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Hypomethylation of DNA with 5-Azacytidine Alters Chromosome Replication Patterns in Cultured Human Lymphocytes

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The DNA replication of human lymphocytes was studied using Bromodeoxyuridine incorporation. The characteristic patterns of dynamic banding were analysed. Human chromosomal DNA was synthesized in a segmental but highly coordinated fashion. Each chromosome replicates according to its innate pattern of chromosome structure (banding). R-positive bands are demonstrated as the initiation sites of DNA synthesis, and G-bnads initiate replication after it has been completed in the autosomal R-bands. Many researchers demonstrated that developmental or induced methylation of DNA can inactivate the associated gene loci. Such DNA methylation can be reversed and specific genes reactivated by treatment with 5-azacytidine. We treated the hypomethylating agent 5-azacytidine and tested for changes of DNA replication pattern. Treatment with 5-azacytidine causes an advance in the time of replication. These observed changes in timing of replication suggest that DNA methylation may modify regional groups of genes in concert.

KEY WORDS: Methylation, 5-Azacytidine, Replication bands

In previous studies, [^{3}H]-thymidine incorporation, followed by autoradiography, has been frequently used in the study of mammalian chromosome replication (Odartchenko and Pavillard, 1970; Kofman and Chandley, 1970). Later, the methods that permit detection of chromosomal incorporation of the thymidine analogue 5-bromodeoxyuridine were developed (Dutrillaux, 1977; Toshiaki and Watanabe, 1988; Drouin and Richer, 1989). Consequently, these techniques and in particular the BrdU-Hoechst 33258-Giemsa method have produced a higher resolving power allowing analysis of patterns of chromosome replication at the metaphase band level (Kaluzewski, 1982; Yerle *et al.*, 1991; Just *et al.*, 1993).

Many researchers demonstrated that developmental or induced methylation of DNA can inactivate associated gene loci (Lester *et al.*, 1982; Graves, 1982; Riggs and Pfeifer, 1992). In most higher organisms, DNA is modified after synthesis by the enzymatic conversion of many cytosine residues to 5-methylcytosine (Razin and Riggs, 1980). In mammalian DNA, 2 to 7 percent (depending on the species) of the total cytosine is converted to 5-methylcytosine. Methylation occurs enzymatically after DNA synthesis by methyl transfer from S-adenosyl methionine (SAM) to position 5 of cytosine. Such DNA methylation can be reversed and specific genes reactivated by treatment with 5-azacytidine (Mohandas *et al.*, 1981). 5-Aza-C is an analogue of cytosine that is incorporated into DNA and then dramatically inhibits DNA-methyltransferase activity, probably by a covalent attachment mechanism (Jones and Taylor, 1980; Friedmen, 1981).

After a short treatment period, the cytosine

analogue can be removed from the culture medium, yet the new state of differentiation is maintained. Incorporation of 5-aza-C leads to hypomethylation of DNA at the sites of substitution with 5-aza-C. That is a result of the replacement of nitrogen in place of carbon at the fifth position in the pyrimidine ring and then 5-aza-C causes somatically heritable changes in the cellular phenotype.

Therefore, human lymphocyte cultures were treated with 5-aza-C for at least one cell cycle and then allowed to complete subsequent cycles in the absence of this analogue. The early to late S period between treated and untreated cells were then compared to determine if 5-aza-C causes any alteration in replication patterns.

Materials and Methods

Cells and growth conditions

Peripheral lymphocytes of chromosomally normal woman were seperated by gradient centrifugation and used in all experiment. Cells were routinely cultured in the standard medium (RPMI 1640) supplemented with 15% fetal calf serum at 37°C in a humidified 5% CO_2 incubator.

5-Azacytidine treatment

5-Aza-C was added to growing cells (final conc. 7×10^{-6} M) and incubated for 24 hours. Treatment with the drug was terminated by washing the cells three times with PBS and 5-aza-C free medium.

Replication banding

Non-treated (control) and 5-aza-C-treated cells were incubated with 5-bromodeoxyuridine (BrdU), final concentration 2×10^{-5} M, for 3-13 hours prior to harvest and were then treated with colcemid (0.05 ug/ml final concentration) for 30-60 min. before harvest to arrest cells in metaphase. The cells were harvested by the standard techniques and replication bands were obtained by the Hoechst-Giemsa technique introduced by Perry and Wolff (1974) with minor modification.

