Apoptosis, or programmed cell death, plays a central role in tissue remodeling during development and differentiation as well as in the normal adult animal (5). Moreover, it is becoming apparent that apoptosis may be involved in the pathophysiology of several disease states. Apoptosis differs from necrosis in several important molecular, biochemical and morphological features that serve as the basis for the detection and quantitation of these two mechanisms of cell death (2). One important feature of apoptosis is the random internucleosomal cleavage of the chromosomal DNA, yielding fragments of DNA with a size corresponding to multiples of 200 bp; i.e., the size of a nucleosome. DNA isolated from tissues or cultured cells can be size-separated on agarose gels to observe the hallmark pattern of DNA bands, or apoptotic ladder. Initially, large amounts of DNA were required for the analysis of these ladders because the DNA was detected by ethidium bromide staining. Tilly and Hsueh (3) developed a microscale autoradiographic method for the qualitative and quantitative analysis of apoptotic DNA fragmentation. The procedure uses terminal transferase to uniformly add one molecule of [α-32P]dideoxyribonucleotide to the 3’ end of the DNA fragments. This method increased the sensitivity of apoptotic DNA detection at least 100-fold over that observed with the use of the ethidium bromide staining method.

While this procedure is valuable for the analysis of apoptosis in minute quantities of tissues and cultured cells, radioactive techniques require expensive laboratory equipment and result in costly disposal fees. Moreover, these techniques are time-consuming and potentially hazardous to laboratory personnel and the environment. Recently, we have developed a new method for the detection of DNA ladderig using a nonradioactive labeling technique. This method, a modification of the method used by Tilly and Hsueh, involves the labeling of cellular DNA with digoxigenin-11-2’3’-dideoxy-uridine-5’-triphosphate (Catalog No. 1 218 603, DIG-11-ddUTP; Boehringer Mannheim, Indianapolis, IN, USA). Following agarose gel electrophoresis and Southern blotting, the DNA was detected immunochromatically using an anti-DIG antibody conjugated with alkaline phosphatase and then visualized with...
the chemiluminescent substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.13,7]decan}-4-yl)phenylphosphate (CSPD®; Boehringer Mannheim). Enzymatic dephosphorylation of CSPD by alkaline phosphatase leads to a maximum light emission at a wavelength of 477 nm, which is recorded on X-ray films.

Our laboratories have been examining the effects of atrial natriuretic peptide (ANP) on the fate of cardiac myocytes (4). Enriched cultures of myocytes, isolated from neonatal rats (1), were treated with vehicle or ANP (1 µM) for 24 h. The cells were analyzed by nuclear morphology using Hoechst dye H33342 for the presence of apoptosis. Cardiac myocytes treated with 1 µM ANP exhibited a 4.1-fold increase in the percentage of apoptotic cells compared to vehicle-treated cultures (4.78 ± 0.65 vs. 19.1% + 1.45, n = 10, P < 0.05). To confirm this result, an examination of DNA laddering was performed using a microscale nonradioactive labeling method. Cells, plated on 60-mm dishes and incubated for 3 days, were treated with ANP or vehicle for 24 h. To isolate nuclear DNA, the cells were harvested in lysis buffer (300 mM NaCl, 50 mM Tris-Cl, pH 8.0, 25 mM EDTA, pH 8.0, 0.15 mg/mL proteinase K and 100 µg/mL RNase A). The lysate was collected and incubated for 3 h at 37°C, followed by two phenol/chloroform/isoamyl alcohol extractions. DNA was then precipitated by the addition of 1/3 vol of 10 M ammonium acetate and 2 vol of cold ethanol.

The 3′-end labeling of the isolated DNA was performed following the protocol of Boehringer Mannheim with minor modification. The reaction was performed in a 1.5-mL microcentrifuge tube with 1 µg of DNA, 4 µL 5× reaction buffer containing 5 mM CoCl2, 0.05 mM DIG-11-ddUTP and 2.5 U/µL terminal transferase in a final volume of 20 µL. The mixture was incubated for 15 min at 37°C, after which the reaction was terminated by addition of EDTA at a final concentration of 0.2 mM. The solution was brought to 0.5 M LiCl and 50 µg/mL tRNA, and the labeled DNA was separated from unincorporated ddUTP by the addition 2.5 vol of cold ethanol. The DNA was pelleted by centrifugation at 10,000×g and resuspended in 20 µL TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA).

