In Situ Hybridization Using Biotin-Labeled Oligonucleotides: Probe Labeling and Procedures for mRNA Detection

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Summary. Methods for non-radioactive in situ hybridization are rapidly progressing in refinement and application. In this overview, we present our experiences with the use of synthetic oligodeoxynucleotides, labeled with biotin- or digoxigenin-tagged nucleotides by the terminal transferase method.

By in situ hybridization, unique DNA or RNA sequences can be localized in cells by virtue of their specific base-pairing to a labeled complementary DNA or RNA probe. Probes may be manufactured either by cloning methods or by chemical synthesis (Cochlan et al., 1985). As the latter approach does not require access to a molecular biology laboratory and since probes recognizing short, interesting (e.g., hypervariable) regions can be quickly manufactured, it has rapidly gained in popularity (Arentzen et al., 1985; Cochlan et al., 1985; Collins and Hunsaker, 1985; Fuller et al., 1985; Nojiri et al., 1985; Uhl et al., 1985; Lewis et al., 1985; 1986a, b; Ellis, 1986; Jablonski et al., 1986; Morris et al., 1986; Riley et al., 1986; Sherman et al., 1986; Zeff et al., 1986; Hellman and Pettersson, 1987; Larsson et al., 1988). We have used this approach for investigating conditions for hybridization of biotin-labeled probes. Non-radioactive detection of hybridization produces results rapidly and thereby enables us to plan and vary our conditions systematically. In addition, the localization is more precise than that of autoradiography. On the negative side, the sensitivity of non-radioactive detection is sometimes inferior to that of radioactive techniques and certain problems with interfering materials in cells and tissues may be encountered. Nevertheless, these problems do not seem insurmountable and the advantages of non-radioactive detection (rapidity, safety, precision and convenience) make further methodological ventures worthwhile.

GENERAL PRINCIPLES

In situ hybridization requires:

a. Fixation/immobilization of target sequences.

b. Permeabilization of tissue to enhance probe penetration (deleted in some applications).

c. Prehybridization to eliminate unspecific probe binding (deleted in some applications).

d. Hybridization using a labeled probe complementary to the target sequence under specified conditions.

e. Posthybridization washings, during which the stringency of the hybridization is influenced by salt concentrations and temperature.

f. Detection of the labeled probe.

Each of these steps is important for determining the sensitivity, reproducibility and specificity of the hybridization signal. In the following, we will review our experience with short oligodeoxynucleotide probes complementary to proopiomelanocortin (POMC) mRNA. These probes are labelled at their 3'-ends with short tails consisting of biotin-labeled and inter-spaced unlabeled nucleotides (Larsson et al., 1988). The signal detection is obtained by use of alkaline phosphatase, used either in conjunction with streptavidin/avidin or with monoclonal anti-biotin in the APAAP (alkaline phosphatase-anti-alkaline phosphatase) system (Cordell et al., 1984).

FIXATION

Tissue specimens should be fixed as rapidly as possible, preferably by perfusion-fixation. Aldehyde-based fixatives are believed to immobilize mRNA's by physical trapping in a fixed protein network. The more effective the fixative, the more inaccessible the target sequences may become. With weak fixation (e.g., in 1-4% freshly depolymerized paraformaldehyde,
combined with cryostat sectioning), penetration of small oligodeoxynucleotide probes into the tissue may be successful, but stronger fixatives (commercial formalin, Bouin’s fluid and glutaraldehyde) and paraffin embedding require effective permeabilization by proteolytic enzymes (GRIFFIN and MORRISON, 1985; LARSSON et al., 1988). We often use Bouin’s fluid and paraffin embedding. This choice is to some extent dictated by our alkaline phosphatase detection system. Thus, fixation in Bouin’s fluid inhibits intestinal alkaline phosphatase activity, which otherwise is difficult to inhibit. For non-gastrointestinal specimens, other fixatives, like phosphate-buffered 10% formalin, serve well (BRIGATI et al., 1983; MCALLISTER and ROCK, 1985). Thus, the technique can be applied to conventional surgical pathology material. Alternative procedures include the use of freeze-dried, paraformaldehyde vapour-fixed and paraffin-embedded tissue (COGHLAN et al., 1985; LARSSON et al., 1988). Alcohol-based fixatives have also been used with success (MCALLISTER and ROCK, 1985), but are usually not compatible with immunocytochemistry of the neurohormonal peptides we are working with.

