In Situ Hybridization Histochemistry

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INTRODUCTION

The technique of *in situ* hybridization histochemistry has become increasingly popular with scientists in a number of fields over the last few years. The unique ability of this method to allow for the detection of specific messenger RNAs in single cells makes it a method of choice to study the regulation of gene expression in small or heterogenous tissue samples. Technical advances have greatly increased the scope of *in situ* hybridization histochemistry and made it accessible to a variety of investigators with minimal expertise in molecular biology. The goal of this book is to present and discuss some widely used methods to perform *in situ* hybridization histochemistry, illustrate their potential with chosen examples and provide an update on some of the newest developments in the field. Most examples are drawn from the field of neurobiology, but the principles developed have much wider applications. It is not our intention to cover every study or method related to the use of *in situ* hybridization histochemistry, but to provide enough information to allow investigators to apply this new approach to a particular scientific question.

In the first two chapters, Lewis and Baldino and Bloch discuss the critical issue of probe choice and preparation, including the most recent advances in the development of nonradiolabeled probes. Jordan then provides detailed information on the different requirements for *in situ* hybridization in cells and tissue sections, and illustrates both approaches in studies of myelin gene expression in the central nervous system. The potential of *in situ* hybridization histochemistry for the study of normal and abnormal gene expression in the brain is further illustrated in the chapters by Frantz and Tobin and by Murray on the use of the technique to study mutant mice and mRNAs encoding growth factors and oncogenes.

The chapters by Eberwine et al. and by Soghomonian describe some of the newest and most promising developments of *in situ* hybridization histochemistry; *in situ* transcription and electron microscopic detection of mRNAs in tissue sections. The first method is expected to allow not only for the localization of mRNAs, but also for the determination of the translational state of specific mRNAs. The second method will provide unique information on the location of various mRNAs within the cell.

As illustrated in these chapters, *in situ* hybridization histochemistry has been most useful in determining the pattern of specific gene expression in a number of tissues. It is now clear from work in tissue homogenates and cell cultures that a number of factors, including developmental events, neuronal activity, and pharmacological treatments can modify gene expression, resulting in a change ni the level of specific mRNAs. In order to study these phenomena with the anatomical resolution characteristic of *in situ* hybridization histochemistry, it will be necessary to develop a means of quantifying the results obtained with this method. This crucial question is addressed by Smolen and Beaston-Wimmer in Chapter 8. Several laboratories have now reported changes in mRNA levels in

identified cell populations under a variety of experimental conditions. One may expect that future use of quantitative *in situ* hybridization histochemistry will greatly contribute to our understanding of factors regulating gene expression in complex tissues. Together with the development of more refined ways to use *in situ* hybridization for the localization of mRNAs into cells, improvement of quantification methods will most certainly broaden the use of *in situ* hybridization histochemistry in the near future. It is our hope that the methodological information and data reported in this volume will help and encourage more investigators to use the potential of *in situ* hybridization histochemistry to answer critical biological questions.

THE EDITOR

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Dr. Chesselet has membership in the American Society for Neurosciences and the International Narcotic Research Council and has published elsewhere extensively.

to

Allan J. Tobin
Michael J. Brownstein
Hans-Urs Affolter

and all who helped us get started

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Chapter 1

PROBES FOR *IN SITU* HYBRIDIZATION HISTOCHEMISTRY

Michael E. Lewis and Frank Baldino, Jr.

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I. INTRODUCTION

Prior to the development of *in situ* hybridization histochemistry, pedigree analysis or somatic cell genetics was required to estimate the location of genes on chromosomes. However, these traditional methods were superceded by the discovery that biosynthesized, radioactively labeled RNA could be used for hybridization to homologous DNA in cytological preparations of chromosomes to indicate the locus of the corresponding gene.[1-3] This technology was rapidly exploited to determine the chromosomal localization of genes encoding 18 and 28S ribosomal RNA, transfer RNA, and histone messenger RNA (mRNA) in a wide variety of species,[4] but was limited by the unavailability of probes for many sequences of interest. This limitation was overcome by the introduction of recombinant DNA technology, which made available a wide variety of complementary DNA (cDNA) probes of known sequence, which were then used to map single copy genes on chromosomal preparations.[5]

The introduction of recombinant cDNA probes also greatly facilitated the cytological study of mRNA, which had previously required the isolation of genome templates for the synthesis of radiolabeled cDNA probes,[6,7] a procedure with very limited applicability. This chapter is devoted to a discussion of the various types of probes which are now available for the study of mRNA *in situ*, as well as the use of various radioactive and nonradioactive probe labeling methods.

