A Simplified *In Situ* Hybridization Protocol Using Non-radioactively Labeled Probes to Detect Abundant and Rare mRNAs on Tissue Sections

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Abstract
The article describes a simplified and efficient protocol for non-radioactive *in situ* hybridization experiments on tissue sections. The sensitivity of the method allows the expression of genes to be studied with transcript levels ranging from very low (20–30) to high (several thousands) copy numbers per cell. The immediate procession of cryosections to the ISH protocol after sectioning, coupled to the active DEPC treatment of slides before hybridization, are shown to improve considerably the mRNA detection. Moreover, we also improved the detection of rare transcripts such as PPARα mRNA by increasing the hybridization time up to 40 h. The resolution obtained is such that distribution gradients of mRNAs present at less than 30 copies within the cells are detected, both on cryosections and paraffin sections.

Introduction

In situ hybridization (ISH) techniques have been applied increasingly to localize gene expression at the cytological level [1, 2]. Since the first (ISH) experiments [3, 4], mainly radioactive nucleotides have been used to synthesize probes [5]. The advantage of radiolabeled probes is their ability to detect very low levels of transcripts, whereas their major limitations are very long exposure times and a relatively poor resolution, depending on the radioisotope used. Moreover, the photographic emulsion revealing the hybridization signals is not at the same focus as the tissue section, which hampers resolution and microscopic observation. The more recent use of non-radioactively labeled nucleotides has considerably improved ISH with both a shortening of the development time and excellent histological resolution [6, 7, 8]. Of all the non-radioactive labeling methods developed, digoxigenin based detection has proved the most appropriate for rare transcripts. Since digoxigenin is synthesized only in plants of the genus *Digitalis*, background problems in cells of other organisms are avoided [7]. However, ISH protocols allowing the nonradioactive detection of rare transcripts have not been available hitherto. Our aim was thus to develop a refined non-radioactive ISH protocol for detecting rare transcripts that would prove reliable for both cryosections and paraffin sections, and that would be suitable for all kinds of tissues. Therefore, we critically assessed the experimental ISH protocols described to date. The usefulness of each step was evaluated and each solution or buffer was simplified where possible. The remaining steps were then improved, one by one, for the detection of a relatively abundant transcript, albumin mRNA, on cryosections of adult rat liver. As a result, a simplified and efficient ISH protocol was obtained, which was then tested on the expression of the peroxisome proliferator-activated receptor alpha (PPARα) gene, which encodes a transcription factor belonging to the nuclear receptor superfamily [9, 10]. This protocol is sensitive enough to allow the detection of approximately 25 copies of PPARα mRNA per hepatocyte in rat liver. Finally, we chose one of the most difficult tissues to prepare, white adipose tissue, in order to show that this ISH method can also be employed for detecting rare transcripts on paraffin sections.

Materials and Methods

Cloning of albumin and PPARα cDNAs

Two different rat serum albumin cDNAs were cloned in order to synthesize one short (101 nt) and one long (1169 nt) ISH riboprobe. The first cDNA was obtained from rat liver total RNA by reverse transcription coupled to PCR by the use of the primers albumin-up-1 (5'-GAAGGGGATCCTCCTGCCTGCTACGGCAC-3') and albumin-down-1 (5'-CTCTCCAAGCTTCTCGTAAAGCTCACAG-3').

The resulting fragment, 101 nt long and corresponding to nt 1165-1265 of the rat serum albumin cDNA (11), was digested with *Bam* HI and *Hin*d III and cloned into the pBluescript-SK+ and –KS+ vectors (Stratagene) to obtain the pSK+/ALBUMIN (1165-1265) and pKS+/ALBUMIN (1165-1265) plasmids. The second cDNA fragment was obtained by RT-PCR with the use of the primers albumin-up-2 (5'-GCC-ACCCTTCTAGAGGCCGGAGGCT GAGGCC-3') and albumin-down-2 (5'-CAGCTTTGAATTCTTTGGGGA-CATATGTCTC-3'). The resulting fragment, 1169 nt long (nt 413 to 1581, ref. 11), was digested with *Xba* I and *Eco* RI and cloned into the pBluescript-SK+ and -KS+ vectors to obtain the pSK+/ALBUMIN (413-1581) and pKS+/ALBUMIN (413–1581) plasmids. This albumin cDNA was further restricted to nt 1261 to 1581 by digestion with *Hin*d III and *Eco* RI and cloned into the pBluescript-KS+ vector to obtain the pKS+/ALBUMIN (1261–1581) plasmid, which was used to synthesize riboprobes for RNase protection assay. A rat PPARα cDNA fragment of 390 nt was obtained as described (12,13) and cloned into the pBluescript-KS+ and -SK+ vectors to obtain the pKS+/PPARα (1377–1766) and pSK+/PPARα (1377-1766) plasmids.

