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Report

Replication Timing Profile Reflects the Distinct Functional and Genomic Features of the MHC Class II Region

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KEY WORDS

replication timing, Major Histocompatibility Complex, transcription, DNA sequence, gene density, interferon, nascent DNA

ABSTRACT

The timing of DNA replication generally correlates with transcription, gene density and sequence composition. How is the timing affected if a genomic region has a combination of features that individually correlate with either early or late replication? The major histocompatibility complex (MHC) class II region is an AT-rich isochore that would be expected to replicate late, but it also contains coordinately regulated genes that are highly expressed in antigen-presenting cells and are strongly inducible in other cell types. Using cytological and biochemical assays, we find that the entire MHC replicates within the first half of S-phase, and that the class II region replicates slightly later than the adjacent regions irrespective of gene expression. These data suggest that despite AT-richness, an early-to-middle replication time in the class II region is defined by an open chromatin conformation that allows rapid transcriptional activation as a defence against pathogens.

ABBREVIATIONS

MHC, major histocompatibility complex; IFNγ, interferon-gamma; FISH, fluorescence in situ hybridization

INTRODUCTION

DNA replication in eukaryotes begins at multiple origins across the genome, with each origin firing at a specific time during S phase.[1] Although the mechanisms that regulate when an origin fires are not understood well, intrinsic genomic features that correlate with replication timing have been identified. These include gene density and DNA sequence composition, since gene dense/GC-rich DNA generally replicates early and gene-poor/AT-rich DNA replicates late during S phase.[2] The clearest manifestation of this relationship is seen on metaphase chromosomes where clustered early- and late-firing replicons form replication bands that are usually at the positions of R- and G-bands.[3,4]

The best-studied correlate of replication timing, however, is transcriptional status. In general, studies on individual genes, chromosomes, and whole genomes show that active genes replicate early in S phase while silent genes and heterochromatin replicate later.[5-8] For example, developmentally regulated genes that play a role in cell fate restriction adopt an early replication pattern when they become expressed during neural differentiation. In contrast, genes that are associated with maintenance of pluripotency in stem cells shift to a later replication status after commitment.[9] Examples are also seen in imprinted and other monoallelically-expressed genes where the active alleles replicate earlier than the inactive alleles.[10] This relationship does not hold in all genomic regions, since a highly transcribed and late-replicating region was recently identified on chromosome 22.[11]

The association of replication timing with fixed genomic features, on the one hand, and with variable transcription or chromatin conformation, on the other, poses a problem for our understanding of the organisation of replication across the genome. The patterns and sizes of replication bands in metaphase chromosomes appear to remain unchanged in different cell types. Yet studies at individual genomic locations usually show variations in replication timing according to transcriptional activity. Which of these factors are the most significant correlates of replication timing? Does replication timing change following transcriptional activation of coordinately regulated gene clusters in larger genomic regions?

What is the replication time of a genomic region with features that individually are associated with either early or late replication? Here, we have addressed these questions by examining replication timing in human cells derived from tissues that exhibit different expression profiles. The MHC has been selected as a model for these studies, as it contains clusters of co-regulated and inducible genes positioned within distinct isochores.

The MHC is one of the most gene-rich regions of the genome, and spans 3.6 Mb on chromosome 6.[12] It is divided into the class I (telomeric), III, and II (centromeric) regions. Over 30% of genes in the MHC encode immune-related proteins. The classical class I (*HLA-A*, *-B*, and *-C*) and the class II (*HLA-DP*, *-DQ*, *-DR*) genes encode proteins that present self and foreign peptides to T-cells, while the class III region encodes complement factors. MHC class II genes are expressed constitutively in antigen presenting cells such as B-lymphocytes, macrophages and dendritic cells, but not in fibroblasts.[13] Interferon-gamma (IFNγ) treatment of fibroblasts, however, coordinately induces expression of the classical MHC class II genes, and upregulates several other genes across the MHC. In fact, up to 15 genes are induced or upregulated within ~600 Kb of the class II region, many of which are clustered. Of particular interest to this study, the neighbouring classical class II and class III regions are distinct isochores with GC contents around 42% and 53%, respectively.

