ACTA HISTOCHEM. CYTOCHEM. Vol. 19, No. 6, 1986

NON-RADIOACTIVE IN SITU HYBRIDIZATION PROCEDURES USING MERCURY- AND ACETYLAMINOFLUORENE LABELLED PROBES

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Two methods for nucleic acid hybridization are discussed in which, instead of radioactive markers, labels like fluorochromes or cytochemically detectable enzymes are applied. In one method mercury is covalently bound to the probe in order to allow, after the hybridization, coupling of a ligand which in addition to the mercury-binding thio-group, contains a fluorochrome or a hapten. In another procedure the probe is haptenized before the hybridization by treatment with N-acetoxy-2-acetylaminofluorene, resulting in the covalent attachment of AAF-groups.

In both methods, the haptens in the hybrids are visualized by immunocytochemical procedures.

Our efforts so far, resulted in methods which in addition to speed and a high topological resolution, reached a sensitivity that allows the detection of unique mammalian sequences in both *in situ* and in Southern blot hybridization experiments. With these procedures the detection of separate target DNAs simultaneously within one microscopic preparation is possible, as is *in situ* hybridization on interphase nuclei in suspension. Several aspects of these procedures as well as results obtained so far are discussed.

The development of reliable, sensitive and rapid cytochemical procedures which can be applied to tissue section, cell and chromosome preparations, is extremely important both in basic research and in diagnostic medicine. Such procedures provide for the classical morphological disciplines of histology, cytogenetics and pathology, but also for clinical virologists, access to the rapidly expanding tools of molecular biology, immunology and genetic research.

Hybridocytochemistry or *in situ* hybridization is an approach which allows the detection, localization and even quantitation, of specific nucleic acid sequences in microscopic preparations. The method stems from the fact that the two strands of nucleic acid duplexes can be separated by denaturation and then reannealed (hybridized) with (modified) complementary strands under standardized conditions.

Two types of hybridization are used frequently for the analysis of specific nucleic acid sequences in biological samples. For the first, a more biochemical procedure, DNA or RNA is isolated from the specimen and immobilized on nitrocellulose (if necessary after restriction enzyme cleavage and electrophoresis—Southern blotting),

after which hybridization and detection is performed. In hybridocytochemical procedures, modified DNA or RNA, which is used as a probe to localize the gene sequences of interest (the target sequences) is hybridized directly to tissue section, cell or chromosome preparations. This latter technique was first developed by Gall and Pardue (8) and originally called "*in situ*" hybridization. Until recently, only radioactive-labelled probes were used; the specific nuclear acid hybrids being detected by autoradiography. In 1975 our group started a project to replace the radioactive labelling of the hybridization probe by a fluorochrome or an enzyme. Direct terminal labelling of complementary RNA with a fluorochrome, led to a successful method for the localization of viral DNA in adenovirus-5-infected cultured fibroblasts, kinetoplast DNA in Crithidia, and several genes in Drosophila polytene chromosomes (1–3). In an indirect approach, antibodies are used for the detection of the hybridized target sequences. For these indirect approaches some structural element in the probes or the hybrid has to function as an antigen. One method involves the covalent attachment of AAF groups to the probe by treatment with N-acetoxy-2-acetyl-aminofluorene (14).

In a more recently developed procedure, mercury-labelled nucleic acid probes are used and (after the hybridization) a ligand is introduced which contains in addition to a mercury-binding sulfhydryl group, a hapten group for immunocytochemical detection or a fluorochrome for direct visualization (10, 11). The AAF- as well as the mercury-labelled probes are stable for prolonged periods of time. The possibility to detect separate target DNAs simultaneously within one microscopic preparation was recently realised by combining the detection of one target sequence by use of a mercury- or AAF-labelled probe, with the detection of another with a biotinylated probe. Using two fluorochromes or two cytochemical enzyme procedures for the visualization, the results show sharp localization and clear color distinction between two types of target DNA present in the preparation (12).

The non-radioactive procedures for probe modification mentioned and several other ones (25, 27) are all based on chemical incorporation of markers. Ward and coworkers (18) developed a biotin modification of DNA, which is based on the fact that *in vitro* DNA molecules can be labelled through enzymic incorporation of biotinylated deoxynucleotide triphosphates.