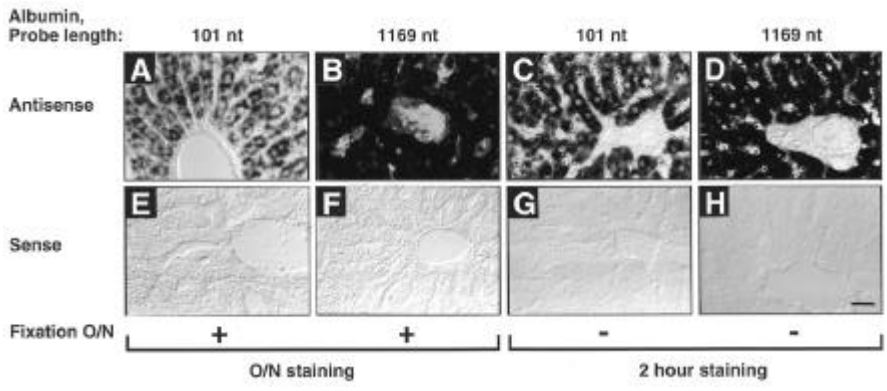

Figure 1 **Expression of the albumin gene: effect of fixation and probe length on mRNA detection sensitivity.** Adult rat liver was either fixed O/N in paraformaldehyde-PBS or frozen immediately after dissection as indicated. 12 µm thick cryosections were then prepared, immediately postfixed 10 min in paraformaldehyde-PBS, then treated 2x 15 min in 0.1% active DEPC-PBS, and equilibrated in 5x SSC. The ISH protocol was then applied as described in Materials and Methods. Sections were hybridized with 400 ng/ml of albumin antisense and sense short (101 nt, panels A, C, E, G) or long (1169 nt, panels B, D, F, H) riboprobes. For fixed sections staining was O/N (panels A, B, E, F), whereas for non-fixed sections it was for only 2 h (panels C, D, G, H). Bar: 20 µm; nt= nucleotides.

ISH riboprobe synthesis

The cDNA-containing plasmids were linearized as follows: pSK+/PPARα (1377-1766) and pSK+/ALBUMIN (413-1581) with *Xba* I, pKS+/PPARα (1377-1766) and pSK+/ALBUMIN (413-1581) with *Eco* RI, pKS+/ALBUMIN (1165-1265) with *Hin*d III, and pSK+/ALBUMIN (1165-1265) with *Bam* HI. The linearized plasmids were then gel-isolated and used as templates for antisense and sense Digoxigenin (DIG)- and [α-^{32}P] UTP-labeled riboprobe synthesis (Boehringer Mannheim, and Amersham Corp., respectively). The transcription mixture (50 µl) included 1 µg of linearized template cDNA, ATP, GTP, and CTP at 1 mM each, UTP 0.7 mM, DIG-UTP 0.3 mM, [α-^{32}P]UTP 30 nM, DTT 10 mM, RNase Inhibitor (1 unit/µl of transcription mix), and T3 or T7 RNA polymerase (1 unit/µl of transcription mix). Transcription was performed for at least 2 h at 37°C. The template cDNAs were then digested by RNase-free DNase (2 µl at 1 unit/µl, 30 min at 37°C), and all reactions were stopped by adjusting the reaction volume to 100 µl with Tris/EDTA (10/1 mM, pH 8.0). The riboprobes were then purified through two precipitation steps by addition of 100 µl NH_4-acetate 4 M and 500 µl EtOH 100%, and centrifugation for 30 min at 4°C in a microfuge. The pellet was resuspended in 200 µl DEPC-treated water. Low-level [α-^{32}P]UTP incorporation was used in order to determine the amounts of riboprobes synthesized and to control the length and integrity of the probes by gel electrophoresis. 5 to 10 µg of synthesized riboprobe was obtained from 1 µg of cDNA matrix. The DIG-incorporation into the probes was controlled by dot spots, according to [14]. DIG was visualized with an anti-DIG antibody coupled to alkaline phosphatase.

Preparation of tissue sections

Liver pieces of adult male Sprague-Dawley rats (300 g, BRL) were rapidly rinsed in diethylpyrocarbonate (DEPC)-treated PBS, and either immediately embedded in Tissue Freezing Medium (Jung) and then frozen in isopentane and dry ice, or fixed overnight (O/N) in 4% paraformaldehyde-PBS (NaCl 0.9% w/v, NaH_2PO_4 12.5 mM, NaOH 10 mM, pH 7.5) at room temperature. The O/N fixed liver pieces were then cryoprotected by incubation for 6 h in 12% sucrose-PBS and O/N in 18% sucrose-PBS at room temperature and frozen as described for the unfixed pieces (see above). All tissues were kept at –80°C until used. 12 µm thick tissue sections were prepared (–35°C; Reichert and Jung frigocut), mounted on poly-L-lysinated slides, postfixed in 4% paraformaldehyde-PBS (10 min at room temperature), and processed immediately for the ISH experiment.

White adipose tissue was fixed O/N in 4% paraformaldehyde-PBS, dehydrated through successive baths of EtOH (70%, 95%, and 100%) and xylol (2x 30 min each), and embedded in three successive baths of Paraplast (58°C, Sherwood medical). After paraffin solidification at room temperature, the tissue was kept at 4°C until used. Paraffin sections were cut (12 µm thick, room temperature), mounted on poly-L-lysinated slides, air-dried O/N, and stored at 4°C in a dry atmosphere until used for the ISH experiment. The sections were rehydrated through successive baths of xylol, 2x EtOH (100% and 95%, respectively), DEPC-treated water, and DEPC-treated PBS (2x 5 min each). They were then post-fixed for 10 min in 4% paraformaldehyde-PBS and processed for ISH.