II. TYPES OF PROBES

A. cDNA Probes

1. Isolation and Labeling of cDNA Probes

Until recently, hybridization probes were almost invariably obtained from cloned pieces of DNA which are complementary to a particular mRNA species. The complementary DNA (cDNA) clones must be isolated from cDNA libraries of clones which are prepared by enzymatic reverse transcription of isolated mRNA into cDNA copies which are then made double-stranded and inserted into appropriate cDNA cloning vectors.[8] After the positive clone is identified (e.g., by oligonucleotide probe hybridization or antibody binding if an expression system is employed), *Escherichia coli* containing the recombinant plasmid with a cDNA copy of the relevant mRNA are grown in large quantities. The plasmid is then extracted, purified, and digested with a restriction endonuclease to excise the cloned DNA from the vector sequence. The DNA fragment is then purified by gel electrophoresis, eluted, and then labeled by nick translation,[9] i.e., using DNase I to generate random nicks and DNA polymerase I to initiate DNA synthesis with radioactive nucleotide triphosphates at the nick sites (Figure 1). Alternatively, mixed sequence hexadeoxynucleotides can be used as "random primers" to prime the synthesis (by the Klenow fragment of DNA polymerase I) of labeled DNA probes from restriction fragments which have been purified by

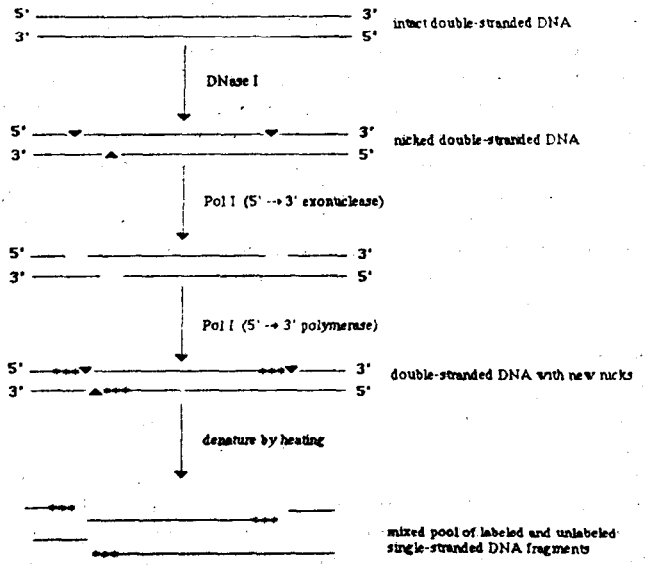

FIGURE 1. Complementary DNA probe labeling by nick-translation (see text for further explanation). Note that method generates labeled and unlabeled fragments of various sizes which can reassociate.

agarose gel electrophoresis following restriction nuclease digestion.[10,11] The radioactive DNA fragments are then purified from unincorporated nucleotide by phenol-chloroform extraction or column chromatography.

2. Advantages and Disadvantages of cDNA Probes

DNA probes prepared as described above will contain many radioactive nucleotides and so can serve as usable probes for *in situ* hybridization, as detailed elsewhere.[12-14] However, as noted before,[15] there are some disadvantages associated with their use, including: (1) difficulty in obtaining the clones from recalcitrant investigators; (2) poor tissue penetration due to excessive probe length; (3) reannealing of the sense and antisense strands during hybridization, effectively reducing probe availability; (4) variable lengths, which precludes Tm studies; (5) the unavailability of particular DNA sequences due to lack of an appropriate restriction site; and (6) the need to establish microbiological and molecular biological methods, which may be particularly daunting to the histochemist who only wants to obtain probes to use as ligands to detect mRNA in tissue sections. Although denatured double-stranded cDNA probes should form hyperpolymers (partially reassociated fragments) which enhance the hybridization signal, such reaggregation appears instead to impair probe pene-

tration to target mRNAs.[16] Some of these difficulties have been overcome by the introduction of a method for synthesizing single-stranded DNA probes from recombinant templates inserted in phage M13 vectors.[17] In this method, an M13 universal primer is used to initiate the synthesis of a radioactively labeled DNA strand which is then purified from the larger template molecule by restriction digestion and gel electrophoresis. While this procedure produces high specific activity single-stranded DNA probes which are usable for *in situ* hybridization,[18] the utility of the method is limited by the low efficiency of transcription (one transcript per template molecule) and possible contamination with vector sequence transcripts.[16] Because of these limitations, investigators have sought more efficient methods of probe synthesis.

