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Temporal Order of DNA Replication of Imprinted *IGF2* Gene and Non-imprinted *TNF* and *RFC5* Genes in Human Lymphocytes

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Abstract

We have analyzed the replication timing of DNA segments of the imprinted *IGF2* (insulin-like growth factor II) gene and non-imprinted genes in human lymphocytes by fluorescence *in situ* hybridization (FISH). In principle, intranuclear FISH signals of each DNA segment are characterized in three groups according to the replication status of each parental allele; two singlets (SS), singlet-doublet (SD), and two doublets (DD). Non-imprinted genes, both of *TNF* (tumor necrosis factor) and *RFC5* (replication factor C subunit 5), showed a smaller percentage of the SD pattern, while *IGF2* gene had a higher percentage of the SD pattern, suggesting that the imprinted *IGF2* gene replicates asynchronously between its homologous alleles. As the results, together with previous observation, we concluded the temporal order of DNA replication of three genes in human lymphocytes to be as follows : Both alleles of *TNF*, paternal allele of *IGF2*, both alleles of *RFC5*, and then maternal allele of *IGF2*.

Key words: DNA replication • fluorescence *in situ* hybridization (FISH) • genomic imprinting • *IGF2* • animal cell genome

Introduction

DNA replication is a very important intranuclear event to inherit a cellular genetic background to the next generation. Replication of DNA in mammalian cell genome takes place in a temporal order, and each DNA segment has a defined time of replication within S phase under developmental control[1,2]. There are some correlations between gene expression and DNA replication timing. Some tissue specific genes replicate late in non-expressing cells, but early in expressing cells[2-4]. It was also reported that a gene's position on the chromosome, rather than its sequence, determines the time of replication[5]. However, little is known what is the meaning of replication timing and how it is controled and involved in a cellular function. One approach to understand these mechanisms is to compare the replication timing of individual DNA segments and discuss their relations to gene expression, genome organization, and cellular function. The DNA replication timing has been conventionally determined by Southern blot analysis to newly replicated bromodeoxyuridine-labeled DNA prepared from the fractionated cells[6]. But this method needs a radioisotopic compound and a cell sorter with troublesome steps. Fluorescence

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in situ hybridization (FISH) has been demonstrated to be a powerful tool for gene mapping as well as visualization of intranuclear genome organization[7,8]. We have developed a convenient method to determine the DNA replication timing by using FISH to interphase nuclei[9]. This approach has been successfully applied to the demonstration of allele specific replication timing of imprinted genes[10,11] and X-linked genes[12,13]. Genomic imprinting is an epigenetic process which distinguishes the parental origin of certain chromosomal regions in mammals. DNA methylation, chromatin structure, and asynchronous replication have been thought to play a role in this imprinting process[14-16]. The several chromosomal regions containing imprinted genes were found to show asynchronous replication between homologues[10,11]. Studies on these allele specific features may also give important information to understand the mechanisms of DNA replication. In this paper, we precisely determined the order of DNA replication of three genes including imprinted and non-imprinted genes by FISH-based replication assay.

Materials and Methods

Chemicals

Phytohemagglutinin, RPMI1640, and fetal bovine serum (Gibco-BRL), bromodeoxyuridine (BrdU) and 4', 6-diamidino-2-phenylindole (DAPI) (Sigma), and anti-BrdU monoclonal antibody (MBL) were purchased from the indicated sources. Reagents used for probe labeling and signal detection except for the above were purchased from Boehringer Mannheim. Other chemicals were from nacalai tesque.

Cell culture and slide preparation

Human peripheral blood from a male healthy donor was cultured in RPMI1640 containing 10% fetal bovine serum for 72 hours after phytohemagglutinin stimulation. The cells were labeled with 25 μg/ml BrdU for 10 min and harvested. After hypotonic treatment for 20 min (0.075M KCl, 37℃), cells were fixed carefully with methanol/acetic acid (3/1) three times, and then dropped onto microscope slides for FISH.

DNA probes and FISH

Human insulin-like growth factor II (*IGF2*) phage clone[17], cosmid clones of *TNF* (tumor necrosis factor)[18] and *RFC5* (replication factor C subunit 5)[19] genes were kindly provided by Drs. G. Bell, H. Inoko, and T. Eki, respectively. These genomic DNA clones were labeled with biotin-16-dUTP or digoxigenin-12-dUTP by nick translation. Biotin- or digoxigenin-labeled probes were hybridized onto denatured nuclear specimen and detected by fluorescein-avidin or rhodamin-anti-digoxigenin Fab fragment, respectively. In case of detection of S phase nuclei, the incorporated BrdU was visualized simultaneously in FISH detection steps by anti-BrdU monoclonal antibody and following either rhodamin- or fluorescein-anti-mouse IgG to give different color combination with a DNA probe. The nuclei were counterstained with DAPI.