In Situ *Hybridization (ISH)*

After postfixation in 4% paraformaldehyde-PBS, sections were incubated for 2x 15 min in PBS containing 0.1% active DEPC (Fluka), and equilibrated for 15 min in 5x SSC (NaCl 0.75M, Na-Citrate 0.075M). The sections were then prehybridized for 2 h at 58°C in the hybridization mix (50% formamide, 5x SSC, salmon sperm DNA 40 µg/ml; 500 µl on each section). The probes were denatured for 5 min at 80°C and added to the hybridization mix (400 ng/ml). The hybridization reaction was carried out at 58°C for 4 h for abundant transcripts (i.e., albumin) and 40 h for rare transcripts (i.e., PPARα) with 20 µl of hybridization mix on each section, covered by a rectangle of Parafilm (American National Can). Prehybridization and hybridization were performed in a box saturated with a 5x SSC – 50% formamide solution to avoid evaporation. After incubation, the sections were washed for 30 min in 2x SSC (room temperature), 1 h in 2x SSC (65°C), 1 h in 0.1x SSC (65°C), and equilibrated for 5 min in Buffer 1 (Tris-HCl 100 mM and NaCl 150 mM, pH 7.5). The sections were then incubated for 2 h at room temperature with alkaline phosphatase-coupled anti-digoxigenin antibody (Boehringer Mannheim) diluted 1:5000 in Buffer 1 containing 0.5% blocking reagent (Boehringer Mannheim). Excess antibody was removed by two 15 min washes in Buffer 1, and the sections were equilibrated for 5 min in Buffer 2 (Tris-HCl 100mM, NaCl 100mM, and $MgCl_2$ 50mM, pH 9.5). Color development was performed at room temperature (30 min to 3 days, depending on the amount of transcripts to be detected) in Buffer 2 containing NBT and BCIP (Boehringer Mannheim). Staining was stopped by a 10 min wash in Tris/EDTA (10/1mM, pH 8.0), and non-specific staining was removed O/N in EtOH 95% with gentle agitation. Sections were rehydrated for 15 min in deionized water to remove the precipitated Tris and then dehydrated through successive baths of EtOH (70, 95, and 100%) and xylol (2 x 15 min each), and mounted in Eukitt resin (O. Kindler GmbH & Co.).

In addition, the following variations of the main steps were analyzed in the course of ISH protocol optimization. After cutting and before postfixation, the sections were permeabilized with Triton® X-100 detergent (see Figure 2). After section postfixation, the active DEPC treatment was replaced by either a rinse in PBS or acetylation (see Figure 2). Prior to equilibration in 5x SSC and prehybridization, the sections were digested with proteinase K (see Figure 2). Probe concentration, hybridization temperature, and stringency of washes were also tested (see Results). As the number of uridines was identical for each pair of corresponding antisense and sense probes, probe concentrations did not need to be adjusted within a specific pair. Finally, blocking reagent treatment during hybridization (Denhardt's reagent versus salmon sperm DNA), and the development process (fetal calf serum versus Boehringer Mannheim blocking reagent) were optimized as well (see Discussion).

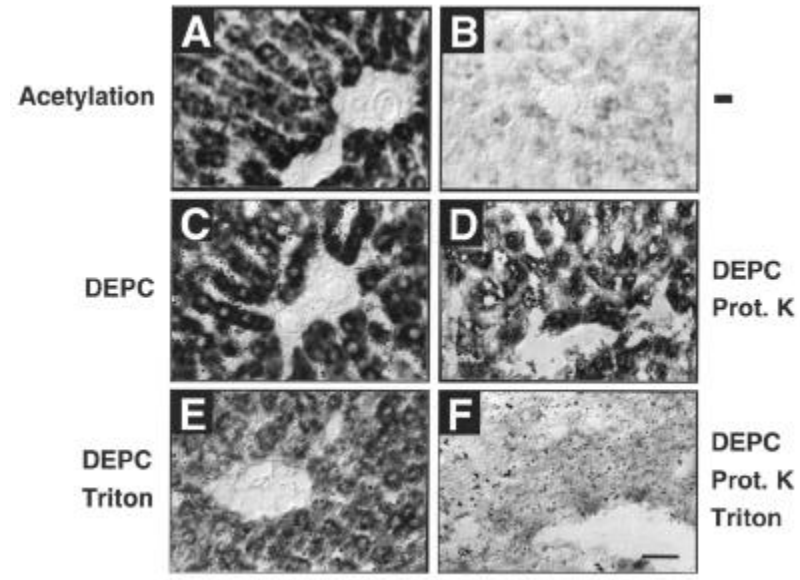

Figure 2 **Expression of the albumin gene: effect of acetylation, carbethoxylation (DEPC), proteinase K digestion and permeabilization with Triton X-100 on mRNA detection sensitivity.** Adult rat liver pieces were frozen immediately after dissection, and 12 µm thick cryosections were prepared. Following postfixation in paraformaldehyde-PBS for 10 min, sections were either acetylated 10 min in 0.25% acetic anhydrid in triethanolamine 10mM (A) rinsed 10 min in PBS (B), or treated 2 x 15 min in 0.1% active DEPC-PBS (C-F). In panels D and F, sections were digested in 5 µg/ml proteinase K in Tris/EDTA 100/50 mM (pH 7.5) for 10 min at 37°C after the DEPC-treatment. In panels E and F, sections were permeabilized in 0.5% Triton X-100-PBS for 5 min after the postfixation step. Sections were then equilibrated in 5x SSC, and the ISH protocol was applied as described in Materials and Methods. Sections were hybridized with 400 ng/ml of the albumin antisense short riboprobe (101 nt). Bar: 20 µm.