B. cRNA Probes

1. cRNA Synthesis

For the synthesis of RNA, plasmids containing specific RNA polymerase promoter sequences (e.g., from phage T7 or the Salmonella phage Sp6) have been prepared with a multiple cloning site (i.e., polylinker) adjacent to the promoter, into which cDNA restriction fragments can be inserted.[19,20] After the recombinant plasmid is grown and amplified in an appropriate bacterial host, and then purified, the plasmid template is linearized with a restriction enzyme that cleaves distal to the promoter and adjacent cDNA insert. An appropriate DNA-dependent RNA polymerase is then used to repeatedly transcribe the cloned sequence (in the presence of radiolabeled nucleotide) to yield the labeled probe (Figure 2). A variety of vectors, such as the pSPT18 and pSPT19 plasmids (Boehringer Mannheim) are designed to have the multiple cloning site flanked on either side by different promoters (e.g., Sp6 and T7). With these plasmids, any DNA cloned into the polylinker is transcribed in one direction with Sp6 RNA polymerase and in the opposite direction with T7 RNA polymerase, yielding labeled probes which will be complementary ("antisense") or identical ("sense", as a control) to the mRNA target.

2. Advantages and Disadvantages of cRNA Probes

cRNA probes have several telling advantages over cDNA probes: (1) they are single-stranded, thus avoiding the reannealing problem; (2) they hybridize with greater stability to mRNA, enabling more stringent posthybridization washes; (3) unhybridized probe can be destroyed by posthybridization treatment with RNase which spares the cRNA-mRNA hybrids; and (4) probes of uniform length can be obtained. Accordingly, it has been reported that cRNA probes hybridize significantly better *in situ* than cDNA probes,[16,21] and have thus begun to see more widespread use.[22-39] (For methodological details on the use of these probes, see References 34 to 36.)

Despite the abundant advantages of cRNA probes, they still require some molecular biological expertise to obtain (e.g., subcloning a cDNA fragment into an SP6/T7 promoter-bearing plasmid, followed by growing and amplifying the

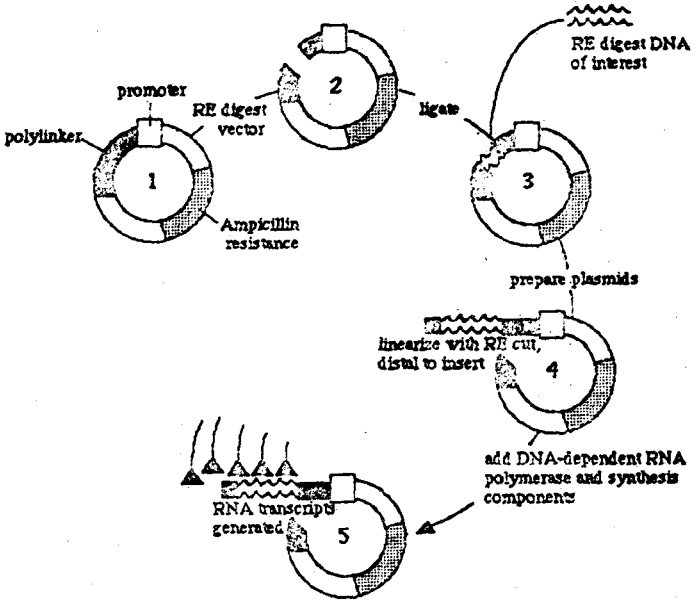

FIGURE 2. Complementary RNA probe labeling using cDNA inserted into specially constructed vector as template for transcription (see text for further explanation).

plasmid in an appropriate bacterial host, etc.), are sensitive to RNases, and may require alkaline hydrolysis into smaller fragments for effective tissue penetration.[21] A further difficulty, which applies to both cDNA and cRNA probes, is that a number of mRNA species are now known to have portions with similar sequences (e.g., the insulin-like growth factors[40-42]). The use of a cloned probe complementary to these homologous regions could readily lead to ambiguous results, a particular difficulty which can be avoided by using probes designed to be uniquely complementary to the nonhomologous regions of mRNAs from a given gene family. Thus, synthetic oligonucleotide probes, as discussed in the following section, may be a useful alternative for some investigators.