To measure the incorporation of DIG-11-ddUTP into the DNA, 2 µL of the labeled DNA were spotted onto a nylon membrane and fixed to the membrane by ultraviolet (UV) cross-linking. The DIG-11-ddUTP-labeled DNA was detected immunologically using an anti-DIG antibody conjugated to alkaline phosphatase using the Luminescent Detection Kit (Genius™ 7, Catalog No. 1 363 514; Boehringer Mannheim).
DIG-labeled DNA was detected immunologically using an anti-DIG antibody and conjugated to alkaline phosphatase using the Genius 7 Luminescent Detection Kit as described above. Following addition of the chemiluminescent substrate CSPD, the blot was exposed to film for 1–5 min.

With DNA isolated from the control myocyte cultures, a low level of DNA fragmentation was observed (Figure 1), consistent with the low incidence of apoptosis determined by nuclear morphology. On the other hand, DNA from the ANP-treated myocytes was shown to be fragmented, yielding the characteristic nucleosomal ladder.

To determine the sensitivity of this assay, a commercially available DNA molecular weight marker, EcoRI- and HindIII-cleaved λ DNA labeled with DIG-11-ddUTP, was used. This DNA consists of 13 fragments of 125–21226 bp (Catalog No. 1 218 603; Boehringer Mannheim). This labeled DNA was size-fractionated on agarose gels, blotted to nitrocellulose and detected as above. Evaluation of the results revealed that as little as 0.5 ng of DNA in a DNA fragment as small as 125 bp can be detected after 3 min exposure to the film (Figure 2). These results suggest that this method is equally sensitive to the previously described procedures of Tilly and Hsueh (3) using radioactive labeling. In addition, since exposure times of a few minutes are usually sufficient for signal detection, multiple images may be acquired.

In summary, we have developed a nonradioactive labeling and detection method for the microscale characterization of DNA laddering. Figure 1 represents the laddering profile from 1 μg of DNA, which is the yield of DNA that we obtain from approximately 10^5 cells. Considering that the cells in Figure 1 typically exhibit 15%–20% apoptosis, as estimated by nuclear morphology, this means that the laddering observed was from approximately 20 000 cells. This must be considered a conservative estimate of the sensitivity because fragmentation from fewer cells should be observable with longer exposure times. This estimate might be inaccurate because the time course for DNA fragmentation, as observed by DNA laddering, might not be the same as that observed for the changes in nuclear morphology (the method by which we quantitate apoptosis). Nevertheless, this calculation demonstrates that the sensitivity is similar to that seen by the Tilly and Hsueh protocol (3), with the advantage that no radioactive material need be handled in this assay. Moreover, multiple images can be acquired in a short period of time.

REFERENCES

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Reliable Detection of DNA CpG Methylation Profiles by the Isoschizomers MspI/HpaII Using Oligonucleotide Stimulators


The increasing interest in methylation patterns of mammalian DNA demands a simple and reliable method to clearly differentiate between cytosine (C) and 5-methylcytosine (5-MeC) within a specific DNA region. The simplest and most commonly used approach is the analysis with restriction endonucleases exhibiting different methylation sensitivities, like the MspI/HpaII pair of isoschizomeric enzymes (New England Biolabs, Beverly, MA, USA). Whereas MspI cleaves the recognition sequence 5′-CCGG independently of the methylation state of the internal C, cleavage by HpaII is blocked by the presence of 5-MeC at this site (3). The method is compromised by the fact that methylation can be detected only in those CpGs that are located within the tetranucleotide recognition sequence.

Another problem is that not all restriction endonucleases cleave their respective unmethylated recognition sequences completely. An incomplete digestion of unmethylated DNA is typically observed when DNA substrates with a low frequency of recognition sites are incubated with restriction endonucleases requiring at least two copies of the recognition site for cleavage activity (2). HpaII seems to belong to this group of restriction endonucleases, because genomes possessing only one or very few recognition sites [simian virus 40 (SV40) (Reference 4 and unpublished) and hamster polyoma virus (unpublished)] exhibit an intrinsic resistance towards this enzyme. This means that unmethylated DNA molecules are not (or are not completely) digested by the enzyme, mimicking a