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Technical Advancement

Differential Analysis of Active and Inactive Genes in Human Neutrophils by Chromosomal *In Situ* Hybridization

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In situ **hybridization has been widely used for localization of specific genes in interphase cell nuclei. The sites of signals, however, do not represent the activity of the gene transcription in the nuclei. To differentially localize genes in active and inactive states, the effect of nuclease digestion prior to *in situ* hybridization using PCR products of DNA fragments from human X-chromosome, as a probe DNA, was examined in female neutrophils. According to the conventional protocol for chromosomal *in situ* hybridization, we detected two dot signals in the nucleus. However, the use of Ca/Mg-dependent endonuclease, which extracts inactive gene DNA, resulted in the loss of the one signal of the outer region of the nucleus, but not that of the inner one. In contrast, when exogenous DNase I, which extracts active gene DNA, was used in place of Ca/Mg-dependent endonuclease, the inner signal, but not the outer one, was almost lost in neutrophils. Considering that the outer signal represents inactive X-chromosome DNA in the site of female neutrophil nuclei, these results indicate that the use of our modified protocol may enable us to discriminate the signals of active and inactive genes in interphase cells by *in situ* hybridization.**

Key words: *in situ* hybridization, active and inactive chromatins, neutrophil

I. Introduction

In situ hybridization has been employed for karyotype analysis, gene mapping, an analysis of chromosome aberration by recombination errors, as well as clinical diagnosis and monitoring of genetic disease [12, 13, 21, 24]. However, it is unknown whether the sites of the signals by conventional *in situ* hybridization procedures are the sites of transcriptionally active or inactive genes. For a further understanding of transcriptional regulation of individual genes, an approach is to demonstrate the differential accessibility of the genomic DNA to the nucleases in individual nuclei [9, 34]. The transcriptional regulation occurs in the DNA domains of chromatins, which contain either a single gene or a group of genes [3]. Inactive chromatin DNA can be biochemically solubilized by Ca/Mg-dependent endonuclease digestion, whereas active chromatin DNA remains in the nuclei [31]. Conversely, transcriptionally active chromatin DNA is sensitive to deoxyribonuclease I (DNase I) digestion, whereas inactive chromatin DNA is not [10]. Accordingly, these nuclease digestions prior to *in situ* hybridization would affect the signals for active and inactive genes. In a human female somatic cell, a pair of sex chromosomes consists of one active X (Xa)-chromatin and another inactive X (Xi)-chromatin [14]. In the nuclei of female neutrophils, the outer heterochromatin region with the Barr body or drumstick appendage, which contains Xi-chromatin, is distinguishable from the inner euchromatin region even at the light microscopic level [20, 29]. Moreover, the neutrophils from human peripheral blood express specific genes [23]

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and contain Ca/Mg-dependent endonuclease activity in their nuclei [2]. In the present study, we attempted to explore the new protocol of nonradioactive *in situ* hybridization to discriminate the sites of active and inactive genes by examining the effects of the nuclease digestions prior to *in situ* hybridization using PCR products of DNA fragments from human X-chromosomes as a probe in female interphase neutrophils.

II. Preparation of Single Cells for *In Situ* Hybridization

Peripheral blood was drawn from healthy human female adult donors after informed consent. After mixing with heparin sodium salt (1 mg/5 ml), white blood cells were collected with NycoPrep Mixer (Nycomed Pharma AS, Norway). The cells (5×10^4 cells/500 μl) were centrifuged on 3% gelatin-coated glass slides by Cytospin at 1,000 rpm for 10 min and then air-dried for 30 min at room temperature. The cells were fixed with 4% paraformaldehyde in 10 mM phosphate-buffered saline (pH 7.2) (PBS) for 10 min at 4°C, and then washed in PBS containing both 0.5% Triton X-100 and 0.5% saponin for 5 min at 4°C to facilitate penetration of the DNA probes into the nuclei.