Determination of replication timing by FISH

Each DNA probe and BrdU were detected in different colors, and FISH signals were examined only for BrdU positive nuclei with an oil x63 objective on a Zeiss Axioskop epifluorescence microscope fitted with a Zeiss filter set for DAPI, FITC, and rhodamin. The FISH signal patterns of individual nuclei were determined as either two singlets (SS), singlet-doublet (SD), or two doublets (DD). At least two

hundred nuclei were scored and the percentages of each population were calculated.

CCD imaging

Fluorescence images were viewed under a Zeiss Axioskop epifluorescence microscope fitted with a cooled CCD camera (Photometrics PXL 1400) coupled to IPLab software (Signal Analytics Co.), pseudocolored, and merged using Adobe Photoshop 2.5J (Adobe Systems Inc.) on a Macintosh computer[20]. The images were directly printed in black and white by Fuji Pictrography 3000.

Results and Discussion

Detection of S phase nuclei

There are some reports which used a BrdU incorporation method to identify S phase nuclei[10, 21]. They labeled cells by BrdU for 60-90 minutes. Since the S phase of animal cells is generally 6-8 hours, this labeling time seems to be too long. So, we checked a BrdU labeling condition first. Randomly cultured lymphocytes were labeled with BrdU for 5-30 minutes and the incorporated BrdU was detected by either rhodamin or fluorescein in FISH step to identify S phase nuclei. The nuclei in S phase were detected even in 5 minutes' labeling, and the fluorescence intensity increased dependent on the labeling time (data not shown). We concluded that 10 minutes' BrdU labeling is enough for the detection of S phase nuclei. As shown in Fig. 1A and B, the S phase nuclei are easily distinguished with non-S phase nuclei under the fluorescence microscope.

Replication timing analysis by FISH

The individual DNA segments on the animal cell genome generally replicate synchronously between

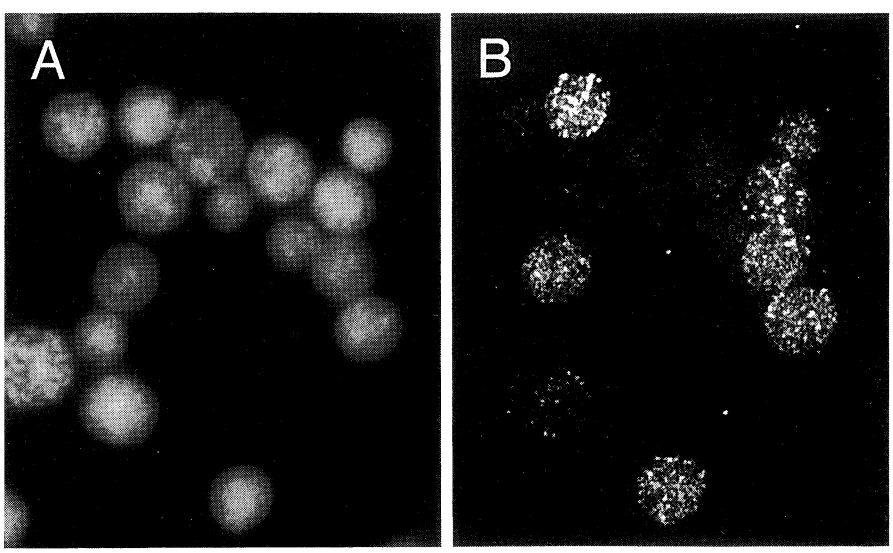

Fig. 1 Identification of S phase nuclei. The nuclei fixed on the microscope slide were detected BrdU by rhodamin in FISH steps as described in Materials and Methods section. (A) The CCD image detected all the nuclei with DAPI staining. (B) The rhodamin image detected S phase nuclei.

the homologous alleles, whereas some fragments including imprinted gene regions have been shown to replicate asynchronously[10, 13, 22]. Human *IGF2*, one of the well-characterized imprinted genes[14, 16, 23], has been shown that the paternal allele replicates earlier than maternal one[10, 21]. We have precisely compared the replication timing of three genes; *IGF2* and two non-imprinted genes, *TNF* and *RFC5*, in human lymphocytes by FISH analysis.

