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**A simplified in situ hybridization protocol using
non-radioactively labelled probes to detect abundant and rare mRNAs on
tissue sections**

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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 radiolabelled probes is the possibility of detecting very low levels of transcripts, whereas their major limitations are very long exposure times or 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 labelled nucleotides has considerably improved ISH with both a shortening of the development time and an excellent histological resolution (6,7,8). Of all the non-radioactive labelling 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 non-radioactive detection of rare transcripts were not available up to now. Our aim was thus to develop a refined non-radioactive ISH protocol applicable to the detection of rare transcripts, reliable for both cryosections and paraffin sections, and 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 when 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 (PPAR α) 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 PPAR α mRNA per hepatocyte in rat liver. Finally, we have chosen one of the most difficult tissues to prepare, white adipose tissue, to show that this ISH method is also applicable for the detection of rare transcripts on paraffin sections.

Material and Methods.

Cloning of albumin and PPAR α cDNAs.

Two different rat serum albumin cDNAs were cloned in order to synthesize one short (101 nt) and one long (1169 nt) ISH riboprobes. 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 BamHI and HindIII 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'-GCCACCCTTCTAGAGGCCGGAGGCTGAGGCC-3') and albumin-down-2 (5'-CAGCTTTGAATTCTTTGGGGACATATGTCTC-3'). The resulting fragment, 1169 nt long (nt 413 to 1581, ref. 11), was digested with XbaI and EcoRI 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 HindIII and EcoRI 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 PPAR α cDNA fragment of 390 nt was obtained as described (12,13) and cloned into the pBluescript-KS+ and -SK+ vectors to obtain the pKS+/PPAR α (1377-1766) and pSK+/PPAR α (1377-1766) plasmids.

ISH riboprobe synthesis.

The cDNA containing plasmids were linearized as follows: pSK+/PPAR α (1377-1766) and pSK+/ALBUMIN (413-1581) with XbaI, pKS+/PPAR α (1377-1766) and pSK+/ALBUMIN (413-1581) with EcoRI, pKS+/ALBUMIN (1165-1265) with HindIII, and pSK+/ALBUMIN (1165-1265) with BamHI. The linearized plasmids were then gel isolated and used as templates for antisense and sense Digoxigenin (DIG)- and [α -³²P]UTP-labelled riboprobe synthesis (Boehringer Mannheim, Germany, and Amersham Corp., Little Chalfont,

UK, respectively). The transcription mixture (50 μ l) included 1 μ g of linearized template cDNA, ATP, GTP and CTP each at 1mM each, UTP 0.7mM, DIG-UTP 0.3mM, [α -³²P]UTP 30nM, DTT 10mM, RNasin (1 unit/ μ l of transcription mix, Promega, Madison, WI) and T3 or T7 RNA Polymerase (1 unit/ μ l of transcription mix, Promega). Transcription was performed for at least 2 hours at 37°C. The template cDNAs were then digested by RQ1-DNase (2 μ l at 1 unit/ μ l, 30 min at 37°C, Promega), and all reactions were stopped by adjusting the reaction volume to 100 μ l with Tris/EDTA (10/1mM, pH 8.0). The riboprobes were then purified through two precipitation steps by addition of 100 μ l NH₄-Acetate 4M and 500 μ l EtOH 100%, and centrifugation for 30 min at 4°C in a microfuge. The pellet was resuspended in 200 μ l DEPC-treated water. The low levels of [α -³²P]UTP incorporation was used to determine the amounts of riboprobes synthesized and to control the length and integrity of the probes by gel electrophoresis. 5 to 10 μ g of synthesized riboprobe was obtained routinely from 1 μ g of cDNA matrix. The DIG-incorporation into the probes was controlled by dot spots, according to ref. 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, Basel) were rapidly rinsed in diethylpyrocarbonate (DEPC)-treated PBS, and either immediately embedded in Tissue Freezing Medium (Jung, Nussloch, Germany) and then frozen in isopentane and dry ice or fixed overnight (O/N) in 4% paraformaldehyde-PBS (NaCl 0.9 % w/v, NaH₂PO₄ 12.5 mM, NaOH 10 mM, pH 7.5) at room temperature. The O/N fixed liver pieces were then cryoprotected by an incubation of 6 hours in 12% sucrose-PBS and O/N in 18% sucrose-PBS at room temperature and frozen as the unfixed pieces (see above). All tissues were kept at -80°C until used. 12 μ m thick tissue sections were prepared (-35°C, Reichert and Jung frigocut, Nussloch, Germany), mounted on poly-L-lysinated slides, postfixated 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 (2x30 min each) and embedded in three successive baths of paraplast (58°C, Sherwood medical, Athy, Ireland). 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, 2xEtOH (100% and 95%, respectively), DEPC-treated water and DEPC-treated PBS (2x 5 min each). They were then postfixed 10 min in 4% paraformaldehyde-PBS and processed for ISH.