III. Effects of Nuclease Digestion on DNA Distribution Detected by Feulgen Staining

To assess DNA distribution after the nuclease digestion, Feulgen staining, which quantitatively stains DNA [11], was performed on the neutrophil preparations. Ca/Mg-dependent endonuclease extraction of inactive chromatin DNA was followed by Stratling's procedure [31]. Briefly, white blood cells were incubated in a solution containing 10 mM Tris-HCl buffer (pH 7.5), 100 mM sucrose, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.14 mM spermidine for 30 min at 37°C, and then immersed in 5 mM Tris-HCl buffer (pH 7.5) containing 5 mM KCl, 0.4 mM $MgCl_2$ and 2 mM EGTA for 1 hr at 4°C. DNase I digestion of active chromatin DNA was performed by a slight modification of Garel's method [10]. Briefly, white blood cells were incubated in a solution containing 10 mM Tris-HCl buffer (pH 7.5), 3 mM $MgCl_2$, 10 mM NaCl and bovine pancreatic DNase I (0.2 Kunitz units/ml=100 ng/ml, Boehringer Mannheim, GmbH) for 10 min at 37°C, and then washed in 10 mM Tris-HCl buffer (pH 7.5) containing 5 mM EDTA. After the treatment with or without either the endonuclease or DNase I digestion, white blood cells were incubated with proteinase K (0.02 units/ml, Sigma Chemical Co., USA) in PBS for 15 min at 37°C [8, 16]. Following each digestion, the cells were incubated in 1N HCl for 15 min at 60°C and then stained with Fuchsin-Schiff reagent (Wako Pure Chemicals, Japan) for 15 min at room temperature. As a result, without any nuclease digestion, the whole nucleus of each neutrophil was intensely stained by Feulgen staining (Fig. 1A–C). On the other hand, endonuclease digestion left the staining in the inner region, but eliminated it in the outer region (Fig. 1D–F). In contrast, DNase I digestion revealed that the outer region was strongly stained whereas the inner region had little or no staining (Fig. 1G–I).

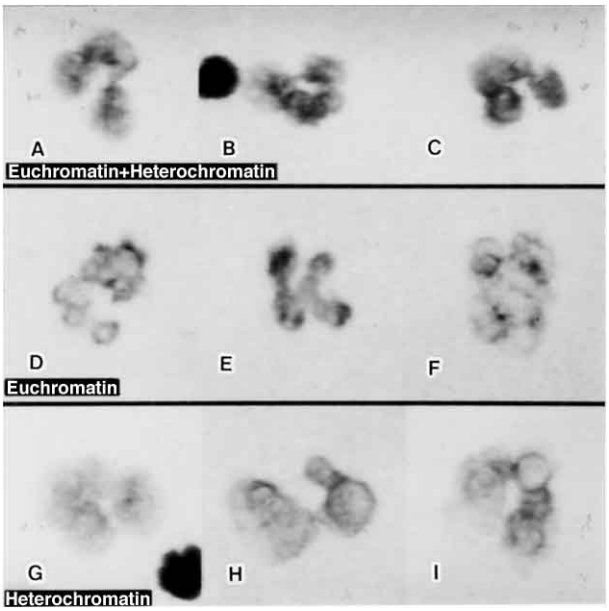

Fig. 1. Photomicrographs of neutrophils by Feulgen staining after proteinase K treatment (**A–C**, Euchromatin+Heterochromatin), after Ca/Mg-dependent endonuclease extraction followed by proteinase K treatment (**D–F**, Euchromatin), or after DNase I digestion followed by proteinase K treatment (**G–I**, Heterochromatin). In **A–C**, both the inner and outer regions of the nuclei are stained. In **D–F**, the inner region of the nuclei is stained. In **G–I**, the outer region of the nuclei is stained.

IV. Effects of Nuclease Digestion on X-chromatin DNA Distribution Detected by *In Situ* Hybridization

For the preparation of probe DNA, three regions, Xq13, Xp21, and Xq21, (approximately 10 mega bases each) from human X chromosomes were micro-dissected [4, 7, 32]. After digestion with Sau 3AI (6 U/μl), the 24-mer primer was ligated to the DNA. For the amplification a polymerase chain reaction (PCR) was performed for 26 cycles with the 24-mer primer in a condition of denaturation at 94°C for 1 min, annealing at 60°C for 30 sec and extension at 73°C for 1 min [7]. The size range of the DNA was between 200 and 700 base pairs (bp) (350 bp on average). PCR products of the DNA fragments were irradiated with ultraviolet light (254 nm) at a dose of 10,000 J/m^2 to introduce thymine-thymine (T-T)-dimers as a hapten [16]. Procedures for *in situ* hybridization using T-T-dimerized DNA probes were described elsewhere in detail [17, 18]. Briefly, after the digestion, the nuclear DNA of the cells was denatured in 70% formamide/2×SSC for 3 min at 75°C. Hybridization medium contained 4×SSC, 4% bovine serum albumin (BSA), 20% dextran sulfate and T-T-dimerized probe DNA (2 ng/μl=40 ng/glass