Histological analysis

In situ hybridization slides were observed and photographed on an Axiophot microscope (Carl Zeiss SA) equipped with Nomarski optics.

RNase protection assay

The plasmid pKS+/albumin (1261–1581) was linearized with *Cla* I and gel-purified. The plasmid pKS+/PPARα (1377–1766) was digested with *Taq* I, and the 717-base pair fragment containing the T7 promoter and the last 287 nt of the PPARα insert (1480 to 1766) was isolated on agarose gel. From the isolated fragments described above, antisense [α-^{32}P]UTP-labeled riboprobes (Amersham Corp.) were synthesized using T7 RNA polymerase. Probes were 392 nt long for albumin and 334 nt long for PPARα. Protected fragments were 321 nt long with the albumin probe and 287 nt long with the PPARα probe. Different ratios of [α-^{32}P]UTP to cold UTP were used: 1:500 for albumin and 1:1 for PPARα. Specific activities were 1.5x 10^6 cpm/µg (1.7x 10^5 cpm/nmol) for albumin and 3x 10^8 cpm/µg (3.4x10^7 cpm/nmol) for PPARα, which allowed signal comparison with the same exposure time. Total RNA from adult male rat liver was prepared by the guanidinium isothiocyanate acid phenol procedure [15]. The RNase protection assay was carried out as

described [16]. 10 µg of total RNA were hybridized with 10 ng of albumin riboprobe and 1 ng of PPARα riboprobe. The amounts of the different probes were tested to be in large excess. Hybridization was performed O/N at 42°C and digestion with RNase A and RNase T1 was carried out for 1 h at 30°C. Protected fragments were separated on a 6% polyacrylamide gel and revealed by exposure to x-ray film (AIF-RX, Fuji). Quantitative analyses were made with a Phosphor Imager (Bio-Rad).

Results

Tissue fixation, hybridization, and washing conditions

Detection of albumin transcripts in adult male liver by ISH on cryosections is presented in Figure 1. After dissection, the tissue was either fixed (panels A, B, E, F) or frozen at once without fixation (panels C, D, G, H). We found that the short (101 nt) and the long (1169 nt) DIG-labeled antisense riboprobes hybridize specifically to albumin mRNA (panels A-D), as validated by the absence of background with the sense control probes (panels E-H). The longer of the two probes appeared to penetrate the tissue as well as the shorter one and thus, allows greater detection sensitivity, which correlates with probe length (compare panels A with B and D with C). However, probes penetrate less easily between the covalently linked structures of tissues fixed O/N after dissection (compare panels A-B with C-D). With fixed tissues, the time of staining had to be lengthened (O/N) to reach the same levels of signal as with unfixed tissues, for which two hours of staining were sufficient.

The following parameters were then tested: probe concentration, hybridization temperature, type of blocking agent, and washing stringency. The results (not shown) are summarized below. Increasing concentrations of probes were tested (100, 200, 400, 800 ng/ml, and 2 µg/ml) with both albumin (101 nt) and PPARα (390 nt) probes. The best specific versus unspecific signal ratio was obtained with these two probes at 400 ng/ml. Hybridization temperature was tested with the albumin-101nt and the PPARα-390 nt probes (antisense and sense) between 42°C and 65°C. For albumin, the antisense signal became specific at 55°C and disappeared at 65°C, whereas specificity was reached at 58°C for PPARα and the signal disappeared also at 65°C. During the prehybridization and hybridization steps, we tested the effect of Denhardt's solution and salmon sperm DNA or a combination of both as blocking agents. Salmon sperm DNA at 40 µg/ml in the prehybridization and hybridization mixtures was sufficient to avoid background problems (a 100-fold excess compared to probe concentration). After hybridization, optimal washing conditions that ensured signal specificity consisted of rinses of 30 min in 2x SSC at room temperature, 1 h in 2x SSC at 65°C, and 1 h in 0.1x SSC at 65°C.

Acetylation, proteinase K digestion, and detergent permeabilization of the tissue

The results of ISH experiments, testing the above mentioned parameters, with cryosections of unfixed adult male liver are shown in Figure 2. Sections were either acetylated, rinsed in PBS, or DEPC-treated (panels A, B, and C, respectively). DEPC-treated sections were also either digested with proteinase K or permeabilized with Triton® X-100 detergent (panels C to F). All sections presented in Figure 2 were hybridized with the short (101 nt) DIG-labeled antisense albumin riboprobe. We found that acetylation of the tissue sections strongly increased the specific ISH signals compared to non-acetylated sections (compare panel A with panel B). We also demonstrated that carbethoxylation with diethylpyrocarbonate (DEPC) is as efficient as the acetylation process (panel C). As proposed by numerous protocols, we have tried mild digestion with proteinase K or permeabilization with Triton X-100 detergent, in order to allow better penetration of the sections by the probes. Both procedures reduced tissue preservation and signal intensity (compare panels D, E, and F with panel C), and thus were not subsequently included in our standard ISH protocol.