MATERIALS AND METHODS

Cells and IFNγ induction. Replication timing was analyzed by fluorescence in situ hybridization (FISH) in the B-lymphoblastoid cell line AHB (kindly provided by the late Lady Julia Bodmer, Oxford, UK), and in MRC5 embryonic lung fibroblasts (ATCC CCL-171) before and after IFNγ treatment. Replication timing was analyzed biochemically in AHB, MRC5, and the myeloid leukaemia cell line HL60 (ATCC CCL-240). AHB and HL60 cells were exponentially grown in suspension in RPMI 1640 supplemented with 10% Fetal Calf Serum (FCS) in a humidified 5% CO_2 atmosphere at 37°C. MRC5 cells were cultured as monolayers in RPMI 1640 with 10% FCS and 2% L-glutamine in a humidified 5% CO_2 atmosphere at 37°C. The MHC class II genes are constitutively active in AHB cells, but are silent in MRC5 cells unless activated by IFNγ. For this study, MRC5 cells were treated with 200 U/ml of recombinant human IFNγ (R&D Systems) for 24 hours to induce MHC class II gene expression. Expression analysis using Affymetrix Human Genome U133 Plus 2.0 arrays confirmed that these cell types showed the expected patterns of MHC class II gene expression (data not shown).

FISH analysis of replication timing. S-phase cells were identified by pulse labelling cultures with 100 μM bromodeoxyuridine (BrdU) (Sigma) for 30 minutes (AHB) or 1 hour (MRC5) before harvesting using standard techniques. DNA cosmid probes covering the MHC (see Supplementary Material) were labelled with biotin (BRL Bionick kit) and FISH performed using standard techniques.[14] Biotinylated probes were detected with avidin-FITC (Vector labs), and BrdU-labeled S-phase nuclei were detected with anti-BrdU followed by anti-mouse-rhodamine (both Boehringer). Slides were counterstained with DAPI and mounted in Citifluor anti-fade mounting solution. Cells were visualized with a Zeiss Axioplan fluorescence microscope. A dual band pass filter was used for the simultaneous visualization of FITC (probe signal) and rhodamine (S-phase nuclei).

Four control probes were used: an early replicating probe, spanning the glycogen phosphorylase gene (*PYGM*) and a late replicating probe (cJ21a) from the cystic fibrosis transmembrane conductance regulator gene (*CFTR*)[6,15] (both probes kindly provided by Prof. Douglas Higgs, Institute of Molecular Medicine, Oxford UK), probe (38C4/A9.5) from a gene-poor region in band 6p24[16] (kindly provided by Dr. Ioannis Ragoussis, Wellcome Trust Centre for Human Genetics, Oxford, UK), and probe RP11-313P11 spanning the β-globin gene (*HBB*) (obtained from the Wellcome Trust Sanger Institute) which is reported to replicate late in non-erythroid cells.[17]

Biochemical analysis of replication timing. Cultures were pulse labelled with 100 μM BrdU for 1 hour before fixation in cold 70% ethanol to label nascent DNA. Two strategies were then used to isolate cells at different stages of S phase so that the relative enrichment of nascent DNA could be examined. In the first strategy, AHB, MRC5 and HL60 cells were FACS-sorted into G_1, G_2/M and four fractions of S phase. The ethanol-fixed cells were then resuspended in phosphate buffered saline (PBS) followed by a 30 minute treatment with RNase A (1 mg/ml) (Sigma) and Propidium Iodide (PI) (50 μg/ml) (Sigma) prior to sorting on a Vantage cell sorter (Becton Dickinson), where 10^5 cells were collected in each fraction. In the second strategy, HL60 cells were synchronised at the G_1/S boundary with two successive treatments of 1 μg and 5 μg of aphidicolin (Sigma) per ml as previously described.[18,19] They were then washed twice with fresh medium to release the block, fixed at timepoints of 0, 1, 3, 5, 7 and 9 hours, and equal numbers of cells collected by flow sorting.