All indirect procedures have in common that the hapten groups in the hybrid are visualized by indirect immunocytochemical procedures or by the potential of biotin to bind strept/avidin.

TECHNICAL ASPECTS

Brief remarks will be given of factors influencing the hybridocytochemical results. For more detailed descriptions of these methods, see Hopman *et al.* (10–12), Landegent *et al.* (14–17) and Raap *et al.* (24), which publications also contain information about hybridization kinetics and the effect of probe size and probe concentration.

Probe modification. AAF-labelling. DNA and RNA probes can be treated with N-acetoxy-2-acetylaminofluorene, a reactive derivative of the well-known and widely studied chemical carcinogen AAF (21). The modification with acetylaminofluorene is relatively easy and fast, and the amount of AAF is proportional to

the relative concentrations of nucleic acid and N-AcO-AAF. AAF binds mainly to the C-8 position of guanosine residues. Routinely a degree of modification of 5% is used. The major substitution product after the reaction is the N-(guanin-8-yl)-AAF adduct.

Mercury modification. DNA probes are mercurated with mercuric acetate and isolated in the form of a cyanide complex. The mercury ions are covalently bound to the 5-C atom of the pyrimidine bases uracil and cytosine, and do not influence the hybridization potential of the nucleic acid probes. Ligands have been synthesized which contain both a sulfhydryl group (to react—after the hybridization procedure—with the mercury in the hybrid) and a hapten group, or biotin or a fluorochrome. When the fluorochrome-bearing ligand is used the hybrids can be detected without further immunological incubations under the fluorescence microscope. When biotin or a hapten has been introduced, visualization requires immunocytochemical procedures (10, 11).

With both procedures it is possible to modify both DNA and RNA, single stranded as well as double stranded, reproducibly in both small and large quantities. As stated before, the modified probe can be stored for longer periods without loss of its ability to hybridize, and the AAF after its reaction with nucleic acids is no longer carcinogenic.

Coating of glass slides. For tissue sections, severe problems were encountered when gelatin-chrome alum (GCA) coated slides were used. Especially after an overnight hybridization, most often the sections were lost from the slides. Therefore, various coating procedures have been tested, and good results were obtained with GCA glass, that had been activated with glutaraldehyde, or with polylysine-coated glass. Tissue sections on slides coated in this way can be treated with proteinase K to improve permeability and accessibility of target without being detached.

Metaphase chromosome preparations are made from cell line or peripheral blood cultures according to conventional methods. For these preparations, and for cell smears, it is not necessary to use the glutaraldehyde-coated glass slides: slides are only cleaned with ethanol/ether (1/1). A so far unclarified observation is the difference of results sometimes obtained with different blood cultures.

Fixation. As with any cytological procedure in which intracellular components are to be detected, fixation is a prerequisite to preserve cellular or chromosomal morphology. In principle any fixation which does not react with the functional groups involved in base pairing is compatible with *in situ* hybridization. Because in double stranded DNA these groups are protected in the double helix, there is a large choice of fixatives. Research will have to be continued to find the best fixation protocols.

Effect of formalin fixation time. As it can be expected that retrospective hybridocytochemical analysis of tissue sections prepared from material from formalin archives will become important, the influence of formalin fixation time was investigated with AAF or Hg-labelled DNA's as probes. For the employed denaturation and hybridization protocol (24) it was shown that long (>24 hr) formalin fixation periods have an adverse effect on the *in situ* hybridization signal. By increasing the protease treatment, however, in some cases improvement can be obtained. The period of time that the tissue is in paraffin, is not important in this

respect.

Accessibility of target. Some of the reactants used in non-autoradiographic *in situ* hybridization are of high molecular weight (probe, antibodies). To increase the accessibility of several targets, the preparations can be treated with protease. This treatment will, however, easily result in loss of morphology.

It is advisable to titrate the protease concentration initially. The protease treatment, which results in the best compromise between optimal *in situ* hybridization signal without losing too much of the morphology, should be used. A general protease protocol cannot be given as the optimal treatment may depend on the type of tissue, the type of fixation, the fixation time and the nature of the target sequences and its surroundings. For instance, for visualization of mouse satellite DNA in formalin-fixed liver tissue, a considerable higher protease treatment is necessary than for the detection of viral sequences in infected tissue sections.