Results and Discussion

By exposing the cells to BrdU for increasingly longer periods, the frequency of darkly stained bands tends to decrease. Consequently, five different stages were identified. Replication is clearly biphasic in the sense that late replicating elements such as G-bands, initiate replication after it has been completed in the R-bands. Table 1 shows the average distribution of five different stages of BrdU incorporation from samples of chromosomally normal subject.

The effect of 5-aza-C on the characteristic late replication of the inactive X chromosome was determined in the treated cells. The results shown in Figure 1 and Table 2 demonstrate that 5-aza-C treatment has a profound effect on the onset of replication of the late replicating X chromosome, shifting it to an early time in S phase. The delay in the onset of replication in late replication regions of several autosomes is also shortened following 5-aza-C treatment but this effect is much less dramatic than that seen for the inactive X chromosome (data not shown). No detectable effect on the onset of replication of the early replicating active X chromosome is found with 5-aza-C. In both 5-aza-C treated and control cells this chromosome replicates early in S phase.

Table 2 shows that in 5-aza-C treated cells the delay in the onset of replication of the inactive X was shortened significantly in 35% of the cells while in another 53% it was abolished altogether. It should be noted that 5-aza-C induced

Table 1. Distribution (in percentage) of stages of replication among mitotic samples treated for 3-13 hours with BrdU.

HRs in BrdU	Stages of replication				
	I	II	III	IV	V
3	–	–	12.3	45.3	41.3
5	–	–	65.1	35.3	–
7	4.5	14.6	75.3	5.7	–
9	42.4	17.6	40.0	–	–
11	67.5	13.6	18.9	–	–
13	80.3	5.0	14.7	–	–