Immunoprecipitation of nascent DNA was performed as previously described.[20,21] The efficiency was monitored by analysis of mitochondrial DNA, which replicates independently of nuclear DNA throughout the cell cycle,[22] and also by spiking each fraction with a fixed amount of BrdU-containing Chinese hamster DNA. The following conditions were used for PCR analysis on a DNA Engine thermal cycler (MJ Research): 95°C for 2 minutes, followed by 19 cycles for mitochondrial DNA and 26 cycles for all other primer sets, of 95°C for 35 seconds, 55°C for 30 seconds and 72°C for 30 seconds and final extension at 72°C for 5 minutes. For each PCR reaction, 0.5 μM primer and 1000 cell equivalents were used. Locations of primer sequences are shown in Figure 2 and details given in Suppl. Table S1. PCR products were sequenced to confirm correct amplification products.

Agarose gels (2% agarose w/v) were run and prepared for southern transfer according to the Hybond N+ protocol (Amersham Biosciences). After transfer, the membranes were UV-crosslinked (Stratalinker, Stratagene) and prehybridized in Rapid-Hyb buffer (Amersham Biosciences) at 65°C for 30 minutes. Radioactively labelled probes were prepared from the gel extracted (Qiaquick, Qiagen) PCR products of the primer sets with random labelling (Prime-It II, Stratagene). Hybridization overnight at 65°C was followed by two 10-minute washes in prewarmed 2x and 1x SSC with 0.1 SDS. Data were generated by autoradiography or phosphorimager detection with accompanying software (Molecular Dynamics).

RESULTS

Replication timing was first examined across the classical MHC and the extended class II region[12] by FISH using 42 cosmid probes (Suppl. Material) in AHB B-lymphoblastoid cells, which have constitutive expression of the classical MHC class II genes. In S-phase nuclei, loci which have not yet replicated are visualised as single fluorescent signals, called "singlets" at each chromosome homologue. After replication, loci are visualised as double fluorescent signals, called "doublets", at each homologue. The proportion of singlets in a population of S-phase nuclei gives the replication time for each locus tested.[6] At least 500 chromosomes were scored for each probe.

Most of the MHC was found to replicate early, but the class II region between *HLA-DRA* (32.5 Mb) and the *TAP/PSMB* cluster (32.8 Mb) replicates later (Fig. 1, blue line). Sharp transitions to earlier replication timing are seen on either side of the later replicating domains. The transition at the class II/III boundary has been reported as a replication "switch" region in HL60 cells,[23] and is at the site of a pronounced shift in GC content. The earliest replication time is observed in the class III region close to the *HSPA1* gene cluster where a replication origin has been identified.[24]

The FISH analysis was then extended to MRC5 fibroblasts before and after IFNγ treatment, focusing on the MHC class II and III regions using 30 of the cosmid probes. The overall pattern of replication timing was very similar to that in AHB cells. In untreated MRC5 cells in which the classical MHC class II genes are silent, DNA replication occurs slightly later than in AHB cells for most of the class II and III regions (Fig. 1, green line). When the fibroblasts are treated with IFNγ for 24 h to induce expression of the classical class II genes, a small shift to earlier replication is found around the class II/III boundary and in the class II region from *HLA-DQB* to *TAPBP* (Fig. 1, orange line). However, treated and untreated fibroblasts show a remarkably similar replication time in the class II region from *HLA-DRA* to *HLA-DQA* and in most of the class III region. Control probes show expected replication times, except for *HBB* (Table 1), which showed early replication in MRC5 but is reported to be inactive in non-erythroid cells and is therefore commonly used as a late replication control. Further analysis revealed low levels of expression of *HBB* in MRC5 cells (data not shown), which probably accounts for the early replication of this locus.