Denaturations. Double stranded target DNA has to be denatured in order to allow hybridization of the labelled probe. Heat, acid and alkaline treatments can be used. Denaturation usually results in loss of morphology and in addition, considerable amounts of DNA can be lost from the cells. As the strands of the *in situ* denatured DNA are in close proximity they will renature rapidly when brought under hybridizing conditions. The probe hybridization will have to compete with this process (23). Several denaturation protocols were investigated. The ethanolic alkaline denaturation, which works well with metaphase chromosomes and isolated methanol/acetic acid fixed mouse liver nuclei, resulted in less intense *in situ* hybridization signals than thermal denaturation when applied to tissue sections. No big differences were observed between simultaneous denaturation of probe and target with 70% formamide/2SSC at 100°C or with 50% formamide/2SSC at 80–90°C. Simultaneous denaturation of probe and tissue has the theoretical advantage that *in situ* renaturation has a stronger competition with *in situ* hybridization.

For formalin fixed tissue sections we found that thermal denaturations using formamide/SSC mixtures give better results than alkaline denaturations and that simultaneous heat denaturation of probe and tissue section under a coverslip is better than separate denaturations.

In situ hybridization procedure. The optimum hybridization temperature is about 25°C below the melting temperature of the DNA. However, incubations at 60–75°C for longer periods of time would cause unacceptable deterioration of morphology. To solve this problem, formamide is added to the hybridization mediums (usually 50% v/v) so that the hybridization can be performed at 35–45°C. Next to formamide, the hybridization medium should contain sufficient monovalent cations to shield the negative charges of the phosphate/sugar backbone of DNA or RNA, a chelator of divalent cations, sufficient carrier DNA or RNA and buffering ions. The probe concentration and hybridization time can be varied according to the copy number of the target sequences, and may range from 3 ng/μl and 5 min, for high copy number sequences to 20 ng/μl and 16 hr for unique sequences.

Addition of 5–10% dextran sulphate may lead to increased signals because of its probe concentrating effect by exclusion of volume. The use of ds DNA probes which also contain sequences that are not complementary to the target but can reanneal (e.g. plasmid sequences), can favor the formation of concatenates and lead to higher hybridization signals.

When DNA probes of ds origin are applied, renaturation and hybridization reactions tend to compete with each other. In order to prevent this problem, ss DNA probes are advantageous, like sequences cloned in the ss DNA phage M13. Similarly, ss RNA probes (e.g. SP6 RNA polymerase-generated RNAs from transcription vectors) can be used. Furthermore, these probes can be applied under high stringency hybridization conditions, thus eliminating *in situ* DNA renaturation.

After the hybridization, the excess of probe is washed away. Also probe that is bound to sequences which only have partial homology with the probe will be removed in this way. The extent to which this occurs depends on the stringency. Stringency can be manipulated by varying temperature, formamide, and salt concentration of the washing fluid.

Immunocytochemical detection. After the hybridization procedures, and for the mercury approach after the introduction of the ligand containing a hapten, visualization is performed by indirect immunocytochemistry. The final marker can be a fluorochrome like FITC or TRITC, a precipitate from a cytochemical enzyme reaction (alkaline phosphatase or horseradish peroxidase) or an electron dense precipitate (13). When the peroxidase DAB/H202 protocol (with imidazole addition) is used on chromosome preparations, it allows visualization of the hybridization signal with reflection-contrast microscopy, which can be advantageous with respect to sensitivity (17). It is also possible to intensify those signals (for both absorbance and reflection contrast microscopy) by gold-silver enhancement procedures, as suggested by Manuelidis & Ward (20), and Burns *et al.* (6).

On the other hand, pigments present in certain tissue sections sometimes make it difficult to distinguish the brown/black DAB precipitate. In such material the alkaline phosphatase BCIP/NBT protocol and Kern Echt Rot counterstaining, which results in blue signals against a red background, is to be preferred. We have shown that both immunoenzyme cytochemical protocols are of equal sensitivity. For double hybridization to be visualized by absorbance microscopy, a combination of peroxidase-labelled and alkaline phosphatase-labelled hybrids is excellent.

The detection of hybridization signals by fluorochrome-marking of the hybrids is also a very sensitive procedure, and the combination of FITC-marked hybrids and propidium iodide as counterstain, which allows visualization of both fluorochromes at the same time, is not only being specific, but also esthetically rather interesting.