SECTION PRETREATMENT

Sections are mounted on aminoalkylsilane-treated slides (in order to withstand subsequent treatments: RENTROP et al., 1986) and are then hydrated and exposed to a variety of treatments for reducing background and increasing probe penetration. We routinely acetylate our sections in order to reduce the background (HAYASHI et al., 1978), but this step is not invariably necessary. In fact, the most important and critical step in the pretreatment schedule is the digestion with proteinase K (BRIGATI et al., 1983; GRIFFIN and MORRISON, 1985; LARSSON et al., 1988). Without this step, very little signal is generated from tissues fixed in strong fixatives. Unfortunately, over-digestion can extract mRNA. Therefore, each new batch of enzyme should be carefully titrated to determine the optimal concentration and time of digestion. In order to inhibit possible contaminating nucleasea, the proteinase K solution contains EDTA and is “autodigested” for 2 h at 37°C (LARSSON et al., 1988). We prepare aliquots of “autodigested” enzyme and freeze-dry and store these at –20°C. The proteinase K digestion is stopped by brief formaldehyde fixation (BRIGATI et al., 1983), and extensive rinsing.

Subsequently, sections are prehybridized in a cocktail containing irrelevant DNA and RNA to preblock unspecific probe binding. In our applications, both the digestion and the prehybridization considerably enhances the signal-to-noise ratio. With more weakly fixed material, the risk of loosing target sequences during these steps must be considered (cf. LAWRENCE and SINGER, 1986).

PROBE CONSTRUCTION AND LABELING

Synthetic oligodeoxynucleotides are synthesized by automatic machinery and their purity checked by HPLC or PAGE. The enzyme terminal deoxynucleotidyl transferase catalyzes the incorporation of nucleotides at the 3'-end of DNA sequences. We employ this enzyme for incorporating biotin-labeled dUTP, containing an 11-carbon atom spacer between the nucleotide and the biotin (Bio-11-dUTP, Enzo Biochemicals, New York) (LARSSON et al., 1988). Reagents for this labeling are available as a kit from Enzo. This kit is for labeling cloned probes and our modifications for labeling oligodeoxynucleotides are given in Table 1.

There are many important considerations with respect to probe construction and labeling. These are best understood against the background of a mathematical formula for approximating the melting point (Tm, i.e., the temperature at which 50% of the hybrids dissociate) (see FITZPATRICK-MCELLIGOTT et al., 1988).

Table 1. Labeling of oligodeoxynucleotide probes using terminal transferase

<table>
<thead>
<tr>
<th>Mix, in the following order, in a plastic Eppendorf tube:</th>
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<tr>
<td>1) 0.1–0.7 nmol Bio-11-dUTP (or other labeled nucleotide, see text) in 0.2 M potassium cacodylate buffer containing 0.9 mM 2-mercaptoethanol.</td>
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<tr>
<td>2) 0–0.7 nmol dCTP in the same buffer.</td>
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<tr>
<td>3) 20 units terminal deoxynucleotidyl transferase, contained in 5 µl 0.05 M potassium cacodylate pH 7.0, containing 5 mM 2-mercaptoethanol and 1 mg/ml nuclease-free BSA.</td>
</tr>
<tr>
<td>4) 0.1–0.2 nmol oligodeoxynucleotide (20- to 30-mer), contained in 5µl redistilled water.</td>
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<tr>
<td>5) Adjust the final volume to 45 µl with redistilled water.</td>
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<tr>
<td>6) Start the reaction by adding 5 µl CoCl₂ (10 mM).</td>
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<tr>
<td>7) Allow reaction to proceed for 60 min at 37°C.</td>
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<tr>
<td>8) Stop reaction by adding 5 µl 100 mM EDTA in redistilled water, whereafter the probe is ready for use.</td>
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T_m = 16.6 \times \log [\text{Na}^+] + (41 \times \text{fraction GC base pairs}) + 81.5 - 1 \degree C / 1\% \text{mismatch} - 675 / \text{probe length}