Detection of abundant (albumin) and rare (PPARα) transcripts

We then compared the efficacy of the ISH protocol for the detection of abundant (albumin) and rare (PPARα) transcripts in the hepatic tissue. Next, PPARα detection was also tested in the white adipose tissue, which was chosen in order to demonstrate that our method can be used to study rare transcripts not only on cryosections but also on paraffin sections. Indeed adipose tissue is very difficult to process by cryosection and requires hydrophobic embedding.

First we performed an RNase protection assay to compare the levels of albumin and PPARα mRNAs in the adult male rat liver (Figure 3A, lane 1). For comparison purposes, antisense undigested probes for PPARα and albumin were present in lanes 2 and 3, respectively. On four independent samples of total RNA used in this assay, the signal of the albumin protected probe was 8.5 +/– 2.5 stronger than the signal of the PPARα protected probe. The specific activity of the albumin unprotected probe was 105 times lower than that of the PPARα unprotected probe (see Materials and Methods). In order to calculate the actual ratio of albumin:PPAR mRNAs, a correction factor was included to take into account the relative number of labeled uridines in protected versus unprotected probes for both albumin and PPARα. With this correction factor, the specific activity of the albumin protected probe was 175 times lower than the PPARα protected probe. Thus, based on both the signals obtained and the correction factor, albumin transcripts are 1,500 $\pm$ 440 times more abundant than PPARα transcripts in adult male rat hepatocytes. The estimated number of albumin mRNA molecules is 40,000 per adult rat hepatocyte [17], and thus there are approximately 25 PPARα mRNA molecules in this cell type.

To demonstrate the usage flexibility of the ISH protocol, we present the results obtained in two very different situations in Figure 3B, cryosections of unfixed adult male liver (panels 1 and 3) and paraffin sections of fixed adult male white adipose tissue (panels 2 and 4). The sections were hybridized with the 390 nt antisense (panels 1 and 2) and sense (panels 3 and 4) PPARα riboprobes. Panels 1 (liver) and 2 (white adipose tissue) demonstrate that our ISH protocol allows excellent signal res-

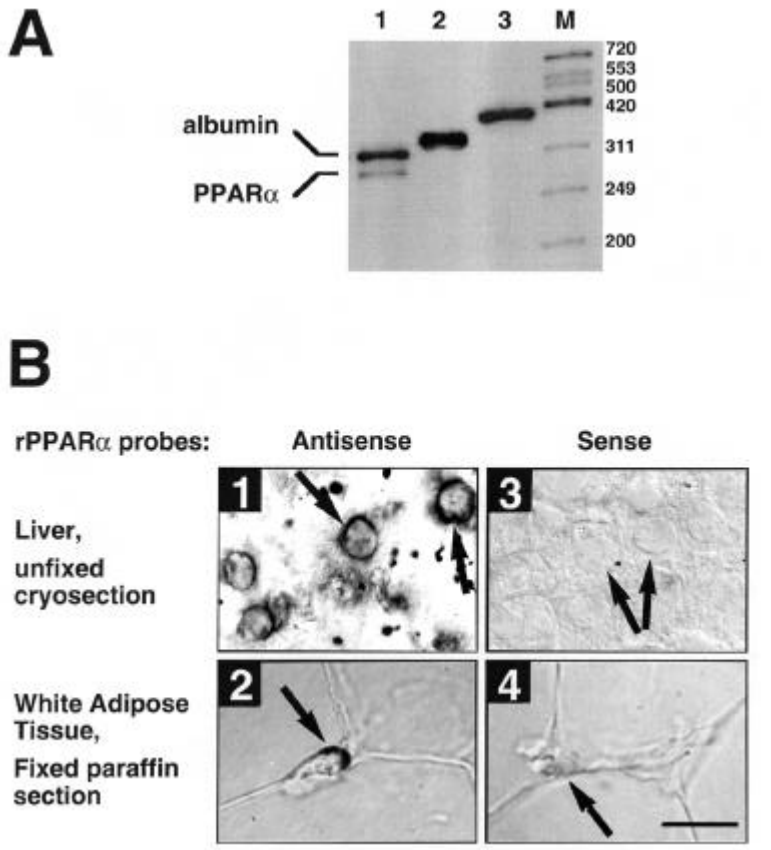

Figure 3 **Sensitivity and resolution of the ISH simplified protocol.**

A: Measure of steady state levels of albumin and PPARα transcripts in adult rat liver by RNase protection assay. Lane 1: 10 µg of liver total RNA were hybridized with 10 ng of albumin and 1 ng of PPARα antisense probes. Lanes 2 and 3: undigested antisense probes for PPARα and albumin respectively. The albumin signal is 8.5 +/– 2.5 times (4 experiments) higher than the PPARα signal, whereas the PPARα probe has a specific activity 175 times higher than the albumin probe (see Materials and Methods, and Results).

