labeling with either polymerase, with similar or greater fluorescence relative to the probes transcribed from clones.

Riboprobes from PCR-generated Template Demonstrated Equivalent ISH Results to Plasmid-generated Probes

We then performed ISH for TNF mRNA in nasal polyp tissue, comparing plasmid-generated probe hydrolyzed from 800 b to 250 b with both antisense probes produced from PCR template (Figure 2). Equal amounts of probe label, as determined by electrophoresis and ImageQuANT analysis, were used, with conventional single-Ab detection. Probes from PCR-generated template showed similar or better signal-to-noise ratios compared with the hydrolyzed probe, demonstrating that for moderately expressed targets, a single probe of the optimal size provides adequate sensitivity.

Detection of ISH Using an Ab Sandwich Yielded Superior Morphology to Single-Ab AP Detection

Detection of ISH for TNF mRNA in EBBx with the conventional single-Ab AP technique resulted in interstitial damage to the subepithelial lamina propria. This occurred as a result of extended incubation in substrate-chromogen at pH 9.5, which was required for at least 3 hr to achieve adequate signal intensity (Figure 3A). Poor cellular localization of signal was also a problem because of diffusion of the chromogen over this time. We therefore expanded the single-layer detection to a four-layer ABC-peroxidase technique. The greater sensitivity thus provided ensured that the tissue was exposed to substrate at pH 5.5 for only 10 min, thereby preserving morphology and yielding superior signal localization (Figure 3B).

Discussion

We aimed to improve ISH in airway tissues by analyzing alternative methods of probe production and detection of hybridization. Therefore, we assessed whether synthesis of riboprobes for ISH could be achieved more efficiently from PCR-generated DNA template compared with traditional plasmid clones and whether tissue morphology could be improved using a multi-layer Ab sandwich for detection of hybrids, rather than the traditional single-Ab AP detection.

Initially we synthesized DNA template for *in vitro* transcription of TNF riboprobes by highly specific nested PCR, incorporating SP6 and T7 RNA polymerase promoters. Primers were selected to generate a template that yielded an optimal length probe for ISH of 250 b (Angerer and Angerer 1992) so that hydrolysis was not required. This is advantageous because hydrolysis can be difficult to control and may give rise to large amounts of small probe fragments that may readily hybridize to nucleolar RNA (Baumgart et al. 1997) or may not hybridize under standard stringency conditions, leading to low signals (Wilkinson 1998).

Fluorescein-labeled riboprobes were then *in vitro* transcribed and relative probe labeling quantified by electrophoresis and laser-based fluorimetric scanning. This ensured that the probes were the correct size and also enabled quantification of relative probe labeling, because the presence of various amounts of unincorporated labeled and unlabeled nucleotides prevents efficient quantification of labeled probes by spectrophotometry (Baumgart et al. 1997) or fluorimetry. Determination of relative yield in this manner also provided a means of ensuring that equal amounts of sense and antisense probe were used in ISH experiments. This resolved the problem of positive hybridization signals we had previously observed with sense probes when their transcription was more efficient than antisense.

Results indicated that riboprobes can be efficiently transcribed from nested PCR-generated template with either SP6 or T7 RNA polymerases in either orientation, providing that there are no stable hairpin loops in the primer sequences. We concluded that transcription from our initial T7 primer was poor because the sequence was likely to form a stable hairpin loop, as demonstrated by our primer design software. This was also confirmed by the fact that minimal base substitution completely disrupted the loop, except when the terminal bases of the hairpin stem were removed, which enabled the loop to remain relatively stable with a T_m of 42°C.

Not surprisingly, larger transcripts appeared with the plasmid-generated transcripts, indicating incomplete restriction enzyme digestion despite rigorous overnight treatment. We have consistently observed this problem with plasmid clones and aimed to resolve it using the PCR-based technique, thus preventing incorporation of undesirable plasmid sequences into the probe.

Overall, synthesis of fluorescein-labeled riboprobes from PCR-generated template was a simple procedure, with transcription efficiency and hybridization sensitivity comparable to or better than traditional plasmid-generated probes. We have successfully used these probes for the analysis of TNF mRNA in EBBx tissue, nasal polyp tissue, and cytopins of BAL cells, and the