C. Synthetic Oligonucleotide Probes

1. Probe Design, Synthesis, and Labeling

The design of synthetic oligonucleotide probes has been discussed elsewhere,[15] and will not be repeated in detail. If the target mRNA sequence is known, probe design is straightforward. The published sequence is generally written 5′ to 3′ (left to right, e.g., 5′-GTCA-3′), so the probe sequence will be complementary from 3′ to 5′ (e.g., 3′-CAGT-5′) although written in the reverse order (e.g., 5′-TGAC-3′). Optimal probe length has not been determined exactly,

but 30 to 50 base sequences should form thermally stable hybrids and, in practice, usually give excellent results. The percent G+C content is also relevant since low content (less than 45% G+C) will tend to reduce the thermal stability of the hybrid, while very high content (greater than 65% G+C) may lead to elevated background labeling of the tissue. The selected mRNA region should be compared to other known nucleotide sequences (via commercial DNA database services) to ensure as far as possible that the probe is uniquely complementary to the target mRNA. If several different animal species are being investigated, and the mRNA sequences are known for each species, regions of perfect sequence homology should be utilized in order to obtain a uniformly efficacious probe.

However, if only the amino acid sequence is known, probe design is greatly complicated by codon degeneracy, i.e., the fact that amino acids are generally encoded by more than one RNA base triplet. Consequently, reverse translation of the amino acid sequence into a corresponding nucleic acid results in a set of several possible coding sequences rather than one unique sequence. Instead of synthesizing a mixture of probes reflecting all codon combinations, investigators have devised multiple strategies for designing what should be an optimal probe.[43] Selecting stretches of amino acids with minimal codon degeneracy (particularly methionine and tryptophan, which are uniquely coded), together with the use of species-dependent codon utilization data, are fundamental strategies. Deoxyinosine can be used to replace other deoxynucleotides at several ambiguous sites (e.g., at A/T or G/T ambiguities) in a probe sequence to enhance duplex stability,[44] and thus should be considered in the design of an optimal probe for an ambiguous target sequence. A hypothetical example of the application of some of these strategies is given elsewhere,[15] but their use to date has been limited to the design of oligonucleotide probes to detect target coding sequences in libraries of cloned DNA segments. Computer programs, such as PROBE (Intelligenetics, Inc.), which incorporate known oligonucleotide design strategies, should facilitate the application of this approach to developing probes for *in situ* hybridization histochemistry. In the event that multiple "optimal" probes are used, *in situ* hybridization might be useful in screening for the correct sequence since hybridization conditions could be adjusted to prevent hybridization of a probe with even a single base mismatch.[45,46] The synthesis and purification of synthetic oligonucleotides has been discussed elsewhere,[15,47,48] and suffice to note here that many research institutions, universities, and commercial organizations now have suitable facilities and trained personnel to carry out a custom synthesis and purification for a reasonable fee.

After the oligonucleotide probe is obtained, several labeling options are available: 5′ end-labeling, primer extension, and 3′ end-labeling (Figure 3).

The first method, 5′ end-labeling, uses the enzyme T4 polynucleotide kinase to transfer the terminal phosphate from [γ-^{32}P]ATP to the free 5′ hydroxyl group of the oligonucleotide. Although the specific activity is limited by the addition

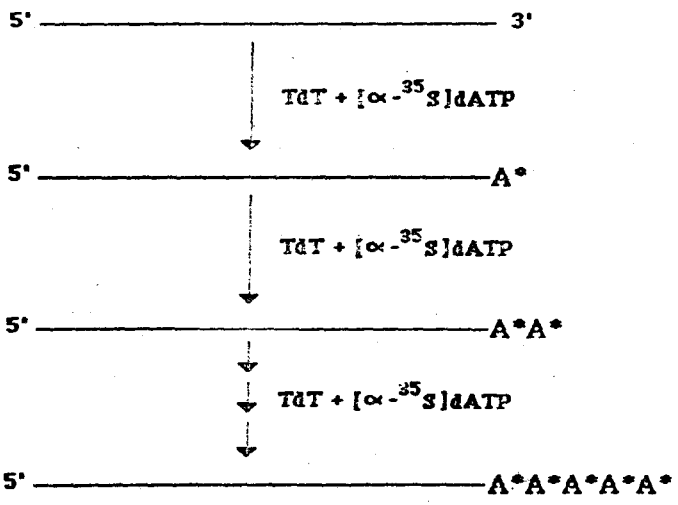

FIGURE 3. Synthetic oligonucleotide probe labeling by progressive enzymatic addition of labeled nucleotide to the 3′ end (see text for further explanation).

