การศึกษาตำแหน่งของการเกิด DNA replication โดยวิธี Non-isotopic

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บทคัดย่อ การศึกษาตำแหน่งของการเกิด replication ของ DNA โดยการใช้วิธี BrdU pulse labelling ร่วมกับการเตรียม interphase halo จากเซลล์ที่ได้ผ่านการติดฉลากด้วย BrdU จะพบว่า DNA จะมีลักษณะเป็น loop ที่อยู่ล้อมรอบ nuclear matrix 90% ของ DNA ที่สร้างขึ้นในระยะเวลาสั้น ๆ จะอยู่ในบริเวณของ matrix ของนิวเคลียส แต่เมื่อเวลาผ่านไปนานขึ้น DNA ที่ติดฉลากด้วย BrdU จะกระจายไปทั่วบริเวณของ DNA loop ซึ่งแสดงให้เห็นว่า replication ของ DNA นั้นจะเกิดขึ้นที่ matrix ก่อนแล้วจึงเคลื่อนต่อไปยังส่วนของ halo ที่อยู่รอบนอกของ DNA matrix

Abstract **Delineation of DNA Replication Site by Non-isotopic Method**

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The site of DNA replication was studied by using BrdU pulse-labelling. The interphase halos were prepared after pulse labelling. With this method, DNA loops could be visualized as a halo surrounding a nuclear matrix. 90% of the labelled newly synthesized DNA was confined in DNA matrix after 15 min incubation with BrdU. In contrast, with longer pulse times, the BrdU-labelled DNA was seen throughout the halo regions. These findings indicated that DNA replications occurs first in nuclear matrix and then travel outward to the halo region.

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Introduction

DNA is organized in higher order in nucleus. Wraping of DNA around histone core causes condensation of DNA. When the chromatin is released from histone, by using detergent and high salt solution to deplete soluble protein and histone (1). Loops of DNA can be visualized as a halo surrounding nuclear matrix which is networks of fiber. It is suggested that matrix protein i) serve as a nuclear infrastructure, ii) keep various molecule in their places and iii) organizes the transcription and replication process (2). Two types of chromatin loop was

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identified (3,4). One is thought to result from an attachment of the DNA to the peripheral nuclear lamina structure. Second type, the DNA appear to be stabilized by a complex set of proteins that forms the residual network in the nuclear matrix. These structural aspects of DNA related to nuclear process as transcription and replication. It is found that the replication occurs first at the nuclear matrix and then travels outward from the nuclearmatrix into the halo region (5). Every gene sequence has a defined time of replication with in the cell cycle. Almost all house keeping genes replicate within the first half of S phase in many cell types and the same is true for some tissue specific genes (6). Other tissue specific gene sequences have a developmentally regulated pattern of replication whereby they undergo DNA synthe sis early in expressing cells, but late in non-expressing cell types. Recently only method using radioactive labelling is reported (1). In this menuscript, a method using to label newly synthesized DNA is described. This method had been used to demonstrate replication time zone and the advantage over radioactive labelling method.

Materials and Methods

Cells labelling

To study the DNA replication by non-radioactive method, pulse labelling of EBV transformed cell lines from healthy male was used. The lymphocytic cell lines were grown in RPMI medium supplemented with 20% fetal calf serum. Cells in exponentially growth were synchronized with methotrexate for 17 hr. After incubation, methotrexate was removed by washing cells 2 times with Hank's basic salt solution. Cells were resuspended in RPMI medium contained 10^{-5} M BrdU (Bromodeoxyuridine) for 15, 30 and 60 min. Cell medium was removed by centrifugation at 1200 rpm for 5 min. The cell pellet was used for halo preparation.

Halos preparation

To visualize the halos of DNA surrounding the nuclear matrix lymphoblastoid cell lines were performed as described previously (7). The supernatant was removed by centrifugation at 1200 rpm for 5 min. The cell pellet was washed twice with 1XPBS buffer and dropped on slides. The air dried slides were first dipped in extraction buffer (50 mM Hepes pH 7.8, 10 mM MgCl2, 10 mM CaCl2, 0.22 M sucrose and 0.5% NP 40) for 5 min. They were then sequentially dipped for 30 sec each, in solutions of 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 M NaCl all containing 0.2 mM MgCl2 and 10 mM Tris (pH 7.4). The slides were irradiated with UV light (254 nm) for 1 min and fixed in 1% formaldehyde/1XPBS for 10 min. The slides were dehydrated through an ethanol series (50%, 70%, 90%, 100%) for 3 min each. Finally, the slides were air-dried and kept at 4°C until used.

Detection of newly synthesized DNA

The slides were washed in 1XPBS for 3 times 5 min each. To visualized of BrdU incorporated DNA, fluorescein conjugated mouse monoclonal antibody (sigma) was applied to the slide and incubated at 37°C for 30 min. At the end of the incubation period, the slides were washed for 5 min in prewarmed 1XPBS for 3 times. The counterstain was performed by using DAPI (4',6'-diamidino-2-phenylindole). 50 cells in each case were analysed.

