A peek at the future through histological preparations

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ABSTRACT When histologists and pathologists examine histological preparations, they can often predict the future of tissues that have not been excised from bodies. This is possible because, unlike chemical and physical matter, living organisms travel in time on a genetically mandated fixed path. To recognize a portion of the path that tissues have already traveled, histological methods such as histochemistry and immunohistochemistry are effective. In this regard the development of the peroxidase-labeled antibody method (Nakane and Pierce, *J. Histochem. Cytochem. 14:* 929-931, 1966) contributed immensely. For the portion of path which cells and tissues were traveling when they were removed from bodies, the method of choice is localization of mRNA by *in situ* hybridization. Specific methods designed to predict the future path of tissues are still at the drawing board phase. However, by discerning the past and current portions of the path and by referring to the paths that other tissues have traveled, one may deduce the future path of the tissues in question. For some time now, it has been my dream to develop methods enabling us to *peek at the future* path of tissues more concretely. To accomplish this one requires new procedural approaches. Thus, we would like to introduce *in situ* nick translation and oligonucleotide histochemistry.

KEY WORDS: *immunohistochemistry, oligonucleotide histochemistry, in situ nick translation*

In situ nick translation

In living cells and tissues, it has been demonstrated that genes that are ready to be activated in a near future may be activated when single stranded breaks are introduced into the genomic DNA by ultraviolet rays or by certain chemicals (Tanno *et al.*, 1987). Hence, by exposing cells or tissues to ultraviolet light and observing what genes are activated, one may predict some future activity of living cells. However, this approach is not applicable for fixed cells or histological preparations. To identify genes that were either active or ready to be activated in fixed cells or tissues, one of the methods we are developing (Koji *et al.*, 1989) is based on the assumption that single stranded DNA breaks accumulate during cell differentiation and maturation and those breaks in «active nuclear chromatin» act as initiation points of active genes and ready-to-be-activated genes. On the other hand, those in «inactive chromatin» will not participate in the future gene activities. The method is designed to discriminate those genes in the active chromatin from those in the inactive chromatin by utilizing *in situ* nick translation. In fixed cells and tissues, active chromatin is loose in the nucleus and DNA polymerase used in nick translation is able to make contact with the breaks, where it quickly initiates translation; whereas inactive chromatin is tightly netted and covered with basic nuclear proteins such as H1 histone. Since the DNA polymerase does not gain access to the breaks, these genes will not be translated. Once identified, the genes translated by this method indicate the active genes and genes ready to be activated. Thus, it is possible to deduce the present as well as future activity of the cells.

Details of *in situ* nick translation include the following. In this method, a mixture of DNA polymerase I, dATP, dGTP, dCTP and biotin and biotin-11-dUTP (or TTP) was first applied to sections of fixed tissues. In this step, the DNA polymerase I was expected to act upon the single stranded DNA breaks and incorporated biotin-11-dUTP. The biotin incorporated into DNA was then detected immunohistochemically using HRP-labeled anti-biotin antibody. Various experiments were performed to confirm that the DNA polymerase acted on the breaks in the active chromatin. On the assumption that the chromatin which is accessible to the DNA polymerase is also accessible to DNase, formaldehyde-fixed cells were treated extensively with DNase prior to the *in situ* translation. It was found that most of the DNA breaks which were accessible to DNA polymerase had been removed. However, when the DNase

Abbreviations used in this paper: T-T, thymine-thymine; mRNA, messenger RNA; R anti-T-T, rabbit anti-T-T dimer DNA antibody; HRP-G anti-R IgG, goat anti-rabbit IgG conjugated with HRP; PCNA, proliferating cell nuclear antigen; HRP, horseradish peroxidase; REBP, regulatory element binding proteins; RE, regulatory element; CREBP, cyclic AMP responsive element binding protein; CRE, cyclic AMP responsive element; GREBP, glucocorticoid receptor protein; GRE, glucocorticoid regulatory element.

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0214-6282/93/$03.00

Printed in Spain

treatment was followed by proteinase K digestion, additional DNA breaks were exposed. Using PCNA genomic DNA as a marker, formaldehyde-fixed lymphocyte and granulocyte nuclei were subjected to the DNase treatment. It was found that most of the PCNA gene (Nakane *et al.*, 1988) was digested in the lymphocyte nuclei, whereas the PCNA gene in the granulocyte nuclei remained. An experiment with mouse ear skin was carried out on the assumption that if exogenous DNA polymerase is accessible to active chromatin, then endogenous DNA polymerase should also be. By first introducing T-T dimers into nuclear DNA of squamous cells by ultraviolet irradiation and monitoring the fate of the T-T dimers, it was found that most of the dimers were repaired quickly by endogenous DNA polymerase, whereas the T-T dimers that remained after two days were not extractable by DNase. The latter dimers, however, became accessible to exogenous DNase and DNA polymerase after proteinase K digestion. These results suggested that the DNase treatment removed the active chromatin and that the subsequent proteinase K treatment exposed breaks in the inactive chromatin. Using a combination of DNase treatment, proteinase K treatment and *in situ* nick translation, it was found that cells with potential to differentiate, such as peripheral lymphocytes and basal cells in skin, contained more breaks than terminally differentiated cells, such as nerve cells and chondrocytes in active chromatin. On the other hand, terminally differentiated cells contained more single stranded DNA breaks in their inactive chromatin than those in the inactive chromatin of proliferating cells.