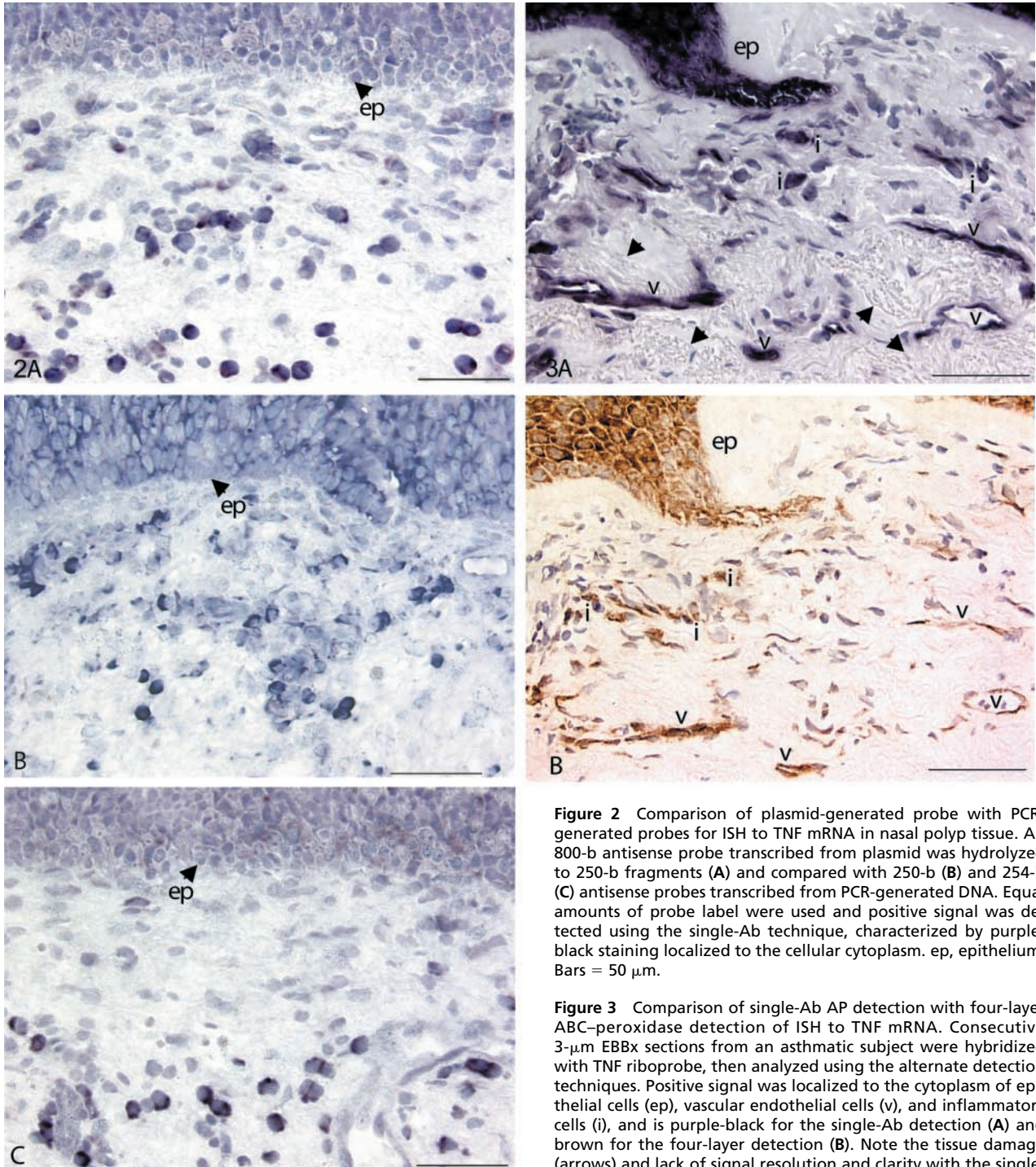


Figure 2 Comparison of plasmid-generated probe with PCR-generated probes for ISH to TNF mRNA in nasal polyp tissue. An 800-b antisense probe transcribed from plasmid was hydrolyzed to 250-b fragments (A) and compared with 250-b (B) and 254-b (C) antisense probes transcribed from PCR-generated DNA. Equal amounts of probe label were used and positive signal was detected using the single-Ab technique, characterized by purple-black staining localized to the cellular cytoplasm. ep, epithelium. Bars = 50 μ m.

Figure 3 Comparison of single-Ab AP detection with four-layer ABC-peroxidase detection of ISH to TNF mRNA. Consecutive 3- μ m EBBx sections from an asthmatic subject were hybridized with TNF riboprobe, then analyzed using the alternate detection techniques. Positive signal was localized to the cytoplasm of epithelial cells (ep), vascular endothelial cells (v), and inflammatory cells (i), and is purple-black for the single-Ab detection (A) and brown for the four-layer detection (B). Note the tissue damage (arrows) and lack of signal resolution and clarity with the single-Ab technique, especially in the epithelium. Conversely, the four-Ab technique preserved tissue morphology, affording good signal localization and clarity, and provided good contrast with the hematoxylin counterstain. Note the obvious localization of TNF to the cytoplasm of epithelial cells and the visible intercellular spaces. Bars = 50 μ m.

layer technique preserved tissue morphology, affording good signal localization and clarity, and provided good contrast with the hematoxylin counterstain. Note the obvious localization of TNF to the cytoplasm of epithelial cells and the visible intercellular spaces. Bars = 50 μ m.