In Situ Hybridization (ISH).

After postfixation in 4% paraformaldehyde-PBS, sections were incubated 2x15 min in PBS containing 0.1% active DEPC (Fluka, Buchs, Switzerland), and equilibrated 15 min in 5xSSC (NaCl 0.75M, Na-Citrate 0.075M). The sections were then prehybridized for 2 hours at 58°C in the hybridization mix (50% formamide, 5xSSC, salmon sperm DNA 40µg/ml; 500µl on each section). The probes were denatured 5 min at 80°C and added to the hybridization mix (400ng/ml). The hybridization reaction was carried out at 58°C for 4 hours for abundant transcripts (i.e. albumin) and 40 hours for rare transcripts (i.e. PPAR α) with 200µl of hybridization mix on each section, covered by a parafilm rectangle (American National Can, Greenwich, CT). Prehybridization and hybridization were performed in a box saturated with a 5xSSC - 50% formamide solution to avoid evaporation. After incubation, the sections were washed for 30 min in 2xSSC (room temperature), 1 hour in 2xSSC (65°C), 1 hour in 0.1xSSC (65°C) and equilibrated 5 min in Buffer 1 (Tris-HCl 100 mM and NaCl 150 mM, pH 7.5). The sections were then incubated for 2 hours at room temperature with alkaline phosphatase-coupled anti-digoxigenin antibody (Boehringer-Mannheim) diluted 1:5'000 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 5 min in Buffer 2 (Tris-HCl 100mM, NaCl 100mM and MgCl₂ 50mM, pH 9.5). Colour 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 15 min in deionised water to remove the precipitated Tris and then dehydrated through successive baths of EtOH (70, 95 and 100%) and xylol (2x15 min each) and mounted in Eukitt resin (O. Kindler GmbH & Co., Freiburg, Germany).

In addition, the following variations of the main steps were analysed in the course of ISH protocol optimization. After cutting and before postfixation, the sections were permeabilized with Triton X100 (see Figure 2 for details). After section postfixation, the active DEPC treatment was replaced by either a rinse in PBS or acetylation (see Figure 2 for details). Prior to equilibration in 5xSSC and prehybridization, the sections were digested with proteinase K (see Figure 2 for details). Probe concentration, hybridization temperature and stringency of washes were also tested (see Results). As for each pair of corresponding antisense and sense probes the number of uridines was identical, 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).

Histological analysis.

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

RNase protection assay.

The plasmid pKS+/albumin (1261-1581) was linearized with ClaI and gel-purified. The plasmid pKS+/PPAR α (1377-1766) was digested with TaqI, and the 717-base pair fragment containing the T7 promoter and the last 287 nt of the PPAR α insert (1480 to 1766) was isolated on agarose gel. From the isolated fragments described above, antisense [α -

³²P]UTP-labeled riboprobes (Amersham Corp.) were synthesized using T7 RNA polymerase (Promega). Probes were 392 nt long for albumin and 334 nt long for PPAR α . Protected fragments were 321 nt long with the albumin probe and 287 nt long with the PPAR α probe. Different ratios of [α -³²P]UTP to cold UTP were used: 1:500 for albumin and 1:1 for PPAR α . Specific activities were 1.5×10^6 cpm/ μ g (1.7×10^5 cpm/nmol) for albumin and 3×10^8 cpm/ μ g (3.4×10^7 cpm/nmol) for PPAR α , 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 μ g of total RNA were hybridized with 10 ng of albumin riboprobe and 1 ng of PPAR α 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 (Sigma) and RNase T1 (Life Technologies, Inc.) was carried out 1 hour 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 show that the short (101 nt) and the long (1169 nt) DIG-labelled 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 appears to penetrate the tissue as well as the shorter one and thus, it allows a higher sensitivity of detection 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 have then been tested first : 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 the albumin (101nt) and PPAR α (390nt) 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 PPAR- α -390nt 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 PPAR α and the signal disappeared also at 65°C . During the prehybridization and hybridization steps, we have 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 insured signal specificity consisted of rinses of 30 min in 2xSSC at room temperature, 1 hour in 2xSSC at 65°C and 1 hour in 0.1xSSC 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 X100 (panels C to F). All sections presented in Figure 2 were hybridized with the short (101 nt) DIG-labelled antisense albumin riboprobe. We show that acetylation of the tissue sections strongly increased the specific ISH signals compared to non-acetylated sections (compare panel A with panel B). We demonstrate also that carbethoxylation with diethylpyrocarbonate (DEPC) is as efficient as the acetylation process (panel C). As proposed by numerous protocols, we have tried a mild digestion with proteinase K or a permeabilization with Triton X100, to allow a better penetration of the probes into the sections. Both procedures reduced tissue preservation and signal intensity (compare panels D, E and F to panel C), and thus were not included further in our standard ISH protocol.