B: PPARα-expression in the adult liver and white adipose tissue. Liver was frozen immediately after dissection and prepared for cryosection, whereas white adipose tissue was fixed O/N in paraformaldehyde-PBS after dissection, dehydrated, and prepared for paraffin section. 12 µm thick sections were prepared. Paraffin sections were rehydrated, then all sections were postfixed 10 min in paraformaldehyde-PBS, treated 2x15 min in 0.1% active DEPC-PBS, and equilibrated in 5x SSC. The ISH protocol was then applied as described in Materials and Methods. Sections were hybridized with 400 ng/ml of PPARα antisense and sense riboprobes (390 nt). The arrows point to the perinuclear distribution of the PPARα transcripts. Bar: 20 µm.

olution. In particular, it reveals high concentrations of PPARα transcripts in perinuclear regions of hepatocytes and adipocytes (arrows). In contrast, there was no signal with the sense PPARα control probe (panels 3 and 4).

Discussion

Based on the results of our experiments, we established an optimized *in situ* hybridization protocol for detecting abundant and rare transcripts with DIG-labeled RNA probes, both on cryosections and paraffin sections. To this end, we have tested, compared, combined, and optimized various experimental parameters of a classical ISH protocol. We managed to simplify and improve many of the steps.

ISH probe synthesis and labeling

Various kinds of probes can be used for the detection of mRNA in ISH experiments. However, *in vitro* transcribed riboprobes are the best choice on tissue sections [18]. These probes are single-stranded, may span hundreds of nucleotides, and consequently comprise numerous labeled nucleotides, which results in specific antisense probes with high detection sensitivity. Moreover, *in vitro* transcription allows the synthesis of ideal test antisense and control sense probes, both having the same length and G+C content, defining similar properties of hybridization. After synthesis of the probes, we did not hydrolyze them into smaller pieces, as this treatment leads to elevated background signals. Moreover, we demonstrated that intact probes up to 1,200 nucleotides in length penetrate the tissue effectively, even if it has been fixed for a long period of time (see Figure 1). ISH riboprobes usually comprise short flanking sequences, corresponding to the poly-cloning site of the plasmid vector used, that do not participate in the hybridization process. These "floating" sequences could potentially contribute to unspecific signals, especially with short probes, such as the one used here in albumin mRNA detection. Under our conditions however, these flanking sequences did not affect the specificity of hybridization, as illustrated in Figure 2.

Sample preparation and cutting

Paraformaldehyde in PBS has been shown to be one of the best fixatives for preserving tissue quality, keeping RNA within the tissue and allowing a good recognition of the RNA by the probes [1, 6, 19]. With cryosections on relatively homogeneous tissues (adult liver for example), one may omit fixation after dissection without loss of the histological quality in the sections. In this case, the tissue has to be frozen immediately and stored at –80°C until used, as only a short postfixation is necessary immediately after sectioning and mounting on slides. In the case of heterogeneous samples (whole embryo for instance), fixation is often necessary immediately after dissection and before freezing in order to ensure good preservation of tissues during sectioning. Tissues like white adipose tissue require embedding in a hydrophobic medium such as paraffin to avoid alteration of their morphology. In this case, fixation has to take place immediately after dissection to avoid transcript extraction during the dehydration and rehydration processes. With tissues fixed after dissection, probes penetrate less easily between the covalently-linked structures of the tissue, and the hybridization and staining times may have to be lengthened (see Figure 1). Moreover, depending on the tissue, over-

fixation can lead to the generation of background [2, 20]. Thus, the fixation time needs to be optimized in each case when postfixation after sectioning is not sufficient. Fixation inactivates RNases and therefore paraffin sections may be stored at 4°C until used for ISH. By contrast, to keep rare transcripts intact in cryosections for their subsequent detection, we find it very important to proceed to the ISH protocol immediately after cutting. This procedure allows a single individual to process up to 200 ISH slides per week.

ISH protocol

Acetylation is recommended in ISH protocols for decreasing background (21). Although we did not observe this effect with the albumin probe, acetylation strongly increased specific ISH signals compared to non-acetylated sections (see Figure 2). Acetylation most probably inactivates RNases in the sections, which is consistent with the observation that it can be replaced by the carbethoxylation resulting from diethylpyrocarbonate (DEPC) treatment (see Figure 2C) (22). However, carbethoxylation is easier to perform than acetylation and we therefore recommend the former. Numerous protocols propose permeabilization of the sections with Triton X-100 detergent or mild digestion with proteinase K to achieve better penetration of the probes. Both procedures reduced tissue preservation and signal intensity, and thus were not subsequently included in our standard protocol for cryosections (see Figure 2). It should be noticed that, in contrast with unfixed cryosections, strongly fixed paraffin embedded sections can be mildly digested with proteinase K and/or permeabilized with Triton X-100 detergent to ensure effective penetration of the section by the probe.