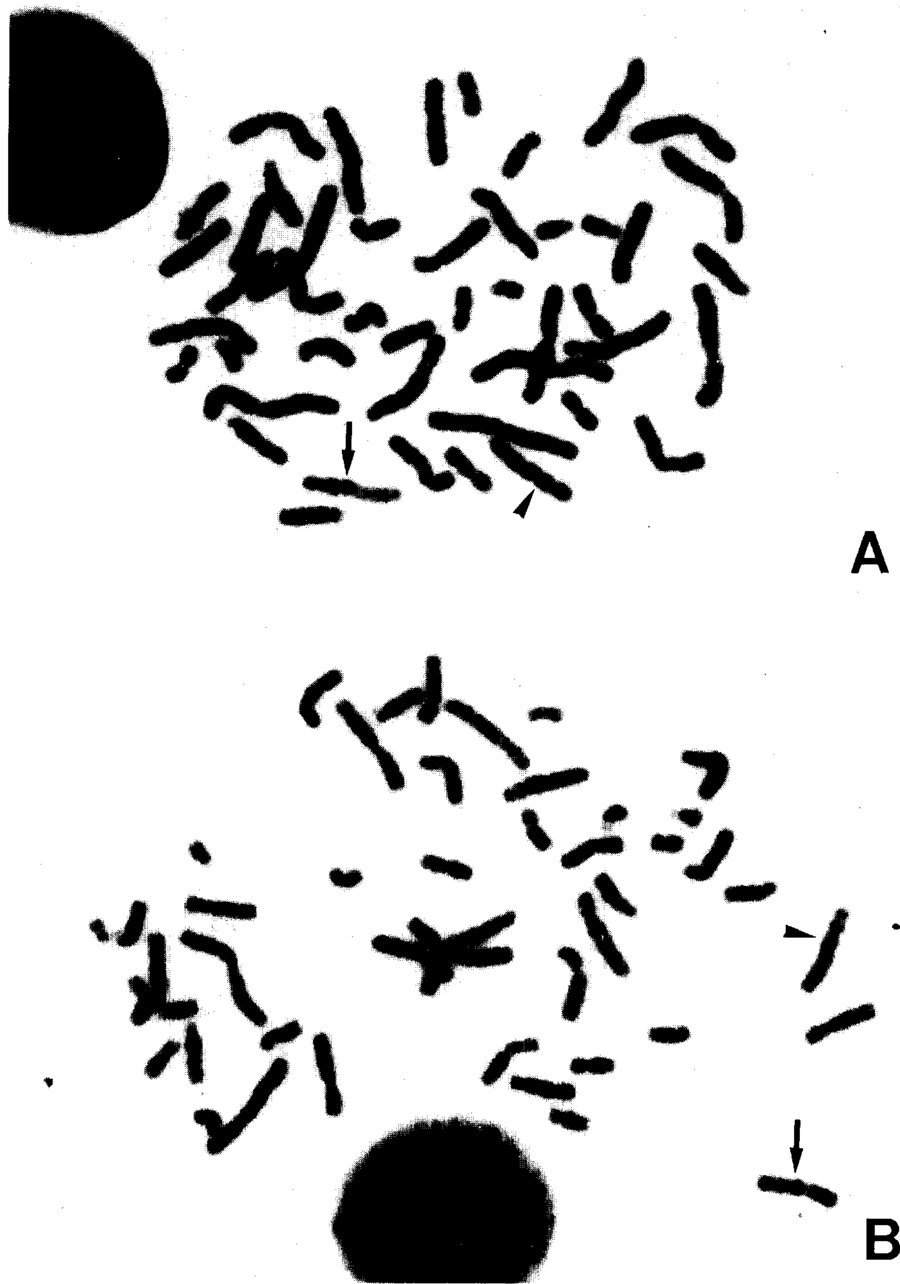

Figure 1. A: Replication banding patterns of a control cell. Early replicating X chromosome (arrowhead) and late replicating X chromosome (arrow) are shown. B: Replication banding patterns of a 5-aza-C treated cell. Late X chromosome changes in timing of replication. So, two X chromosome (arrow and arrowhead) are both early replicating.

Table 2. Effects of 5-aza-C on time of replication in pairs of X-chromosome.

Treatment	Replication of X-chromosome			
	ER/ER	ER/IR	ER/LR	Total
Control	8	29	183	220
	3.6%	13.2%	83.2%	100%
Treated	127	84	29	240
	52.9%	35.0%	12.1%	100%

*ER: Early replicating X-chromosome
IR: Intermediate replicating X-chromosome
LR: Late replicating X-chromosome

hypomethylation appears to alter the timing of initiation of X chromosome replication without affecting the well-defined order of appearance of the replication bands.

R-bands are the segments in which DNA replication is initiated at the onset of the S phase (Holmquist *et al.*, 1982). Therefore, R-bands constitute the basic units of DNA synthesis. Methaphase bands are generally composed of subbands or a mixture of G and R subbands. Consequently, these chromosomal segments richer in R-bands might simulate earlier replicating regions more likely than those segments relatively richer in G-bands (Holmquist, 1987).

The inactive X chromosome in animal cells *in vivo* is characterized by a lack of transcriptional activity of a large number of X linked genes (Lyon, 1972), a change in the chromosomal structure of these genes (Wolf *et al.*, 1984; Yen *et al.*, 1984) and a general delay in the time of replication of the entire chromosome (Takagi and Oshimura, 1973). In this study we analysed that hypomethylation of DNA causes a dramatic change in the time of replication of the entire inactive X chromosome.

Delayed onset of replication in the inactive X is shortened by 5-aza-C. This finding consists with the hypothesis that hypomethylation may be concurrently associated with induction of earlyer replication as well as with reactivation of the inactive X.

Treatment of cells with 5-aza-C altered the time of initiation without affecting the order of replication, suggesting that only the initiation event is under the control of DNA methylation. These studies demonstrate the existence of control mechanisms for regulating the time of replication of large chromosomal domains.

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배양 인체 백혈구의 chromosome replication에 미치는 DNA hypomethylation의 영향

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배양 인체 백혈구의 DNA replication 양상을 BrdU 치환을 통한 dynamic banding을 이용하여 분석하였다. 인체 염색체에서의 DNA복제는 분절상으로 일어나나 매우 협동적 양상을 보인다. 각각의 염색체는 그들 구조(banding)의 내재양상에 따라 복제된다. R-band 부위가 DNA 합성이 시작되는 부위로 보이며 G-band 부위는 R-band 부위의 복제가 완료된 후에 복제된다. 한편 발생과정에서 혹은 인위적으로 유도된 DNA의 methylation이 연관된 유전자좌들을 불활성화 시킨다는 것이 알려져 있다. 이러한 DNA methylation은 5-azacytidine을 처리함으로서 undermethylation 시킬 수 있으며 특정 유전자들을 재활성화 시킬수도 있다. 이러한 hypomethylating agent인 5-azacytidine을 처리하여 DNA 복제양상의 변화 여부를 분석 하였다. 5-azacytidine의 처리는 전반적인 복제시간의 단축을 유도하였으며 특히 불활성 X 염색체에서 두드러졌다. 이러한 복제시간의 변화는 DNA methylation이 지역적인 유전자좌들을 협동적으로 조절한다는 것을 시사한다.