The later replication of the MHC class II region, irrespective of gene expression, suggests that the high AT-content is the predominant factor influencing replication timing. Since the FISH assay depends on the discrimination of singlet and doublet signals in populations of unsynchronized cells, an erroneous interpretation of late replication can arise from poor hybridization efficiency of the probe giving an artificially low doublet score.[25] However, in the control experiments, all the probes used hybridized with an efficiency of ≥98% (data not shown). Another reason for a possibly erroneous interpretation of late replication is delayed chromatid resolution at certain replicated loci giving an abnormally high score of singlets. This has been shown previously when cells were fixed with a method that preserves 3D nuclear protein integrity, but not when fixed with the method used here.[20]

In order to verify these findings and confirm the transitions in replication timing, a biochemical approach was then taken for ten MHC class II and III loci, by determining the enrichment of BrdU-labelled nascent DNA in different cell cycle fractions of

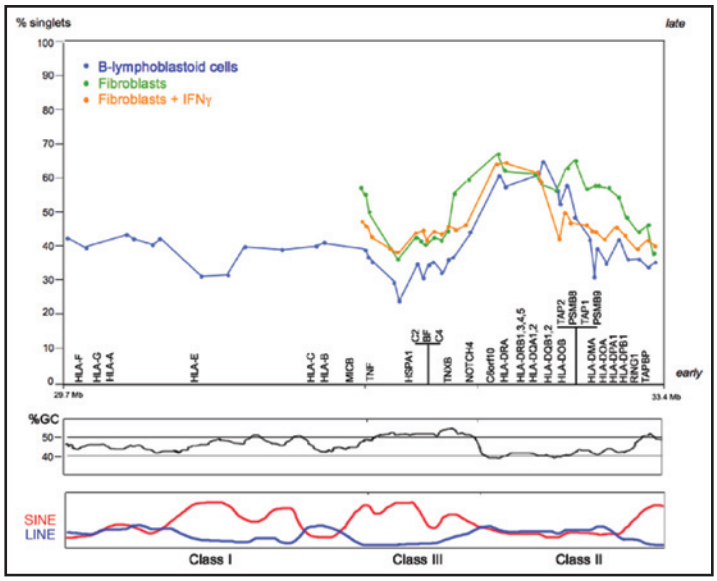

Figure 1. FISH analysis of replication timing in the MHC. The data points in the top panel show the relative replication time, expressed as percentages of singlet FISH signals, of probes tested in AHB cells (blue line), MRC5 cells (green line) and MRC5 cells induced with IFNγ (orange line). The middle panel shows the %GC,[31] while the lower panel shows the LINE and SINE repeat content across the MHC.

Table 1 **Replication timing of control probes in AHB and MRC5 cells assessed by FISH**

	% Singlets	
Probe Name	**AHB**	**MRC5**
PYGM	34	37
cJ21a	71	70
A9.5	76	74
HBB	80	49

PYGM is an early replicating control probe. cJ21a, A9.5, and HBB are late replicating control probes. HBB replicated earlier than expected in MRC5 cells.

AHB, MRC5, and a third cell type, HL60 myeloid leukaemia cells. *HLA-DRA*, a classical MHC class II gene, was found by immunofluorescent flow cytometry not to be expressed in HL60 (data not shown), as previously reported.[26] As the class II genes are coordinately regulated, they can all be assumed to be silent in HL60.