PCR-based technique has proved reliable for the in-house synthesis of several different riboprobes for use in ISH studies. In cases where mRNA expression is low, a few PCR-generated templates representing more of the

mRNA sequence may be required to increase the sensitivity of ISH.

Once we established that PCR-generated DNA was the most efficient template for riboprobe synthesis, we

then sought to modify the detection component of ISH and thereby improve the resultant morphology of our tissues. The simplest method for detection of hapten-labeled hybrids formed *in situ* is a single anti-hapten Ab conjugated to AP, with substrate incubation yielding a localized colored reaction product (Herrington et al. 1991; Stahl and Baskin 1993). The advantages of this procedure are ease of use and the stability of the AP enzyme, which allows the color reaction to proceed for many hours to achieve a high amplification of signal (Hougaard et al. 1997; Wilkinson 1998; Kadkol et al. 1999). However, we found that conventional single-Ab AP-based detection of ISH for TNF mRNA was unsuitable for use in delicate tissues such as airway EBBx. EBBx are fragile because they are very small (1–2 mm²) and contain little connective tissue to provide structural support, and therefore are susceptible to damage, particularly by harsh chemical treatments. Using this detection technique, EBBx tissue became damaged by the extended incubation at high pH that was necessary for substrate processing by AP. Diffusion of the chromogen over this time also led to loss of definition and hence to loss of signal resolution (Herrington et al. 1991; Herrington 1998) and was also not easily distinguished from the hematoxylin counterstain. In addition, AP substrate-chromogens that are stable enough for such extended incubations (including NBT-BCIP and iodo nitroretazolium-BCIP) also require the use of aqueous mountant which, although providing permanent chromogen preservation, does not achieve the same microscopic resolution as xylene-based mountant. Although the AP substrate-chromogen new fuchsin is compatible with xylene-based mountant, we have found it insufficiently sensitive for use in single-Ab detection of ISH because it is not stable for the extended periods required.

In an attempt to resolve this problem, we devised a multilayer ISH detection system (Herrington et al. 1991; McQuaid and Allan 1992; Hamilton-Dutoit and Pallesen 1994; Larsen and Mikkelsen 1994) by expanding the single-layer detection to a four-layer ABC-peroxidase protocol. The resultant increase in sensitivity afforded by the number of detection layers enabled a significant reduction in substrate processing time. This resulted in superior morphology and localization of TNF signal to epithelium, inflammatory cells, and vascular endothelium of EBBx. Increased resolution was also enhanced by the xylene-based mountant. Moreover, the entire procedure takes no more time than the single-Ab method.

For relatively robust samples, therefore, a single-Ab AP-based ISH detection is simple and effective, whereas for fragile specimens, such as lung and brain tissues, a multilayer Ab sandwich should be used to maintain good sample morphology. We have since used this multilayer

technique for detection of ISH in EBBx from over 60 subjects, with excellent results.

Increasing the sensitivity of detection also allows the use of either AP or peroxidase reporters because the reduced substrate processing time enables enzymes and/or substrates to remain stable. This has the advantage of allowing different reporter enzymes and substrates to be used for dual IHC–ISH or ISH–ISH protocols. The multilayer ISH detection is also extremely flexible. Many combinations of layers and amplification systems can be used, so long as adequate sensitivity is achieved and substrate incubation is correspondingly short.

In conclusion, we found that sensitive riboprobes can be transcribed more efficiently from PCR-generated template than from plasmid, using either SP6 or T7 RNA polymerase in either orientation, providing that there are no stable primer hairpin loops. This approach enables the investigator to synthesize riboprobes of any sequence by appropriate primer selection, without the problems associated with cloning, plasmid restriction digestion, and probe hydrolysis. Relative fluorescence quantification of probes enabled equal amounts of probes to be used for ISH, thus avoiding the inaccuracy of spectrophotometry or fluorimetry. We also developed an Ab sandwich technique that proved most suitable for the detection of ISH in delicate tissue specimens, such as small human bronchoscopic EBBx. The technique increased sensitivity of detection, thus reducing substrate processing time, thereby maintaining tissue morphology, and providing good signal localization.

Acknowledgments

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