NaCl-aided Hoechst 33258 staining method for DNA quantification and its application

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Summary. We investigated the effect of salt on the fluorescence staining procedure for quantification of the amount of DNA in cell nuclei in situ. For this, NaCl was added at various concentrations to the Hoechst 33258 (Hoe) medium for staining DNA. The fluorescence intensity of free DNA-Hoe solution was not changed by the addition of NaCl, but that of the nuclei-Hoe complex in situ increased 4-fold on increasing the NaCl concentration up to 1 M. SDS polyacrylamide gel electrophoresis showed that histones H1, H2A, and H2B dissociated from cell nuclei in the presence of 1 M NaCl, resulting in increasing accessibility of DNA to the fluorochrome.

The applicability of the NaCl-aided fluorescence staining method was evaluated by measuring the ploidy classes of various cells. The amount of DNA in spermatozoa is half that in 2n hepatocytes, but by the conventional Hoe staining procedure the fluorescence intensity of spermatozoa is higher than that of 2n hepatocytes, due to differences in accessibility of the dye to DNA. In contrast, by the NaCl-aided procedure, the fluorescence intensity of 2n hepatocytes was twice that of spermatozoa. The effectiveness of the NaCl-aided Hoe staining method was checked using cultivated human gingival cells and hepatocytes from LEC rats with hereditary hepatitis. In all cases, reasonable proportionality between the fluorescence intensity and the amount of DNA was observed.

Materials and methods

Fluorochrome. The bisbenzimidazole dye Hoechst 33258 (Hoe, Hoechst, Japan), was used as a 200 μM stock solution in distilled water. Dye solution of the desired concentration was prepared by diluting the stock solution with 10 mM Tris-HC1 buffer (pH 7.2) just before use. The concentration of dye was determined colorimetrically, taking the molar absorption coefficient at 338 nm as 42 200 M⁻¹ cm⁻¹ (Latt and Wohlleb 1975).

Nucleic acid. Calf thymus DNA was prepared by the method of Zamenhof et al. (1954). The concentration of DNA was determined from its absorbance at 260 nm, assuming that the molar absorption coefficient of DNA-phosphorus (DNA-P) was 6200 M⁻¹ cm⁻¹. The base composition (A-T%) of calf thymus DNA was reported to be 61% by Fasman (1976).

Cell preparation. Specimens of rat and, pig hepatocyte, pig spermatozoa, cultivated human gingival cells and hepatocytes from rats with hereditary hepatitis (LEC rats; Matsumoto et al. 1987; Yoshida et al. 1987) were used. The nuclei of rat and pig hepatocytes were isolated from liver tissue by use of 7% citric acid. They were smeared on glass slides as thin layers, dried in air, fixed in absolute methanol for 5 min, and then dried again. The samples of pig spermatozoa and LEC rat hepatocytes were smeared on glass slides by the touch-smear method and treated by the same fixation procedure as that for isolated nuclei. Human gingival cells were cultivated on coverslips in minimal essential medium for 7 days. The culture solution was then removed and the cells were washed with phosphate buffer saline, fixed in absolute methanol for 5 min and dried.

The specimens on coverslips were treated with staining medium (10 mM Tris-HC1 buffer, pH 7.2, containing 2 μM Hoe and 1 M NaCl) for 1 h at room temperature. For this, after addition of one drop of the staining medium, the coverslip was placed on a glass slide and sealed with rubber cement. As the reactivity of Hoe with RNA is negligible (Brunk et al. 1979; Cowden and Curtis 1981), pretreatment of the specimen with RNase was unnecessary.