Detection of abundant (albumin) and rare (PPAR α) transcripts

We next compared the efficacy of the ISH protocol for the detection of abundant (albumin) and rare (PPAR α) transcripts in the hepatic tissue. Then, PPAR α detection was also tested in the white adipose tissue, which was chosen 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 an hydrophobic embedding.

First we performed an RNase protection assay to compare the levels of albumin and PPAR α mRNAs in the adult male rat liver (Figure 3A, lane 1). For comparison, antisense undigested probes for PPAR α and albumin are 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 fold stronger than the signal of the PPAR α protected probe. The specific activity of the albumin unprotected probe was 185 times lower than that of the PPAR α unprotected probe (see Material 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 labelled uridines in protected versus unprotected probes for both albumin and PPAR α . With this correction factor, the specific activity of the albumin protected probe was 175 times lower than the PPAR α protected probe. Thus, based on both the signals obtained and the correction factor, albumin transcripts are $1,500 \pm 440$ times more abundant than PPAR α transcripts in adult male rat hepatocytes. Albumin mRNA molecules are estimated at 40,000 per adult rat hepatocyte (17), and thus there are approximately 25 PPAR α mRNA molecules in this cell type.

To demonstrate the usage flexibility of the ISH protocol, we present 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 long antisense (panels 1 and 2) and sense (panels 3 and 4) PPAR α riboprobes. Panels 1 (liver) and 2 (white adipose tissue) demonstrate that our ISH protocol allows an excellent signal resolution. In particular, it reveals high concentrations of PPAR α transcripts in perinuclear regions of hepatocytes and adipocytes (arrows). In contrast, there was no signal with the sense PPAR α control probe (panels 3 and 4).

Discussion

From the results of our experiments, we established an optimized in situ hybridization protocol applicable to the detection of abundant and rare transcripts, both on cryosections and paraffin sections. To achieve this, we have tested, compared, combined and optimized various experimental parameters of a classical ISH protocol. We have simplified and improved many of its steps.

ISH probe synthesis and labelling

Different 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 labelled nucleotides, which results in specific antisense probes with a high sensitivity of detection. 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 provide evidence that intact probes up to 1,200 nucleotide long penetrate well into the tissue, even if it has been fixed for a long period of time (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 as the one used herein in albumin mRNA detection. Under our conditions however, these flanking sequences did not affect the specificity of hybridization, as illustrated in Figure 1.

Sample preparation and cutting

Paraformaldehyde in PBS has been shown to be one of the best fixatives to preserve tissue quality, to keep RNA within the tissue and to allow 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 that 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 to ensure good preservation of tissues during sectioning. Tissues like white adipose tissue require an embedding in an hydrophobic medium like paraffin to avoid alteration of their morphology. In that 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 (Figure 1). Moreover, depending on the tissue, overfixation 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. In contrast, to keep rare transcripts intact in cryosections to further allow their detection, we find it very important to proceed to the ISH protocol immediately after cutting. This procedure is convenient for a single person to process up to 200 ISH slides per week.

ISH protocol

Acetylation is recommended in ISH protocols to decrease background (21). Although we did not observe this effect with the albumin probe, acetylation strongly increased specific ISH signals compared to non-acetylated sections (Figure 2). Acetylation most likely inactivates RNases in the sections, which is consistent with the observation that it can be replaced by the carbethoxylation resulting from diethylpyrocarbonate (DEPC) treatment (Figure 2 C and ref. 22). However, carbethoxylation is easier to perform than acetylation and therefore, we recommend the former. Numerous protocols propose permeabilization of the sections with Triton X100 or a mild digestion with proteinase K to allow a better penetration of the probes. Both procedures reduced tissue preservation and signal intensity, and thus were not included further in our standard protocol for cryosections (Figure 2). It should be noticed

that contrary to unfixed cryosections, strongly fixed paraffin embedded sections can be mildly digested with proteinase K and/or permeabilized with triton X100 to ensure a good penetration of the probe in the section.