FISH signal profiles of each clone, two singlets (SS), singlet-doublet (SD), and two doublets (DD) shown in Fig. 2 as a typical example, were examined under a fluorescence microscope and scored their ratio only for S phase nuclei detected BrdU. As shown in Fig. 3, *TNF* and *RFC5* had a narrow range of SD pattern, whereas *IGF2* has a wide range of SD. Since most DNA segments replicate on the two homologous chromosomes in a fairly synchronous manner and at most 15% of the nuclei show SD

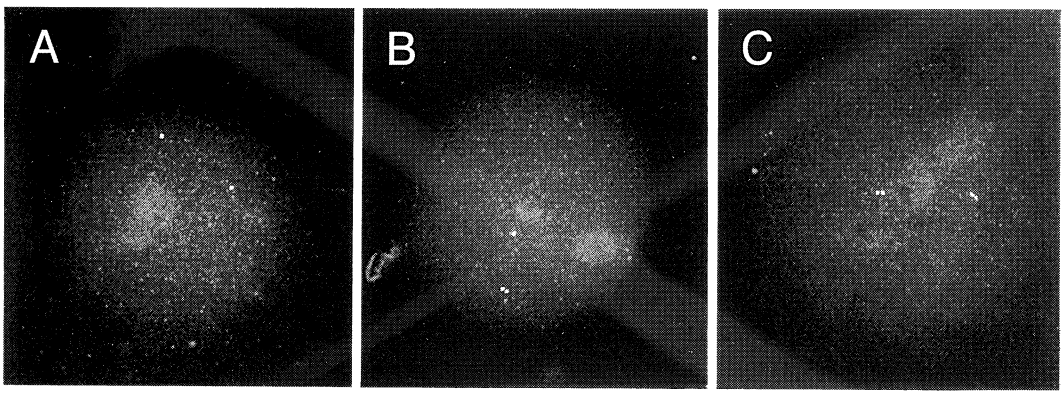

Fig. 2 Signal patterns of individual DNA segments detected by FISH. (A) Two singlets (SS) ; before replication, (B) singlet-doublet (SD) ; only one of the alleles replicated, and (C) two doublets (DD) ; both the alleles replicated.

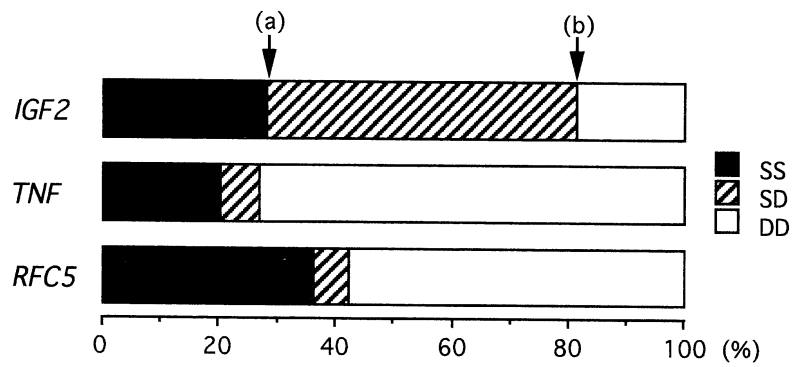

Fig. 3 Replication profiles of each gene. The nuclei in S phase were only scored according to the signal patterns shown in Fig. 2. A smaller percentage of SS pattern shows relatively earlier replication. The arrows (a) and (b) in the profile of *IGF2, i. e.*, SS% and (SS+SD)%, are shown as the point of replication of the first (paternal) and second (maternal) allele of *IGF2*, respectively.

pattern in FISH-based replication assay[9, 10], *TNF* and *RFC5* replicate synchronously (SD<10%), on the other hand, *IGF2* replicates asynchronously (SD>40%). In principle, a DNA segment with a smaller SS% replicates in the earlier stage of S phase, while the segment with a higher SS% replicates in the later stage of S phase, so we can compare the temporal timing of DNA replication based on FISH signal profiles. As shown in Fig. 3, *TNF* which has a smaller SS% replicates earlier than *RFC5*. Imprinted genes including *IGF2* have been shown to have an allelically different replication timing, for example, the paternal allele of *IGF2* replicates earlier than its maternal allele[21]. Thus, we considered the arrow (a) and (b) of Fig. 3, *i.e.*, SS% and (SS+SD)%, as the point of replication of the paternal and maternal allele of *IGF2*, respectively. The comparison of these signal profiles lead to the conclusion of the temporal order of DNA replication of three genes in human lymphocytes as follows: Both alleles of *TNF*, one allele of *IGF2* (paternal), both alleles of *RFC5*, and then the other allele of *IGF2* (maternal).

Delineation of replication order by multicolor FISH

To demonstrate the temporal order of replication of three genes, we employed multicolor FISH; two genes with different labeling were hybridized together onto the same slide and simultaneously detected in different color combination. In case of the combination of *RFC5* and *IGF2*, some nuclei with SD for *IGF2* showed SS (Fig. 4A), but mainly DD (data not shown) for *RFC 5*. On the other hand, when *TNF* and *IGF2* were detected together, nuclei with DD for *TNF* and SS (data not shown) or SD for *IGF2* (Fig. 4B) were main population. These results confirmed the temporal order of replication of three genes determined in Fig. 3.