of a maximum of one label per molecule of probe, this method has been found to be suitable in some studies of relatively high abundance mRNAs.[15,49-51]

The second method, primer extension labeling,[52] uses the Klenow fragment of DNA polymerase I to catalyze the synthesis of the probe (by extension of a synthetic primer oligonucleotide in the presence of labeled deoxynucleoside triphosphates) across a complementary oligonucleotide template. Although this method has been used to create relatively high specific activity probes for *in situ* hybridization studies,[53-56] the procedure is complicated by the need to prepare both template and primer oligonucleotides, and then separate them electrophoretically to isolate the extended, labeled primer from the reaction mixture. Nevertheless, in contrast to the 5′ end phosphorylation method, lower energy radioisotopic labels (from ^{3}H, ^{35}S, or ^{125}I-labeled deoxynucleoside triphosphates) can be incorporated into the probe to facilitate high resolution anatomical studies.

The third procedure, 3′ end-labeling, uses the enzyme terminal deoxynucleotidyl transferase to catalyze the sequential addition of radioactive deoxynucleoside monophosphates (from appropriately labeled deoxynucleoside triphosphates) to the free 3′ hydroxyl end of the synthetic oligonucleotide.[57] Since this enzyme will continue adding deoxynucleoside monophosphates to the 3′ end, the specific activity of the probe (i.e., probe length) can be controlled by reaction conditions such as time and substrate concentration. This labeling

method has been used for the *in situ* detection of mRNAs encoding proopiomelanocortin,[15,58] vasopressin,[55,59-62] oxytocin,[60] somatostatin,[62-65] enkephalin,[25,47,66] dynorphin,[66,67] substance P,[66] neuropeptide Y,[68] corticotropin releasing factor,[69,70] vasoactive intestinal polypeptide,[62,71] cholecystokinin,[72] β-amyloid,[73] calmodulin,[74] and tyrosine hydroxylase,[66,75] among others. Collins and Hunsaker[76] have also used 3′ end-labeling to prepare high specific activity probes for genomic blotting studies. The obviously noncomplementary "tail" does not appear to impair either the stability or the specificity of the hybrids.[15,58,70] Electrophoretic separation of probes with different length "tails" is therefore unnecessary, and the labeled probe can be separated from the reaction mixture by a rapid and simple chromatographic step.[55] Thus, while the primer extension and 3′ end-labeling methods share the advantage of incorporating multiple-labeled nucleotides into each molecule of probe, the latter procedure is technically much easier to perform.

2. Advantages and Disadvantages of Oligonucleotide Probes

In contrast to cDNA and cRNA probes, synthetic oligonucleotides do not require any molecular biological expertise to obtain or label. These probes are designed to be complementary to a known mRNA sequence, and are then chemically synthesized by automated apparatus, and labeled enzymatically (see References 15, 34, and 48 for review). Following their successful use as hybridization probes for isolated mRNA in Northern blots,[77-82] synthetic oligonucleotides were successfully used for the *in situ* detection of many mRNAs, as noted above. These numerous studies indicate that synthetic oligonucleotides are useful probes for the detection of a wide variety of target mRNAs *in situ*, and are therefore a viable alternative for the investigator who prefers to avoid the requirements for obtaining biologically derived cDNA or cRNA probes. Nevertheless, since synthetic oligonucleotides generally cannot be labeled to the specific activity which is readily possible with cRNA probes, the study of very rare mRNAs may be more effectively carried out with cRNA probes which share the advantage of being single-stranded and have the further advantages of increased hybrid stability, higher specific activity, and posthybridization enzymatic removal of unhybridized probe.

III. PROBE LABELING OPTIONS

A. Radioisotopic Labels

Probe labeling reactions have been carried out using substrates incorporating ^{3}H, ^{32}P, ^{35}S, and ^{125}I. Although some investigators have succeeded in obtaining cellular resolution with ^{32}P-labeled probes,[25,51] most high-resolution studies have utilized probes labeled with the other three of the above radioisotopes. While tritium offers the highest resolution possible, prolonged exposure times are necessary. ^{125}I-labeled probes permit particularly short exposure times together with high resolution.[55,61,63,84] While high background labeling may sometimes