Results

Sensitivity of BrdU to detect DNA synthesis was comparable to that of ^{3}H-thymidine. Thus, BrdU was used to study the relationship of DNA replication and the DNA loop prepared by high salt extraction, in which pulse labelling was performed. After a 17 hr treatment of the cells with methotrexate which inhibited the cells in the S-phase, BrdU was added into the lymphoblastoid cell line. After labelling with BrdU as described in materials-methods. The appearance of the DAPI staining halos were the same as those prepared without BrdU pulse (data was not shown). The BrdU substituted regions on DNA loops was detected with antibody to BrdU conjugated with FITC. Examination of labelled nuclei using conventional fluorescent microscopy, the signals of BrdU were seen as green dot. At 15 min BrdU pulse, the green signals of BrdU were confined in the matrix of the 80% of the cells (Fig. 1 a, b) After 30 min labelling, in most of the cells (82%), BrdU was also restricted only in the nuclear matrix. But the intensity of 30 min pulse was stronger than those of 15 min pulse (Fig. 1 c, d). In contrast, nuclei from 80% cells which were treated with BrdU for 1 hr, had signals distributed throughout the halo region (Fig. 1 e, f).These results suggested

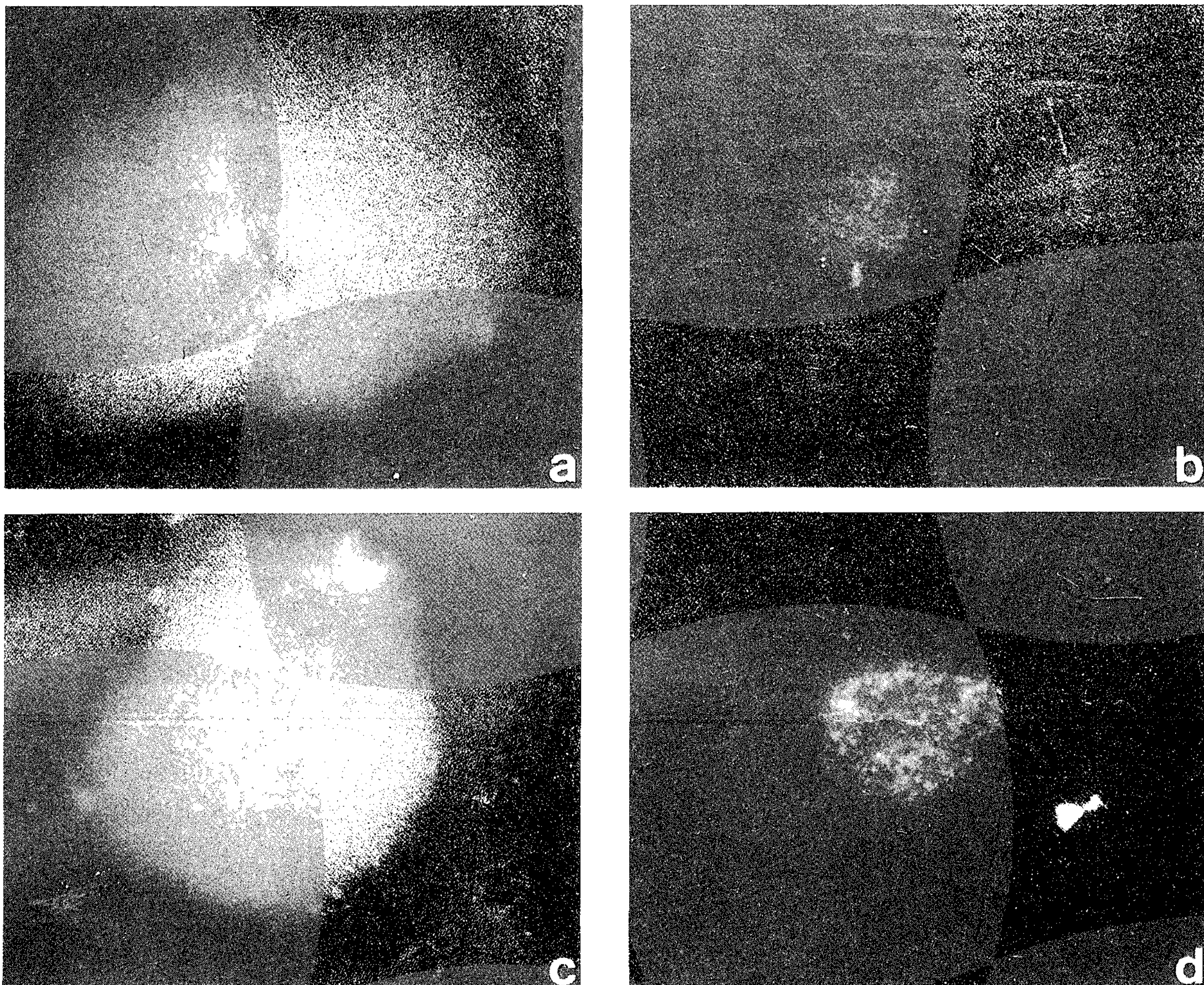
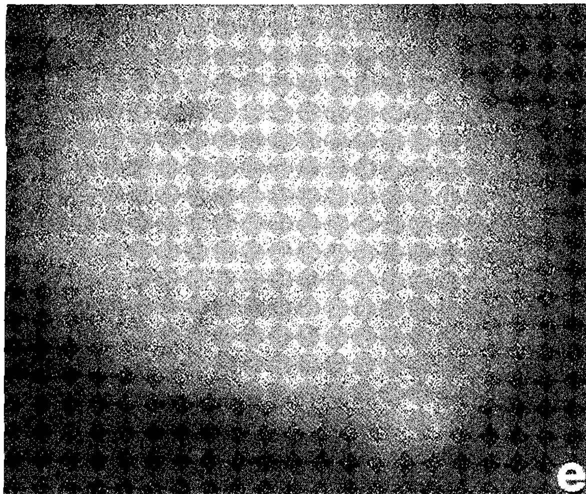
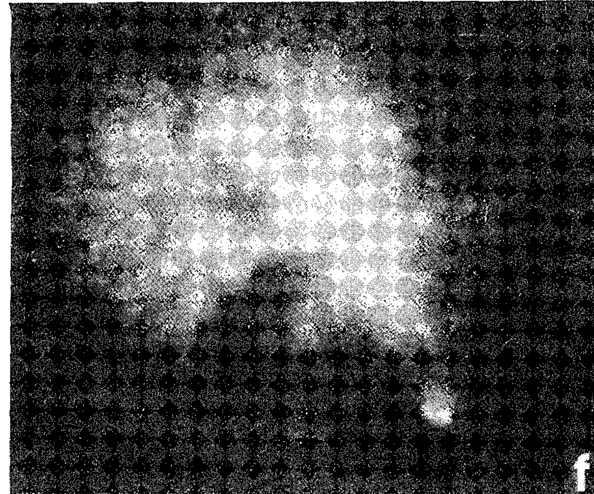