From this formula it is apparent that the strength of the hybrids is determined by the sodium ion concentration [\text{Na}^+], the temperature, the probe length, its degree of correlation to the target sequence and by its frequency of GC base pairs (the latter form stronger bonds than AT or AU base pairs). A number of publications have reported successful results with probes ranging in length from 15 to about 100 nucleotides (15–100-mer) (Arentzen et al., 1985; Collinson and Hunsaker, 1985; Füller et al., 1985; Nojiri et al., 1985; Uhl et al., 1985; Lewis et al., 1985, 1986a, b; Jablonski et al., 1986; Morris et al., 1986; Riley et al., 1986; Sherman et al., 1986; Zeff et al., 1986; Hellman and Pettersson, 1987). Most synthesis machines prepare up to 30- to 40-mer oligonucleotides with a good yield. Poorer yields are obtained with longer sequences, necessitating much purification. Alternatively, shorter sequences can be enzymatically ligated.

We have found a probe size between 20- and 30-mer to be very convenient for our use. If the target sequence is known, such a complementary probe will be very specific. Thus, from the 4 nucleotides (A, T, G, C) a 24-mer can be made in \(4^{24} (= 2.8 \times 10^{14})\) different ways. Such a sequence will correspond to a peptide sequence of 8 amino acids. Most peptide antibodies recognize a much smaller sequence (usually around 4–5 amino acids) (Larsson, 1988). However, assuming that a “superantibody” were specific for a stretch of 8 amino acids, this sequence could be varied in \(20^8 (= 2.56 \times 10^{25})\) ways using the 20 natural amino acids. From this simple mathematical calculation it should be apparent that quite a high degree of sequence specificity can be attained by shorter oligonucleotides. This has also proven to be the case in practice. As in immunocytochemistry, “cross-reactivity” phenomena could occur with \textit{in situ} hybridizations and the short probes could detect identical or similar sequences repeated in the same or in different RNA and DNA sequences. In analogy to sequence-specific immunocytochemistry (Larsson and Rehfeld, 1977), probes recognizing different regions of the same mRNA molecule can be used (cf. Lewis et al., 1985). Application of these probes to adjacent sections reveals congruent staining patterns for POMC mRNA (Fig. 1). Accordingly, the use of several short probes may be more advantageous than a single large one. Moreover, multiple short probes each have a 3’-end suitable for labeling, and a combined use of multiple labeled probes against different parts of the same target mRNA may increase sensitivity. Finally, it is apparent from the earlier formula that the degree of mismatch strongly influences the melting point. Thus, for investigations of closely related sequences, shorter probes will be more sensitive to small changes than larger probes (cf. Hellman and Pettersson, 1987).

Apart from the probe size, the content of G and C residues is also important for the binding strength. In agreement with others (Lewis et al., 1985), we have employed probes with a GC content between 45–70% and have, so far, obtained our best and strongest reactions with the GC-rich variants. A few probes constructed by us have failed to work. Coghlan et al. (1985) also reported difficulties with some synthetic probes. Perhaps, as they suggested, occasional probes may be designed against poorly accessible sequences.

Terminal transferase incorporates biotin-labeled, digoxigenin-labeled and radioactive nucleotides at the 3’-end of synthetic oligodeoxynucleotides. Especially when UTP or TTP are used, it is desirable to avoid long homopolymer tails that could hybridize to poly-A sequences. We use two different strategies (alternatively or combined) for avoiding this: 1) the tails prepared are much shorter than the anti-sense region, making their binding to irrelevant nucleotide sequences weak; and 2) during the terminal transferase reaction, unlabeled nucleotides (e.g., dCTP) are included to break the monotony of the tail (Larsson et al., 1988). With terminal transferase very long tails have been produced; these may result in increased detection efficiency. In most of our applications, however, we prepare relatively short tails (2–7 nucleotides long). This is very conveniently carried out by permitting the nucleotide concentration to be limiting (Larsson et al., 1988). Thus, the excess enzyme will produce a total incorporation of labeled nucleotides into the probe. We have found that such probes do not need purification before use (details in Table 1).