Infinite variations in prehybridization or hybridization solutions are suggested in various ISH protocols, all aiming to achieve the right stringency for favoring specific signals, while avoiding unspecific signals. In addition to different saline solutions, the blocking reagents used also vary considerably and include yeast RNA, salmon sperm DNA, Denhardt's reagent or various combinations of these. In our attempts to simplify prehybridization and hybridization solutions, we found that a 5x SSC solution with 50% formamide, used between a temperature of 55°C to 60°C, confers the desired stringency for RNA-RNA hybrids of 100 to 1,200 nucleotides in length. The use of blockers was also simplified, as a 100-fold excess of salmon sperm DNA was found to be satisfactory. The abundance of albumin transcripts allows a short hybridization time (4 h, Figures 1 and 2). By contrast, we observed that the detection of the rare PPARα transcripts requires a much longer hybridization time (40 h, Figure 3). After hybridization, our washing procedure ensures signal specificity. Many ISH protocols include RNase digestion during the washing steps in order to eliminate incompletely or unspecifically hybridized probes (23). In our case, however, this procedure was not necessary, as no background was obtained in the sense controls. As illustrated in Figure 1, both short and long albumin antisense probes penetrated the tissue section very effectively, and gave specific signals. In both cases, the probe concentration in the hybridization buffer was in large excess. Moreover, the long probe generates a much stronger signal than the short one, as it is composed of a greater number of uridines.

Signal staining and slide mounting

In the signal visualization step we chose casein (Boehringer Mannheim Blocking Reagent) as a blocker during the anti-DIG antibody incubation, without including a pre-blocking step, which we found not to be necessary. When using alkaline-phosphatase signal development, fetal calf serum should be avoided as a blocker, as it contains endogenous alkaline phosphatase activities (data not shown, and ref. [24]). Under our hybridization conditions (58°C, 50% formamide), endogenous alkaline phosphatase activities were inactivated, therefore, alkaline phosphatase inhibitors such as levamisole in the signal visualization mixture were not necessary. After the removal of the unspecific labeling in ethanol, a rapid rehydration step in water was found to be necessary in order to eliminate the tris precipitate from the tissue, which otherwise hampers histological observation.

Conclusions

We have developed a simplified and efficient protocol for non-radioactive *in situ* hybridization experiments on tissue sections, the sensitivity of which allows the expression of genes to be studied with transcript levels ranging from very low (20–30) to high (several thousands) copy numbers per cell. Table 1 summarizes the main steps of this ISH protocol. The immediate procession of cryosections to the ISH protocol after sectioning, coupled to the active DEPC treatment of slides before hybridization are shown to improve considerably the mRNA detection. Moreover, we also improved the detection of rare transcripts such as PPARα mRNA by increasing the hybridization time up to 40 h. The resolution obtained is such that distribution gradients of mRNAs present at less than 30 copies within the cells are detected, both on cryosections and paraffin sections. In a previous report on PPARα, -β, and -γ expres-

Day 1:

- Cryosections
- **Immediate postfixation**, 10 to 30 min in 4% paraformaldehyde in DEPC-treated PBS
- 2x 15 min wash in PBS **with 0.1% active DEPC**
- 15 min equilibration in 5x SSC, DEPC-treated
- Immediate process toward ISH
- Prehybridization, 2 h at 58°C, in 50% formamide, 5x SSC, 40 µg/ml salmon sperm DNA
- Hybridization, **4–40 h at 58°C,** with 400 ng/ml of DIG-labeled probe, **in 50% formamide, 5x SSC, 40 µg/ml salmon sperm DNA**

Day 2:

- 30 min wash in 2x SSC, at room temperature
- 1 h wash in 2x SSC, at 65°C
- 1 h wash in 0.1x SSC, at 65°C
- 5 min equilibration in buffer 1 (Tris 100mM/NaCl 150 mM, pH 7.5)
- 2 h with anti-DIG antibody, AP-coupled, diluted 1:5000 in buffer 2 (buffer 1 with 0.5% Boehringer Blocking Reagent)
- 2x 15 min wash in buffer 1
- 5 min equilibration in buffer 3 (Tris 100 mM/NaCl 100 mM/$MgCl_2$ 50 mM, pH 9.5)
- O/N staining in buffer 3 containing 45 µl NBT and 35 µl BCIP per 10 ml of buffer 3

Day 3:

- Stop staining reaction with TE buffer (Tris 10 mM/EDTA 1 mM, pH 8.0), 15 min
- Removal of non-specific background in EtOH 95%, 1 h
- Rinse in water, 15 min, to dissolve and remove potential crystals due to TE buffer, which would impair further microscopic observation
- Dehydration and mounting of slides

Table 1. **ISH simplified protocol.** Important improvements are shown in bold characters. Tissues are stored at –80°C until used for the ISH experiment. We routinely perform ISH on 100 tissue sections per day of sectioning.

sion, we also demonstrated that our ISH protocol is specific enough to distinguish between the expression of related genes (12). This protocol allows the use of long non-hydrolyzed DIG-labeled riboprobes and the only remaining parameters to be determined on a case by case basis are the tissue fixation time and the hybridization temperature.

