In Situ Hybridization in Endocrine Pathology

In situ hybridization (ISH) is rapidly becoming another powerful tool that is ideally suited for many types of analyses in endocrine and other areas of pathology. This procedure combines molecular biological and morphological techniques and allows the morphologist to study patterns of gene expression in heterogeneous cell populations in tissue sections, single cells, or chromosomal preparations. The early studies of Gall and Pardue (3) anticipated the potential usages of this technique when they hybridized ribosomal RNA to the amplified ribosomal genes in oncocytes of *Xenopus* and showed that the transcripts could be readily detected by light microscopy. Some of the earliest applications of ISH in endocrinology included the demonstration of gene products for growth hormone (GH), prolactin (PRL), and beta-endorphin in the rat pituitary gland (5, 10).

ISH is used frequently in the research laboratory to analyze morphology and function concurrently. The potential uses of ISH in diagnostic pathology are numerous. Although immunohistochemistry has proved to be an excellent method of correlating morphology with the presence of protein and other products in individual cells, many problems cannot be resolved with the immunohistochemical approach. Some of these include (1) separating de novo synthesis from uptake by pinocytosis or other processes, (2) the inability to detect proteins that are not stored in any significant amount in cells, and (3) the absence of specific protein products in cells that may have nucleic acids that are not translated into proteins.

The technical aspects of ISH have been discussed in various reports (2, 7, 12). However, some of the critical methodological approaches should be emphasized. The choice of probes for ISH will largely determine the degree of success in demonstrating specific gene products. When there are abundant copies of the RNA or DNA of interest, such as with many hormones or with viral infections, a relatively insensitive probe or detection system will suffice. However, when there are low levels of nucleic acids, the most sensitive probes and detection systems should be utilized. RNA probes (riboprobes or antisense probes) are generally considered to be the most sensitive. cDNA probes are also effective, but the bonds between the DNA-RNA hybrids are weaker than those of RNA-RNA hybrids. The use of synthetic oligonucleotide probes offers several advantages including the following: (1) The nucleotide sequence is completely known, (2) a large amount of probe can be generated rapidly with a synthesizer, and (3) these smaller probes of 20–50 bases can readily enter tissue sections and penetrate cells, so very little prehybridization treatment or posthybridization washing is needed.

Another variable that helps to determine sensitivity is the preservation of nucleic acids by fixation. Although ethanol-acetic acid was one of the earliest fixatives used for ISH, it is generally recognized that paraformaldehyde is the best all-around fixative and that tissues fixed in neutral buffered formalin rank closely with paraformaldehyde-fixed tissues in the preservation of nucleic acids for ISH analyses. Some fixatives that work well in immunohistochemistry to detect proteins may not preserve nucleic acids as effectively. For example, detection of viral infections such as human papillomavirus in Bouin's-fixed tissue is much more difficult compared to formalin-fixed sections because of the degradation of the nucleic acids (9).

Although non–isotopically labeled probes such as biotin and digoxigenin have been used to detect DNA and messenger RNA (mRNA) by ISH, they are generally less sensitive than radioactive probes and it is more difficult to quantify the results. The disadvantages of working with radioisotopes for ISH must be balanced against the need for greater sensitivity to detect low abundant RNA transcripts. Recent developments such as the use of oligonucleotide probes linked directly to alkaline phosphatase and the use of streptavidin-peroxidase with silver enhancement suggest that more sensitive nonisotopic methods will compete with radioactive probes as the most sensitive methods for signal

detection in the near future. Similarly, recent developments in chemiluminescent detection systems for Northern and Southern hybridization analyses is another promising area in the use of nonisotopic signal detection.

Because of the great degree of sensitivity of ISH and the elaborate methodologies involved in the procedure, the possibilities of obtaining false-positive and false-negative results are high. It is thus necessary to have several positive and negative controls for each set of experiments. Positive controls may include Northern hybridization analysis of the same RNA studied by ISH and detection of the protein product for the RNA transcript of interest. Negative controls can include sense probes for oligonucleotide as well as cRNA probes, use of ribonuclease treatment before hybridization, and use of tissues that do not express the nucleic acids being analyzed in the experiment. Appropriate controls to check the specificity of the detection system, such as positive chemography with radioactive probes and endogenous biotin or alkaline phosphatase with some nonisotopic probes, should also be done.

The uses of ISH in endocrine pathology are expanding daily with the cloning and characterization of new genes. Analysis of the role of oncogenes and growth factors in the development and regulation of endocrine neoplasms promises to be one of the major areas for application of ISH analyses. The presence of RNA transcripts in cells producing ectopic hormones in which very little protein is stored should be another area in which ISH is more effective than immunohistochemical analysis. Although individuals with small-cell carcinoma of the lungs produce many peptides including bombesin, it is often difficult to detect these hormones in the neoplasm by conventional immunohistochemical study. The use of ISH with cRNA probes has enabled investigators in a recent series to localize probes in the cytoplasm of tissue (6 cases), cell lines (2 cell lines), and cytological preparations (3 cases) (4). ISH in endocrine pathology may also be more sensitive than immunohistochemistry in the analysis of protein and gene products that undergo extensive posttranslational processing. For example, chromogranin A is processed to pancreastatin and other peptides. The use of highly specific monoclonal antibodies may produce a weak signal for specific chromogranin A peptide products that have been modified by proteolysis, whereas the presence of chromogranin A mRNA transcripts may be detected with a greater degree of sensitivity by ISH.

Recent ISH studies of the human pituitary gland have provided a great deal of insight about the biological features of pituitary adenomas. Tumors from patients who have acromegaly frequently express prolactin mRNA, whereas most tumors from patients with a clinical diagnosis of prolactinomas have only prolactin mRNA in them (8). These observations fit with recent studies of transgenic mice, which have shown that growth hormone cells in the developing fetus are precursors of most prolactin cells (1), suggesting that the prolactin cell represents the final stage of differentiation of acidophilic cells in the adenohypophysis. ISH hybridization studies of some acidophilic pituitary tumors from patients without acromegaly have helped to characterize a silent GH-producing adenoma subtype. These tumors contain some immunoreactive GH as well as the mRNA for this hormone, but there is only minimal serum elevation of GH and these patients do not have acromegaly (6).

The use of ISH in the analysis of endocrine tissues can sometimes produce more questions than answers. The pituitary transcription factor Pit-1/GHF-1 protein is expressed in a subset of anterior pituitary cells—namely, the lactotrophs, somatotrophs, and thyrotrophs—and is believed to be important in the ontogeny of lactotrophs and somatotrophs. However, recent ISH hybridization analyses have shown the Pit-1 RNA transcripts in all five pituitary cell types, including gonadotrophs and corticotrophs, whereas only lactotrophs, somatotrophs, and thyrotrophs expressed the Pit-1 protein (11). This represents an enigma regarding the role of Pit-1/GHF-1 in lineage-specific anterior pituitary cell expression of mRNA transcripts and protein products.

Although the applications of ISH to diagnostic molecular pathology have begun to be discovered only recently, ISH should become a powerful tool for the analysis of gene products in tissue sections and cytological specimens. The rapid advances being made in the description of the structure and location of new genes will provide many more tools for ISH analyses at an ever-

increasing pace. Some of the major problems in endocrine pathology, including regulation of neuroendocrine differentiation, the progression from hyperplasia to neoplasia, the ability to distinguish between hyperplasia and neoplasia, and the ability to characterize the malignant potential of some endocrine tumors before they metastasize, will become more amenable to rigorous analyses with these new advances.

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