For the fluorescent approach, the slides are dehydrated and mounted in PBS/glycerol (1 : 9, v/v) containing 1,4-di-azobicyclo-(2,2,2)-octane (4).

RESULTS

The procedures described in this report are reliable, fast, show a good topological resolution and have reached a sensitivity that comes close to the most sensitive hybridization procedures using radioactive labelled probes (10, 15).

The indirect non-radioactive hybridization procedures developed—see also Brigati *et al.* (5), Forster *et al.*, (7), Rudkin and Stollar (26), Shroyer and Nakane (27), Tchen *et al.* (28), Leary *et al.* (19), Renz and Kurz (25) and Van Prooijen-Knegt *et al.* (22)—are in principle of equal sensitivity, as long as the modification of the probe does not influence the hybridization efficiency. In practice there are

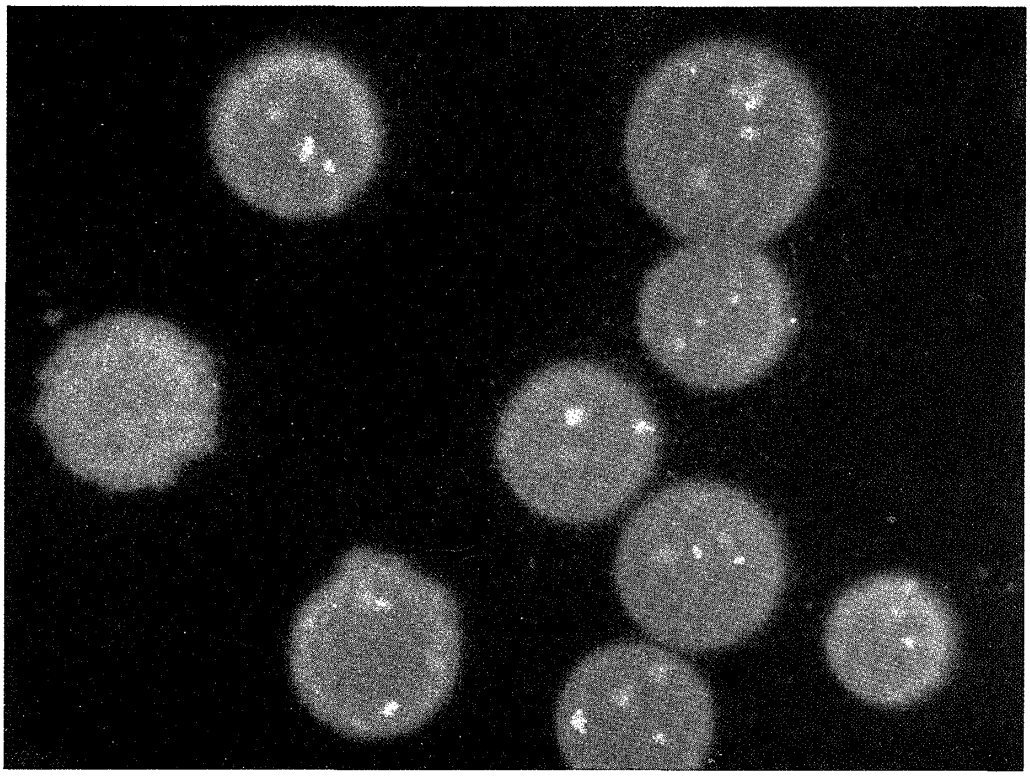

FIG. 1. Human interphase nuclei after double-hybridization. The blue (DAPI) fluorescence is due to the overall DNA staining of the nuclei. Red fluorescence (TRITC) shows the centromeric regions of chromosome nr. 1, and green fluorescence (FITC) is the result of hybridization with a chromosome nr. 15 specific probe. The probe specific for chromosome nr. 1 was biotinylated; the nr. 15 specific probe was mercurated.

differences depending on the target sequences, the type of preparation and the hybridization procedure. Practical differences may be due to the quality of the detection system used. At this moment, in our hands, the mercury approach when applied *in situ*, is the most sensitive more often than the others.

The non-radioactive hybridization procedures do not yet reach the sensitivity achievable with radioactive hybridization procedures. Nevertheless, the sensitivity of the best non-radioactive methods is already quite high. For instance, with AAF-modified DNA probes 1 to 10 pg of specific DNA can be detected in homologous filter hybridizations.