Introduction

In cytochemical studies, an accurate method is required for determination of the amount of DNA in cell nuclei in situ. One method that has been used is fluorometry of DNA associated with fluorochromes. Among the variety of DNA fluorochromes available, Hoechst 33258 (Hoe), which binds to adenine-thymine (A-T) base pairs of DNA, has been widely used, because of its high quantum efficiency and specificity for DNA (Müller and Gautier 1975; Cesarone et al. 1979). However, the fluorescence of the Hoe complex is not stable, and so must be measured as soon as possible after fluorescence staining of the specimen. Furthermore, the fluorescence intensity of the Hoe complex is influenced by the fixative, which may modify the structure of nuclear chromatin. This reaction in Hoe fluorescence is effectively stabilized by addition of 1 M NaCl to the Hoe solution (Araki et al. 1987), which enhances and stabilizes the fluorescence for some reason.

In the present study, we investigated the influence of NaCl to the intracellular chromatin conformation by electrophoresis, we also evaluated the availability of the NaCl-aided Hoe staining method, for measurement of DNA in cells of various ploidy classes.
**SDS polyacrylamide gel electrophoresis.** The nuclei isolated from rat hepatocytes were immersed in 10 mM Tris-HCl buffer (pH 7.2) containing 1 M NaCl for 1 h, and then constructive proteins were extracted by the method of Sugita et al. (1971) and subjected to SDS polyacrylamide gel electrophoresis by the method of Laemmli (1970).

**Fluorometry.** The fluorescence of the nucleus-Hoe complex was measured with a Nikon SPL-FL microfluorometer (×40 objective) at an emission wavelength of 470 nm with excitation at 365 nm of the Hg line. The fluorescence of soluble samples, such as free DNA-Hoe and a nuclear homogenate-Hoe were measured with a Hitachi MPF-2 spectrofluorometer using the same emission and excitation wavelengths as those for microfluorometry.

**Results**

Figure 1 compares the fluorescence intensities of Hoe complexes with DNA, a nuclear homogenate and smeared nuclei, as functions of the NaCl concentration. There was no significant change in the fluorescence intensity of DNA itself in the range of NaCl concentrations tested. But the fluorescence intensity of the homogenate of hepatocyte nuclei increased on addition of NaCl: its fluorescence intensity was enhanced about 30% by 1 M NaCl, but did not increase further at higher NaCl concentrations. The enhancement of the fluorescence intensity of the smeared nuclei was greater than that of the nuclear homogenate, but appeared to increase like that of the nuclear homogenate in the presence of NaCl. From these findings, we conclude that the accessibility of Hoe to the nuclei reaches a maximum at an NaCl concentration of about 1 M. As the influence of NaCl on the Hoe-free DNA complex was negligible, the change in accessibility to dye seemed likely to be due to a conformational change of nuclear components rather than of DNA structure.

To determine the influence of NaCl on nuclear components, we examined the structural proteins of rat liver nuclei by SDS polyacrylamide gel electrophoresis. As shown in Fig. 2, three kinds of proteins (arrows) dissociated from the nuclei on treatment with 1 M NaCl, which caused enhancement of fluorescence. From their molecular weights, these proteins were concluded to be histones H1, H2A and H2B.

The availability of the NaCl-aided Hoe staining method for assay of DNA was evaluated using pig spermatozoa (n) and hepatocytes (2n). When these specimens were stained with Hoe in the absence of NaCl, the fluorescence intensity of the hepatocyte nuclei was only 40% of that of spermatozoa, although hepatocytes contain twice as much DNA as spermatozoa (Fig. 3). On the other hand, on addition of 1 M NaCl to the staining medium, the fluorescence intensities of hepatocyte and spermatozoa increased to 4-fold and 1.6-fold, respectively, resulting in a reasonable proportionality between their fluorescence intensities and DNA contents.

We also checked the availability of the proposed staining method using cultivated human gingival cells. These cells adhere to the surface of a microscope coverslip and form a monolayer, which is an ideal sample for microscopic examination. Histograms of the fluorescence intensities of the gingival cells stained by the proposed method and the conventional method are compared in Fig. 4. In the present study, we used the cultivated cells at the seventh generation, including cells in S phase. Therefore, both 2n and 4n cells...