Figure 1 *: Movement of newly replicated DNA into the halo regions. DAPI counterstain of interphase halos were shown in a, c, and e. and the distribution of BrdU in interphase halos after 15 min (b), 30 min (d) and 60 min (f) labelling.*





(Color picture : page 109)

that the DNA which was located in the matrix, replicated earlier than those in the halo region.

Discussion

Replication of DNA in the animal cell genomes occurs in a temporally ordered fashion during S-phase (8). There are two techniques commonly used to detect individual gene replication, -incorporation of ^{3}H-thymidine followed by autoradiograph (9) or southern blot hybridization to newly replicated BrdU labelled DNA (10). Because the organization of chromatin is the higher order structure and the nuclear matrix is the site of the DNA replication. The relationship between chromatin organization and DNA replication is examined by using histone depleted nuclei prepared after BrdU incorporation. The data shows that matrix DNA is enriched in newly replicated DNA for a short period after synthesis. This result suggests that the DNA matrix preferentially replicated earlier than the DNA in halo. This observation supports previously investigation which used ^{3}H-thymidine (1, 5, 9). BrdU pulse was prefered to ^{3}H-thymidine because of the safety reason and replication time zone of interested clones can be followed when used in combine with in situ hybridization. This method provided an opportunity to assay the replication time of whole chromosome and individual locus. This provides a new approach for determining replication timing in animal cells.

References

1. Vogelstein B, Pardoll DM, Coffey DS. Supercoiled loops and eukaryotic DNA replication. Cell 1980; 22:79-85.
2. Baskin Y. Mapping the cell nucleus. Science 1995; 268:1564-1565
3. Mirkovitch J, Mirault ME, Laemmli UK. Organization of the higher-order chromatin loop specific DNA attachment sites on nuclear scaffold. Cell 1984; 39:223-32.
4. Braunstein JD, Schulze D, DelGiudice T, Furst A, Schildkraut CL. The temporal order of replication of murine immunoglobulin heavy chain constant region sequences corresponds to their linear order in the genome. Nucleic Acids Res 1982; 10:6887-902.
5. Pardoll DM, Vogelstein B, Donald SC. A fixed site of DNA replication in eukaryotic cells. Cell 1980; 19: 527-36.
6. Holmquist GP. Role of replication time in the control of tissue-specific gene expression. Am J Hum Genet 1987; 40:151-73.
7. Tocharoentanaphol C, Cremer M, Schrock E, Blonden L, Kilian K, Cremer T, RiedT. Multicolor fluorescence in situ hybridization on metaphase chromosomes and interphase halo-preparations using cosmid and YAC clones for the simultaneous high resolution mapping of deletion in dystrophin gene. Hum Genet 1994; 93:229-35.
8. Selig S, Okumura K, Ward DC, Cedar H. Delineation of DNA replication time zones by fluorescence in situ hybridization. The EMBO journal 1992; 11:1217-25.
9. Begg AH, Miegeon B. Chromatin loop structure of the human X-chromosome relevance to X inactivation and CpG clusters. Mol Cell Biol 1989; 9:2322-31.
10. Claza RE, Eckhard LA, DelGiudice T, Schildkraut CL. Changes in gene position are accompanied by a change in time replication. Cell 1984; 36: 689-969.