TNF locates on a R-positive band of chromosome 6p21.3 and has a high G+C content[24]. This gene

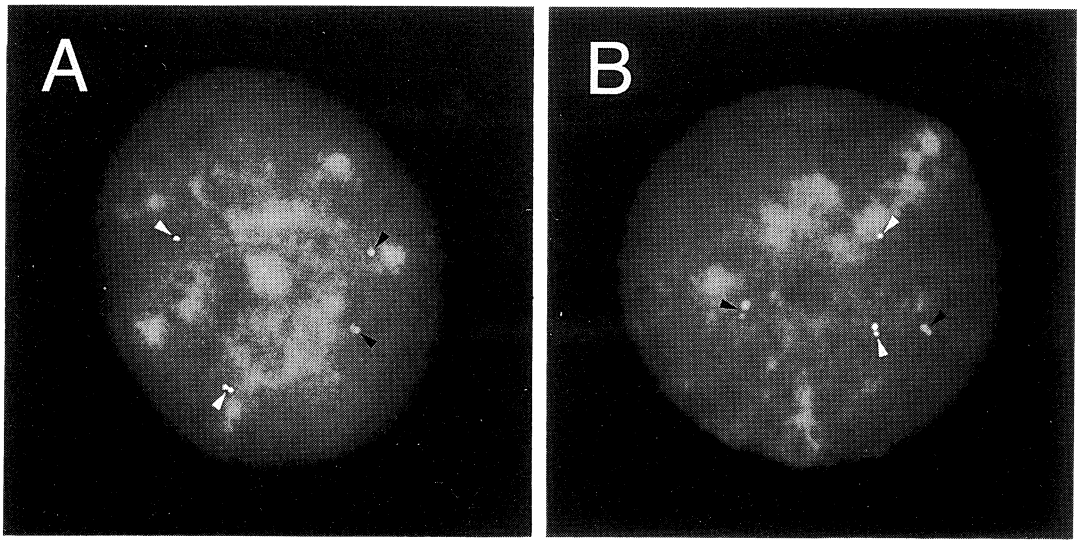

Fig. 4 Demonstration of replication order of three genes by multicolor FISH. *IGF2* was detected by fluorescein, and other genes were detected by rhodamin in each image. (A) Two singlets (SS, black arrowheads) for *RFC5* , and (B) two doublets (DD, black arrowheads) for *TNF*. *IGF2* (white arrowheads) showed SD in both the images.

shows tissue specific expression profiles and may not express in human lymphocytes. *RFC5* is a constitutive gene which expresses in almost all the proliferating cells. Our findings in this paper demonstrate that the active *RFC5* replicates later than inactive *TNF*. This is inconsistent with the general ideas of the relationship between DNA replication and gene expression. In this case, its chromosomal position with higher G+C% can be main effect on this accelerated replication. Thus, replication timing of the genes does not always correlate to their expression status. Moreover, imprinted genes have allele specific timing of DNA replication, in spite that both homologous alleles locate on the same chromosomal position. As for *IGF2*, the active allele replicates earlier than its inactive allele. However, an imprinted H19 gene, which locates on very close to *IGF2* (about 200 kb distance in human), replicates early in its inactive allele. One possible explanation for the allelic differences of replication is that the folding status of the earlier replicating allele of this region may be less compact and proteins required for DNA replication may be easily accessible. Acetylation and deacetylation of histones which consist of nucleosomes also affect on these asynchronous replication[21]. Factors which interact with acetylated or deacetylated histone may be possible to give effects to the folding of this genomic region. Although the control mechanisms of DNA replication timing are not known, many factors, for example, gene expression, chromosomal position, chromatin structure, genome organization, intranuclear arrangement of the genome, may be responsible for this important biological event.

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ゲノム刷り込みを受ける*IGF2*遺伝子および受けない*TNF*と*RFC5*遺伝子のヒト・リンパ球におけるDNA複製順序

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蛍光 *in situ* hybridization（FISH）法を用いて，ヒト・リンパ球におけるDNA複製タイミングをゲノム刷り込みを受ける*IGF2*（インスリン様成長因子型）と受けない*TNF*（がん壊死因子）および*RFC5*（複製因子C第5サブユニット）遺伝子について解析した。各ゲノム断片の細胞核内FISHシグナルは，原理的に，それぞれが由来する親アレルの複製状態に従って，2つのシングルドット（SS），シングルドットとダブルドット各一個（SD），2つのダブルドット（DD）の3つのグループに大別される。ゲノム刷り込みを受けない*TNF*，*RFC5*は，SDパターンの割合が小さいが，刷り込みを受ける*IGF2*はSDパターンが高く，アレル間で非同調的に複製することが示唆された。これらの結果とこれまでの報告とあわせて，これらの遺伝子の複製が，*TNF*の両アレル，*IGF2*の父方アレル，*RFC5*の両アレル，*IGF2*の母方アレルの順に進行すると結論した。