Infinite variations in prehybridization or hybridization solutions are suggested in different ISH protocols, all aiming to achieve the right stringency to favor specific, while avoiding unspecific signals. In addition to different saline solutions, the blocking reagents used vary also considerably and include yeast RNA, salmon sperm DNA, Denhart's reagent or various combinations of these. By trying to simplify prehybridization and hybridization solutions, we found that a 5xSSC solution with 50% formamide used between a temperature of 55°C to 60°C confers the desired stringency for RNA-RNA hybrids 100 to 1,200 nucleotide long. 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 hours, Figures 1 and 2). On the contrary, we have observed that the detection of the rare PPAR α transcripts requires a much longer hybridization time (40 hours, Figure 3). After hybridization, our washing procedure ensures signal specificity. Many ISH protocols include RNase digestion during the washing steps to eliminate incompletely or unspecifically hybridized probes (23). In our hands 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 did penetrate very well into the tissue section, 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 higher signal than the short one, as it is composed of a higher number of uridine.

Signal staining and slide mounting

In the signal visualization step, we have chosen 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 and therefore, alkaline phosphatase inhibitors such as levamisole in the signal visualization mixture were not necessary. After the removal of the unspecific labelling in ethanol, a fast rehydration step in water was found necessary to eliminate the Tris precipitate from the tissue, which otherwise hampers histological observation.

Conclusion

We have developed a simplified and efficient protocol for non-radioactive in situ hybridization experiments on tissue sections, the sensitivity of which allows one to study the expression of genes with transcript levels ranging from very low (20-30) to high (several thousands) copy number per cell. Figure 4 summarizes the main steps of this ISH protocol. The immediate processing of cryosections into 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 PPAR α mRNA by increasing the hybridization time up to 40 hours. 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 PPAR α , - β and - γ expression, we have also demonstrated that our ISH protocol is specific enough to distinguish between the expression of related genes (12). With this protocol, long non-hydrolyzed DIG-labelled riboprobes can be used and the only remaining parameters to be determined from case to case are the tissue fixation time and the hybridization temperature.

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Figure legends:

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 2x15 min in 0.1% active DEPC-PBS, and equilibrated in 5xSSC. 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 hours (panels C, D, G, H). Bar : 20 μm ; nt : nucleotides.

Figure 2: Expression of the albumin gene: effect of acetylation, carbethoxylation (DEPC), proteinase K digestion and permeabilization with Triton X100 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 2x15 min in 0.1% active DEPC-PBS (C-F). In panels D and F, sections were digested in 5 $\mu\text{g/ml}$ 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 X100-PBS for 5 min after the postfixation step. Sections were then equilibrated in 5xSSC, 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 .

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

A : Measure of steady state levels of albumin and PPAR α transcripts in adult rat liver by RNase protection assay. Lane 1: 10 μ g of liver total RNA were hybridized with 10 ng of albumin and 1 ng of PPAR α antisense probes. Lanes 2 and 3: undigested antisense probes for PPAR α and albumin respectively. The albumin signal is 8.5 ± 2.5 times (4 experiments) higher than the PPAR α signal, whereas the PPAR α probe has a specific activity 200 times higher than the albumin probe (see Material and Methods, and Results). **B: PPAR- α 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 μ m 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 5xSSC. The ISH protocol was then applied as described in Materials and Methods. Sections were hybridized with 400 ng/ml of PPAR α antisense and sense riboprobes (390 nt). The arrows point to the perinuclear distribution of the PPAR α transcripts. Bar : 20 μ m.

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.