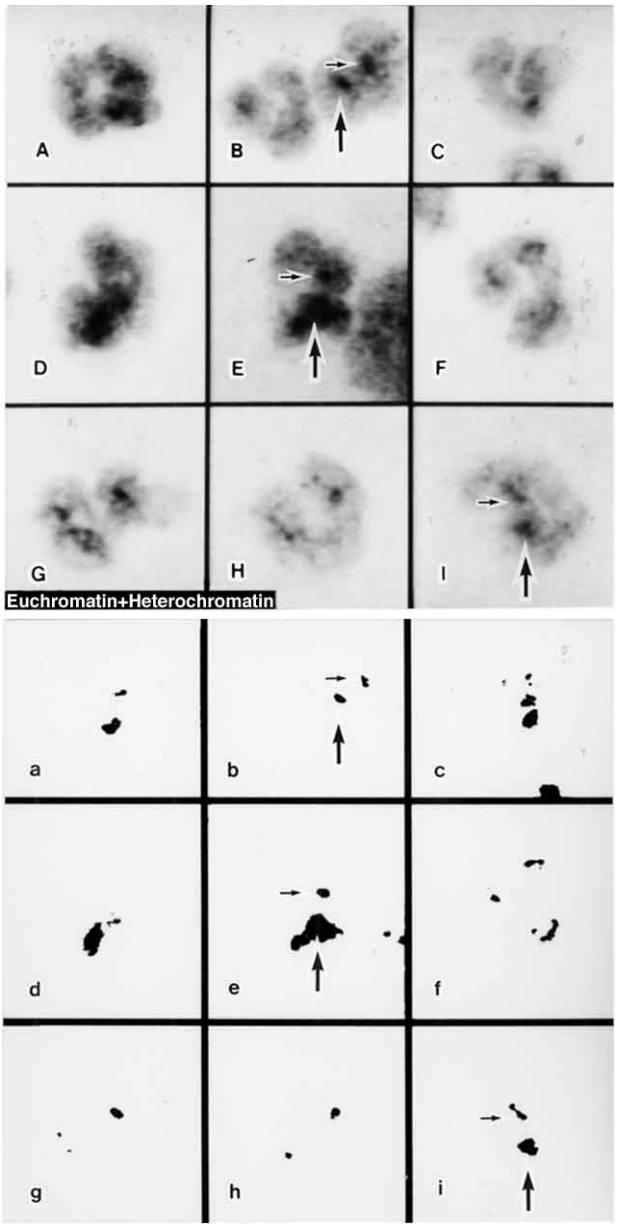

Fig. 2. Photomicrographs of neutrophils by *in situ* hybridization of X-chromosome DNA after proteinase K treatment (**A–I**, Euchromatin+Heterochromatin). Binary images of **A–I** by a computer (**a–i**). Two dot signals are seen in the individual nuclei, one in the inner region and another in the outer. Large and small arrows indicate inner and outer regions, respectively, in representative nuclei.