For non-radioactive *in situ* hybridization the lower limit of sensitivity cannot be defined unambiguously, as it is dependent on the type of object studied and the sample preparation. Working with metaphase chromosomes it is possible to localize unique DNA sequences of about 5–15 kb using AAF-, Hg-, or biotin-labelling of the probes (6, 11, 16).

Both the AAF- and the Hg-procedure have the advantage that no enzyme procedures are necessary to modify the probe. The mercury-modification furthermore is hardly changing the configuration of the probe, so that the hybridization efficiency is not changed. The hapten group in this procedure is introduced after

the hybridization, so that no bulky group can interfere with the hybridization.

We so far have used as test objects, nucleic acids of several types: highly-, middle-, and moderately repetitive, and unique sequences. An example of the first type is mouse satellite (about 10% of the total genome present in the centromeric regions) or human ALU DNA (2%, dispersed all over the human genome). In the middle repetitive group (5000–7000 copies) we took an alphoid DNA sequence, assigned to the human X chromosome using the Hg-approach. Of 25 interphase nuclei of an 4X containing cell line, 50% showed 4 spots, 30% 3 spots and 20%, 2 or less spots. Screening 25 metaphases, 80% of the plates contained 4 chromosomes and 20% contained 3 or less positive chromosomes.

Using 28S ribosomal RNA as a hybridization probe to human metaphase chromosomes we were able to visualize the target sequence on each chromatid of the 5 pairs of acrocentric chromosomes, which contain on average 20–120 kb.

Two unique sequences were used in our experiments; part of the human thyroglobulin gene ($\pm$15.6 kb) and a cosmid free of repetitive sequences closely linked to the Huntington disease locus (15).

With the AAF-procedure both sequences could be localized using reflection contrast after POD/DAB marking of the specific hybrids (15); with the Hg procedure both sequences could also visualized using a fluorochrome as the end marker. The figure shows an example of double hybridization signals in interphase nuclei; and finally-not shown here-attention should be given to the fact that *in situ* hybridization using these procedures can also be performed with cells in suspension.

In general, the sensitivity is still not as high as is obtainable with the best radioactive markers, but on the other hand, the sensitivity of the non-radioactive methods is still increased. The advantage of non-radioactive procedures is obvious and manifold. No radioactive waste, no (or only slight) decomposition of the probes, no decay of the marking substances, better topological resolution, speed of the procedure (important for diagnostic applications), to mention some of the major advantages.

The use of double hybridization procedures for both biomedical and diagnostic purposes is evident too. Again to mention a few examples: screening patient's biopsy material by means of multiple hybridization could speed up detection of various viral sequences or viral co-infections if present. Detection of several mRNA's could give an impression of the relative transcription of separate genes in one and the same cell.

Investigation of the three dimensional topography of two genes in interphase nuclei is another topic, as is the detection of the relative position of two gene sequences in normal and abnormal karyotypes.

CONCLUSION

In situ hybridization is a powerful tool for good localization and visualization of genes and gene transcripts during cell differentiation and for the detection of infectious genomic sequences. The advent of DNA and RNA-labelling techniques which allow non-autoradiographic detection of specific nucleic acid sequences, has opened new possibilities for analysis at the nucleic acid level. In practice the conditions of the various steps of the *in situ* hybridization procedures still have to be

optimized with respect to each other so as to get a satisfactory balance between hybridization signal and morphology.

The hybridization procedures are of tremendous importance for the study of cellular processes at the level of gene activity or gene amplification. A procedure to perform *in situ* hybridization with AAF-modified probes on interphase nuclei in suspension (29) can become of great importance for the flow cytometric detection of cells present in a very low percentage in a total cell population. The non-radioactive hybridization approach may also become a key diagnostic aid, and the method of preference for the detection of the expression of oncogenes and other cancer related genes, and for viruses which for other reasons are difficult to detect. It thus may also complement immunopathology techniques. Both hybridization procedures have been tested on highly and moderately repetitive sequences like mouse satellite DNA, 28S ribosomal human DNA and some probes derived from subclasses of the alphoid sequences, but also on unique genes with the thyroglobulin and c5.5 cosmid probes. At present the sensitivity is so that a 15 kb unique sequence of the thyroglobulin gene can be localized at the end of human chromosome nr 8, but our expectations are that, as a results of further investigations, this still can be improved considerably.

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