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References

1. Angerer, L.M., and Angerer, R.C. (1981) Detection of poly A+ RNA in *sea urchin* eggs and embryos by quantitative *in situ* hybridization. *Nucleic Acids Research* **9**: 2819-2840.
2. Dagerlind, A., Friberg, K., Bean, A.J., and Hökfelt, T. (1992) Sensitive mRNA detection using unfixed tissue: combined radioactive and non-radioactive *in situ* hybridization histochemistry. *Histochemistry* **98**: 39-49.
3. John, H.A., Birnstiel, M.L. and Jones, K.W. (1969) RNA-DNA hybrids at the cytological level. *Nature* **223**: 582-587.
4. Pardue, M.L., Gall, J.G. (1969) Molecular hybridization of radioactive DNA to the DNA of cytological preparation. *Proc. Natl. Acad. Sci. USA* **64**: 600-604.
5. Gerfen, C.R. (1989) Quantification of *in situ* hybridization histochemistry for analysis of brain function. In: Conn, P.M. (ed.) Gene probes. *Methods in Neuroscience*. Academic Press, San Diego, pp. 79-97.
6. Singer, R.H., Ward, D.C. (1982) Actin gene expression visualized in chicken muscle tissue culture by using *in situ* hybridization with a biotinated nucleotide analog. *Proc. Natl. Acad. Sci. USA* **79**: 7331-7335.
7. Farquharson, M., Harvie, R., and McNicol, A.M. (1990) Detection of messenger RNA using a digoxigenin end-labeled oligonucleotide probe. *J. Clin. Pathol.* **43**: 423-428.
8. Morris, R.G., Arends, M.J., Bishop, P.E., Sizer, K., Duvall, E., and Bird, C.C. (1990) Sensitivity of digoxigenin and biotin-labeled probes for detection of human papillomavirus by *in situ* hybridization. *J. Clin. Pathol.* **43**: 800-805.
9. Desvergne, B., Wahli, W. (1995) PPAR: a key nuclear factor in nutrient/gene interactions? In: *Inducible Transcription*. Bäuerle, Birkhäuser, Boston, Basel, Berlin, vol. **1**: 142-176.
10. Wahli, W., Braissant, O., and Desvergne, B. (1995) Peroxisome proliferator-activated receptors: transcriptional regulators of adipogenesis, lipid metabolism and more. *Chem. & Biol.* **2**: 261-266.
11. Sargent, T.D., Yang, M., and Bonner, J. (1981) Nucleotide sequence of cloned rat serum albumin messenger RNA. *Proc. Natl. Acad. Sci. USA* **78**: 243-246.
12. Braissant, O., Foufelle, F., Scotto, C., and Dauca, M., and Wahli, W. (1996) Differential expression of Peroxisome Proliferator-Activated Receptors (PPARs) – Tissue distribution of PPARα, β, and γ in the adult rat. *Endocrinology* **137**: 354-366.
13. Göttlicher, M., Widmark, E., Li, Q., and Gustavson, J.A. (1992) Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. *Proc. Natl. Acad. Sci. USA* **89**: 4653-4657.
14. Boehringer Mannheim (1996) *Nonradioactive* in situ *hybridization application manual*. Boehringer Mannheim Biochemicals.
15. Chomzcynski, P., Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**: 156-159
16. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, pp. 7.71-7.78, Cold Spring Harbor Laboratory, Cold Spring Harbor, Ny.
17. Sell, S., Thomas, K., Michalson, M., Sala-Trepat, J., and Bonner, J. (1979) Control of albumin and α-fetoprotein expression in rat liver and in some transplantable hepatocellular carcinomas. *Biochim. Biophys. Acta* **564**:173-178.
18. Cox, K.H., DeLeon, D.V., Angerer, L.M., Angerer, R.C. (1984) Detection of mRNAs in *sea urchin* embryos by *in situ* hybridization using asymetric RNA probes. *Dev. Biol.* **101**: 485-502.
19. Lawrence, J.B., Singer, R.H. (1985) Quantitative analysis of *in situ* hybridization methods for the detection of actin gene expression. *Nucleic Acids Research* **13**: 1777-1799.
20. Schaeren-Wiemers, N., Gerfin-Moser, A. (1993) A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: *in situ* hybridization using digoxigenin-labeled cRNA probes. *Histochemistry* **100**: 431-440.
21. Hayashi, S., Gillam, I.C., Delaney, A.D., and Tener, G.M. (1978) Acetylation of chromosome squashes of *Drosophila melanogaster* decreases the background in autoradiographs from hybridization with [^{125}I]-labeled RNA. *J. Histochem. Cytochem*. **26**: 677-679.
22. Miles, E.W. (1977) Modification of histidyl residues in proteins by diethylpyrocarbonate. *Methods in Enzymology* **47**: 431-442.
23. Komminoth, P., Merk, F.B., Leav, I., Wolfe, H.J., and Roth, J. (1992) Comparison of ^{35}S- and digoxigenin-labeled RNA and oligonucleotide probes for *in situ* hybridization. Expression of mRNA of the seminal vesicle secretion protein II and androgen receptor genes in the rat prostate. *Histochemistry* **98**: 217-228.
24. Miyasawa, K., Tomoda, I. (1991) Electrophoretical analysis of serum alkaline phosphatase isoenzymes in neonatal calves. *J. Vet. Med. Sci.* **53**: 807-810.

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Hind III	656 313	5000 units
NBT	1 383 213	3 ml
Proteinase K	1 413 783	1.25 ml
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RNA polymerase, T7	881 767 881 775	1000 units 5000 units
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