**HYBRIDIZATION AND WASHING**

A labeled probe (usually at 50–200 ng/ml of 20–30-mer probe, depending upon target sequence abundance) is hybridized to sections in a cocktail containing, i.a., Denhardt’s (1966) solution, dextran sulphate, deionized formamide and carrier DNA/RNA, similar to the prehybridization cocktail (Lewis et al., 1985, 1986a, b; Hoefler et al., 1986; Larsson et al., 1988).
Fig. 1. Adjacent sections of rat pituitary (a, b), submitted to in situ hybridization using 24-mer probes complementary to part of the β-endorphin-coding region (a) or to the α-MSH-coding region (b) of POMC mRNA. For technical details see text and Larsson et al. (1988). Note the congruent reactivity patterns of the two probes with labeling of scattered cells in the anterior lobe (al), of all cells in the intermediate lobe and no labeling in the neural lobe (nl). (c). Higher magnification of the anterior lobe. Note the very distinct labeling of the cytoplasm of corticotrophs. a, b: ×30; c: ×420
Dextran sulphate binds water, thereby increasing the effective probe concentration, while formamide reduces the hybridization temperature (GRiffin and MORRISON, 1985). The hybridization temperature is, of course, dictated by the theoretical melting point of the hybrids and is normally kept 20–25°C below this level (meaning, in most of our applications, 40–50°C). Hybridization is carried out overnight (18–20 h). Subsequently, sections are washed in a series of sodium citrate-sodium chloride (SSC) solutions. As is evident from the formula, an increase in temperature and a decrease in sodium ion concentration results in a weakening of the hybrid binding strength. For practical purposes, washes in 0.1 × SSC at 5–15°C below the theoretical Tm are therefore often employed. Using different hybridization and washing temperatures we have, so far, been able to approximate theoretical melting temperatures using probes with variable degrees of mismatch, although, in practice, exact temperature control is difficult to attain under in situ conditions (LARSSON: studies in progress). Most workers usually employ SSC solutions of descending strengths (ending, e.g., with 0.1 × SSC) for posthybridization washings. However, with the more complex signal detection systems (e.g., double APAAP, vide infra), these long washings are inconvenient. We have observed that direct washings in several changes of 0.1 × SSC at the desired temperature do not sacrifice the low background normally observed.

**SIGNAL DETECTION**

The biotin reporter molecules can be detected either by avidin, streptavidin or by biotin antibodies (BRIGATI et al., 1983). Natural avidin has earned a reputation for stickiness due to its very basic isoelectric point. Most manufacturers now pretreat this protein to reduce its stickiness, and we have very good results using an avidin-alkaline phosphatase conjugate (Dakopatts A/S, Copenhagen, Denmark) (LARSSON et al., 1988). Streptavidin possesses a near-neutral isoelectric point and represents a useful alternative to avidin (BUCKLAND, 1986). Both proteins bind biotin nearly irreversibly. They can be employed as conjugates to enzymes (like alkaline phosphatase or peroxidase) or to particulate markers (like colloidal gold, colloidal silver or ferritin). As these proteins can bind several biotin moieties, they can also be employed as complexes binding biotinylated enzymes or other markers. Such complexes can be made very large, thereby increasing the number of reporter molecules per tissue-bound biotin. Finally, good anti-biotin antibodies have become available. These can be used for biotin detection using any of the many immunocytochemical modifications available (for examples of alternative methods, see HUTCHISON et al., 1982; BRIGATI et al., 1983; LEARY et al., 1983; BURNS et al., 1985; FORSTER et al., 1985; BINDER et al., 1986; LO, 1986; WEBSTER et al., 1987; LARSSON et al., 1988). Recently, we have started using a monoclonal anti-biotin (Dakopatts A/S) for our in situ hybridization studies and have obtained very good results with this. Many different reporter molecules have been tried for non-radioactive in situ hybridization and current interest focusses on the three most sensitive techniques available today: colloidal gold (silver)-silver intensification, silver-intensified peroxidase methods and alkaline phosphatase (LARSSON, 1988). In our hands, alkaline phosphatase surpasses the two other methods in sensitivity and convenience. Alkaline phosphatase is a very durable enzyme, and development times from 10 min to 24 h or longer can be employed, depending upon the detection efficiency needed. In contrast, peroxidase suffers severely from product inhibition and development times exceeding 10–15 min are not useful. As a consequence, however, the precision of the peroxidase-based technique is higher than that of alkaline phosphatase and for specific purposes (e.g., chromosomal studies) peroxidase methods have been used with much success (LANDEGENT et al., 1985; VAN DER FLOEG et al., 1986).