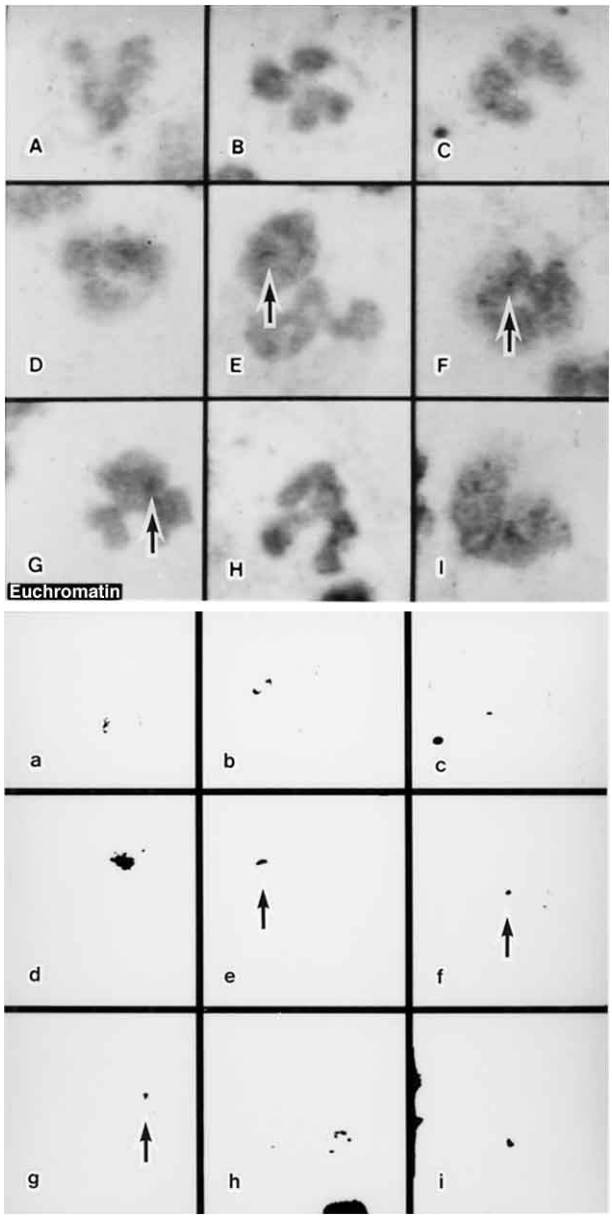

Fig. 3. Photomicrographs of neutrophils by *in situ* hybridization of X-chromosome DNA after Ca/Mg-dependent endonuclease extraction followed by proteinase K treatment (**A–I**, Euchromatin). Binary images of **A–I** by a computer (**a–i**). One dot signal is seen in the inner region of the individual nuclei. Large arrows indicate inner regions in representative nuclei.

slide) [7, 26]. Hybridization was carried out overnight at 37°C. The slide was washed with 50% formamide/2×SSC at 37°C, 2×SSC and 1×SSC at room temperature for 15 min each. The sites of T-T-dimer were detected by indirect enzyme-immunohistochemistry with signal amplification such as multi-layer peroxidase method [22, 30] and the use of 3,3'-diaminobenzidine with cobalt and nickel ions as a chromogen [1]. Light microscopic images of neutrophils were input into a computer. The input conditions were constant: a 100×objective lens and 9.5 V of light source voltage were used. The optical density of the total screen-projected microscopic field in each neutrophil was registered by a computer-assisted image analyzer (IBAS, Carl-Zeiss, Germany) and binary images were obtained at the same level of brightness and contrast among the neutrophils tested. As a result, according to the conventional protocol for chromosomal *in situ* hybridization, two dot signals, one in the inner region and another in the outer, were detected in the nucleus (Fig. 2A–I, Fig. 2a–i). On the other hand, the use of Ca/Mg-dependent endonuclease resulted in the loss

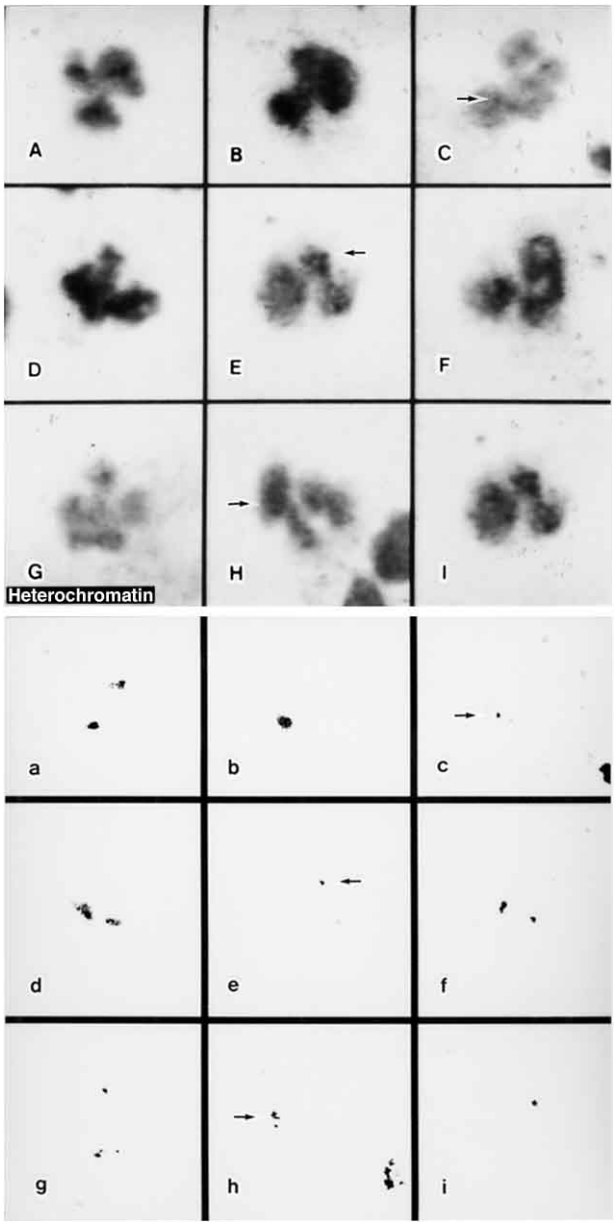

Fig. 4. Photomicrographs of neutrophils by *in situ* hybridization of X-chromosome DNA after DNase I digestion followed by proteinase K treatment (**A–I**, Heterochromatin). Binary images of **A–I** by a computer (**a–i**). Signal is seen in outer region of the individual nuclei. Small arrows indicate outer regions in representative nuclei.