Following *in situ* nick translation, the inactive chromatin was selectively extracted by a method modified after the procedure of Stratling (1987) from liver tissue sections. It was found that biotin-11-dUTP labeled glutathion peroxidase gene, which is always activated in liver, remained on the section, whereas prolactin gene was unlabeled and extracted. This finding, together with the studies with DNase and proteinase K, suggests that our *in situ* translation method mainly incorporated biotin-11-dUTP into DNA in the active chromatin. Thus, by knowing which genes incorporate the marker by *in situ* nick translation, one should be able to identify which genes are either in an active state or will become active, hence the future gene activity of the cells. What is now required to complete the procedure is to develop a quick method of identifying what genes are situated in the active and inactive chromatin.

Oligonucleotide histochemistry

Another method providing a «peek at the future» is a new-comer to the field of histochemistry. The term coined, «oligonucleotide histochemistry» (Koji *et al.*, 1990), denotes a method based on the principle that many gene transcriptions are modulated by the binding of specific protein to specific DNA sequences in nuclei. For example, following cyclic-AMP stimulation, a specific protein, CREBP, binds to CRE on DNA and activates transcription of a wide spectrum of genes. Hence, cells containing REBP have a potential to respond when proper stimuli reach the cell. By capitalizing the specific affinity between REBP and regulatory element (RE), REBP may be localized by reacting synthetic oligo-RE DNA with REBP in cells and tissues. For the localization of CREBP, TTATTATTA nucleotide sequence was added onto the 5' end of TGACGTCA, the CRE nucleotide sequence (Angel *et al.*, 1987), during synthesis. The TGACGTCA being palindromic, when solution containing the oligonucleotide was gradually cooled, the RE segments formed double stranded DNA, whereas the TTATTATTA segment remained single stranded. These reannealed DNAs were then exposed to ultraviolet light to form T-T dimers between the adjacent T-Ts (Nakane *et al.*, 1987, 1991). Excised tissues were sectioned frozen, fixed by formaldehyde and were reacted with the reannealed DNAs without dissociating to single strands. The probe binding sites were recognized by immunohistochemically localizing the T-T dimer using R anti-T-T and HRP-G anti-R IgG dimer (Nakane *et al.*, 1987). It was found that the CRE sequence bound mainly to nuclei of epithelial cells in small intestine, and to nuclei of hepatocytes and littoral cells in the liver of saline injected rat. With rats injected with cAMP the amount of CRE bound to the nuclei of small intestine and liver was reduced dramatically. With rats injected with cortisol, the amount of CRE bound to nuclei of small intestine was reduced slightly, whereas CRE bound focally in the nuclei of hepatocytes. These observations suggest that the amount of free CREBP fluctuates depending on the physiological state of the animal, and following cAMP injection most of the CREBP is bound to nuclear CRE and the exogenous CRE DNA no longer binds. This type of information on the state of CREBP is unattainable by immunohistochemical CREBP detection since free and bound CREBP have a similar antigenicity.

For the localization of GREBP, fragmented pBR 322 DNA containing GRE sequences (Tully and Cidlowski, 1987) was used. The staining with pBR 322 DNA was found in both nuclei and the cytoplasmic area of hepatocytes of the adrenalectomized rat, though the interpretation of the staining was somewhat complicated because pBR 322 DNA contains one CRE sequence as well. The patterns of staining were altered when the rats were either stimulated or suppressed endocrinologically.

Recently many more specific REBP as well as RE have been identified; these are sometimes referred to as tissue specific enhancers, tissue specific promoters and more recently as silencers or dehancers (Hori, 1991). All of these specific interactions between specific protein and specific DNA segments are candidates for oligonucleotide histochemistry. By recognizing what REBP are present and in what state (free or bound), one should be able to assess the cell potential, and hence *peek at the future* of the cell.

Conclusion

By employing these various new histological procedures in combination, one is able to assess the *past*, the *present* and the *future* activities of the preparation. It is therefore possible to trace the dynamic successive events of a biological system in otherwise static preparations.

Acknowledgments

Without the insight and guidance of Professor G. Barry Pierce, I would not be what I am. He provided me with jobs, a place to work and funds. I will never cease to be grateful to him for what he has done for me.

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