Although it is too early to name a superior method, we routinely employ alkaline phosphatase, now usually in conjunction with monoclonal antibiotin and the APAAP system (CORDELL et al., 1984). This method offers the additional advantage of immunological amplification in several steps (double/triple, etc. method) progressively adding more markers per biotin moiety. Many different developing solutions have been suggested for alkaline phosphatase detection, one of the most favored being a β-chloro-indolyl phosphate-nitroblue tetrazolium (BCIP-NBT) medium (LEARY et al., 1983). Studies on endogenous alkaline phosphatase detection have shown that the use of another tetrazolium salt (tetrainitroblue tetrazolium, TNBT) as well as other modifications could be superior (VAN NOORDEN and JONGES, 1987). In our hands, however, the original BCIP-NBT medium works better with reporter molecules linked to calf intestinal alkaline phosphatase, perhaps reflecting differences between the detection efficiency of different enzyme subtypes. Biotin is a vitamin and forms part of several mammalian enzymes. Endogenous biotin-containing
molecules may therefore interfere with avidin, streptavidin and monoclonal antibiotin detection. A procedure for blocking such interfering activities consists of pretreatment of tissues with, e.g., unlabeled avidin (Wood and Warnke, 1981). The avidin will block the available biotin moieties in the tissue and the surplus binding sites on the tissue-bound avidin are then, in turn, blocked by the application of free biotin (which possesses only one binding site for avidin). Subsequently, an immunocytochemical reaction can be carried out. In our in situ hybridization studies we have found that tumours, in particular, are rich in such biotin-like sites. Blocking these sites with avidin and biotin, prior to hybridization, is only partially effective in combination with probe detection using monoclonal antibiotin. This could, theoretically, be due to different binding sites on biotin for avidin and the monoclonal antibodies; further studies are underway to solve this problem.

Ideally, probes should be labeled with molecules that do not exist endogenously in tissue. Several candidates exist, including acetylaminofluorene, mercury-ligands and digoxigenin (cf. Landegent et al., 1985a, b; Van der Ploeg et al., 1986). We have used the latter approach employing digoxigenin-11-dUTP (Boehringer) and have been able to incorporate this nucleotide into oligodeoxynucleotides by terminal transferase (Larsson: unpublished data). However, so far the sensitivity of in situ hybridization has not been as good with digoxigenin-labeled probes as with biotin-labeled probes.

We routinely employ our biotin-labeled probes, which work admirably well in normal tissues. Further studies are needed to determine whether more efficient preblocking methods or alternative labels should be used for special applications.

CONTROLS

A number of control reactions should be performed in order to substantiate the specificity of hybridization (cf. Lewis et al., 1985; Larsson et al., 1988). These may include:

1. Deletion of the labeled probe to exclude the presence of interfering endogenous activities, such as endogenous enzyme activity and endogenous biotin.
2. Prehybridization of sections with an unlabeled probe to show that the labeled probe reacts with tissue with its anti-sense region and not its tail.
3. Pretreatment of tissue with RNase to prove that endogenous RNA is necessary for the reaction.
4. Hybridization with irrelevant, labeled probes.
5. Combinations with immunocytochemistry in order to determine whether the reactive cells express the corresponding protein or peptide.
6. Sequence-specific hybridization using different probes recognizing different regions of the same mRNA.
7. Northern blotting studies to determine the size of the hybridizable sequence.

The relative importance and practicability of these tests will vary from case to case. Thus, in some instances, an mRNA may be expressed without giving rise to the corresponding protein or peptide (McCabe et al., 1986). Thus, if positive, point 5 may corroborate in situ hybridization results, but, if negative, does not definitely argue against the specificity of the hybridization result. In addition, Northern blotting may be impossible to perform on tissues containing only very few and scattered positive cells. For these reasons the controls are not absolute requirements, but have to be adjusted according to the experimental situation. A potent tool, explored in a few studies, is to employ treatments known to result in variations in mRNA abundancy and to correlate such variations to the intensity of the hybridization signal (for examples, see Kelsey et al., 1986; Romano et al., 1987 and Larsson et al., 1988).

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