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/MgCl₂ 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

Figure 1 :

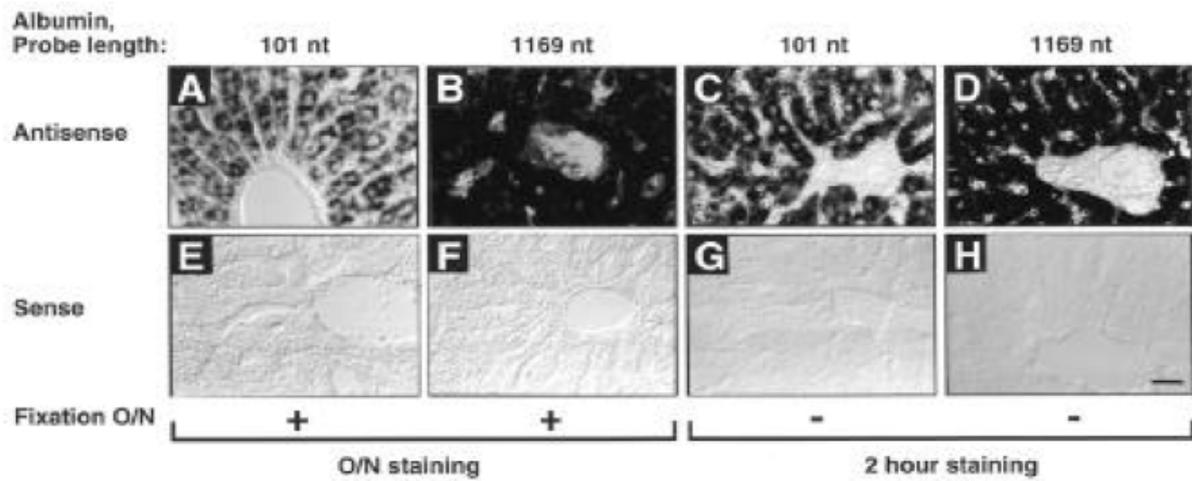


Figure 2 :

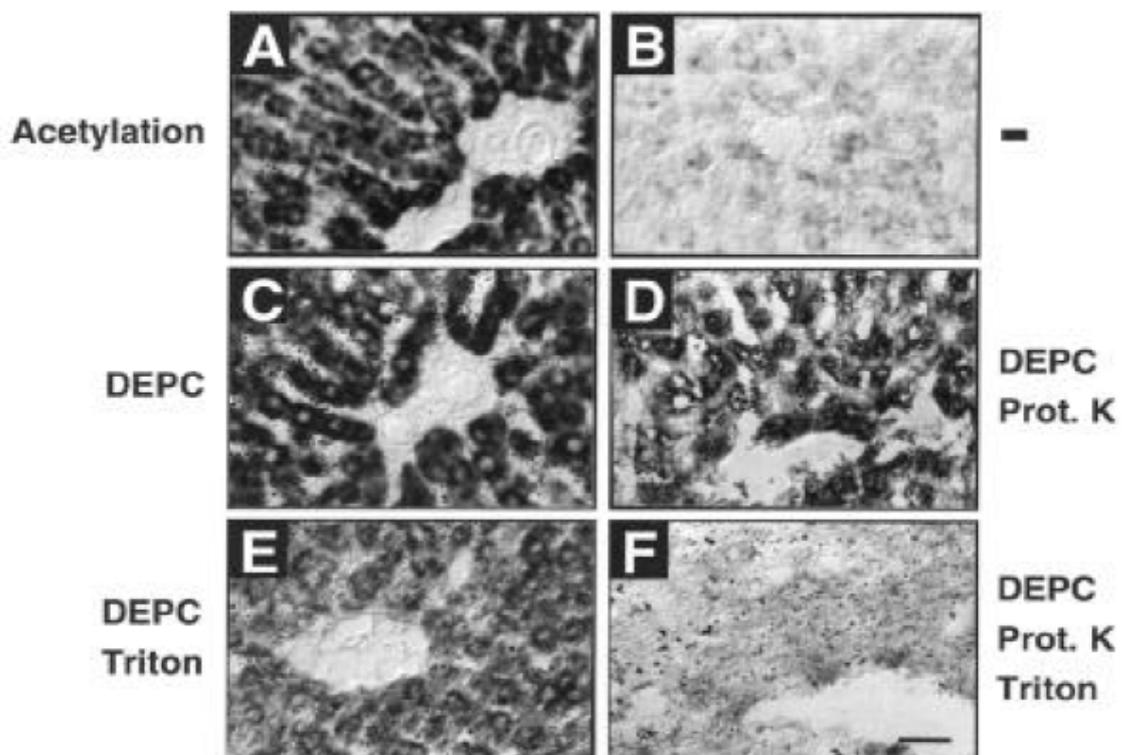


Figure 3 :