of the one signal of the outer region of the nucleus, but not that of the inner one (Fig. 3A–I, Fig. 3a–i). In contrast, when exogenous DNase I was used in place of Ca/Mg-dependent endonuclease, the inner signal, but not the outer one, was almost lost in neutrophils (Fig. 4A–I, Fig. 4a–i).

V. Discussion

In the present study, we attempted to explore the protocol of *in situ* hybridization to discriminate between active and inactive genes by combining use of endogenous Ca/Mg-dependent endonuclease and exogenous DNase I, which are known to extract the DNA of inactive and active chromatin parts, respectively [5, 10, 15, 25, 31, 33]. When the protocol was applied to localize X-chromatin in female neutrophil nuclei, active Xa-chromatin DNA was detected in the inner region and inactive Xi-chromatin DNA was found in the outer region of the nuclei, as was expected. Therefore, the protocol described here should be useful to identify whether the gene to be localized is present in the active or the inactive chromatin part in cell or histological specimens.

Active chromatins have higher sensitivity to DNase I [5, 10, 15, 25, 33]. On the Xa-chromatin DNA, the whole CpG island of the DNA is completely unmethylated. Moreover, it does not contain nucleosomes and is covered by a number of transcription factors. In fact, in the upstream region of the transcription starting site of phosphoglycerate kinase-1 gene on the Xa-chromatin DNA, exogenous DNase I is accessible to the promoter DNA and unmethylated CpG island, which are located among the binding sites of transcription factors [25]. On the transcriptionally inactive Xi-chromatin DNA, DNase I is inaccessible to the Xi-chromatin DNA, since the methylation of CpG island makes putative DNase I sensitive sites to be cryptic to DNase I around nucleosomes. Accordingly, the inner region digested by DNase I in the neutrophil nuclei may contain the Xa-chromatin DNA.

Nuclear chromatin structure is dependent on ion strength [19]. Inactive chromatin particles are held together by histone H1 and mono- and divalent cations. In this study, the medium of inactive gene digestion by the Ca/Mg-dependent endonuclease contained KCl, $CaCl_2$ and $MgCl_2$ as cations, and spermidine as polycations. It has been reported that tetravalent polyamine spermine changed the DNA phosphate charge (–1) into a charge (3+) [27]. Similarly, spermidine, a linear polyamine, is considered to change the charge and compete with histones for the weakly held linker DNA. In addition, the digest in the divalent cations produced smaller sized nucleosomes [19]. Stratling has demonstrated that the endogenous Ca/Mg-dependent endonuclease digestion at the utilized ionic conditions in this study produced a limited rearrangement of H1, with the result that the mononucleosomes in the low molecular weight peak contain 166 bp DNA fragments, but do not contain 170–200 bp DNA fragments [31]. Thus, Ca/Mg-dependent endonuclease seems to nibble off the linker region and to remove an important portion of the binding site of H1 of the Xi-chromatin DNA. On the distribution of Xi-chromosomes, conventional fluorescent *in situ* hybridization (FISH) analysis has demonstrated that Xi-chromosomes are localized in the drumstick appendages (13%) in female neutrophils and in the vicinity of the nuclear envelope or the nuclear outer region (81%) in female neutrophils as well as in male neutrophils [28]. Therefore, our findings, together with other reports, indicate that the outer region digested by Ca/Mg-dependent endonuclease in the neutrophil nuclei may contain the Xi-chromatin DNA.

We propose that the interpretation of the signal sites

detected by *in situ* hybridization should be deliberated on in terms of the nucleosome organization or chromatin structure of the genomic DNA in single interphase cell nuclei. The nuclease digestions prior to *in situ* hybridization may open the way to discriminate the active and the inactive forms of a group of genes or single genes in human single cell nuclei. In a neoplastic cell, for example, hypomethylation of CpG sites is linked with DNA instability and gene activation such as oncogene activation, whereas hypermethylation is linked with gene mutation and gene silencing [6]. Accordingly, the present technique may be useful for applications to discriminate the gene activity in a cell from human neutrophils or peripheral blood cells.

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VII. References

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