Typical FACS profiles for each cell type based on DNA content are shown in Figure 2 (panel A), with the gates on the graphs representing six sorted sub-populations: G_1, G_2/M and four fractions of S phase. All the MHC loci tested showed enrichment in either the S1 or S2 fractions (Fig. 2, panels B and C). AHB showed a pronounced enrichment in the S1 fraction, while HL60 and MRC5 exhibited enrichment across both the S1 and S2 fractions. Therefore, the MHC appears to replicate slightly earlier in AHB cells than it does in the other two cell types. Control probes gave expected patterns of enrichment. Early replication of *HBB* was confirmed in MRC5 cells (Fig. 2, panel C). An additional late replicating control, the factor IX gene (*F9*),[27] was therefore used for MRC5. These findings indicate that the MHC loci examined here replicate within the first half of S phase, suggesting a potential inconsistency with the data

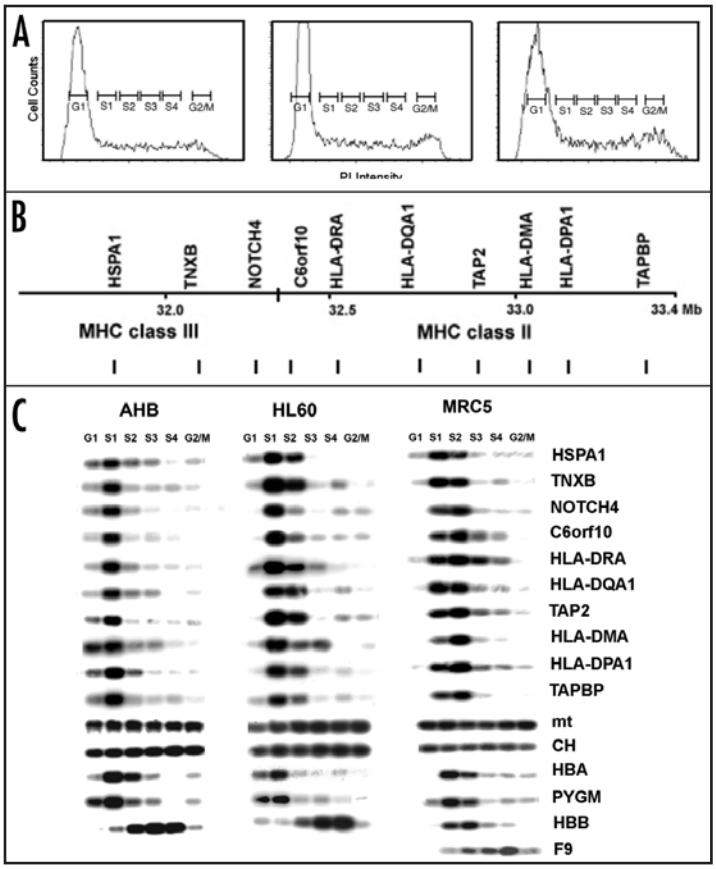

Figure 2. Biochemical analysis of replication timing of the MHC in AHB, HL60 and MRC5 cells. AHB, HL60 and MRC5 cells were pulse-labelled with BrdU and flow-sorted into G_1, S1, S2, S3, S4 and G_2/M fractions. The relative abundance of PCR products in each fraction was then tested after immunoprecipitation. (A) FACS profiles of PI intensity, corresponding to DNA content, in AHB, HL60 and MRC5 cells. The marked gates were used for sorting cells into the different fractions. Gaps were left between the gates to increase the purity of each fraction. (B) Positions of loci examined in the MHC in relation to landmark genes. (C) Relative abundance of nascent DNA from MHC loci and controls in different cell cycle fractions. The MHC loci examined were most enriched in the S1 and/or S2 fractions in all three cell types, indicating that the MHC replicates in the first half of S-phase. Results shown are typical of at least two independent experiments. IP controls: mitochondrial DNA (mt) and Chinese hamster DNA (CH) had bands of similar intensity across the six fractions indicating equivalent efficiency of the immunoprecipitation. Early replicating controls: α-globin gene (*HBA*) and glycogen phosphorylase gene (*PYGM*), were most enriched in the G_1 and/or S1 fractions. Late replicating controls: β-globin gene (*HBB*) was most enriched in the S3 and S4 fractions in AHB and HL60 cells, and surprisingly, in the S2 fraction in MRC5; the factor IX gene (*F9*) was most enriched in the S3 and S4 fractions.

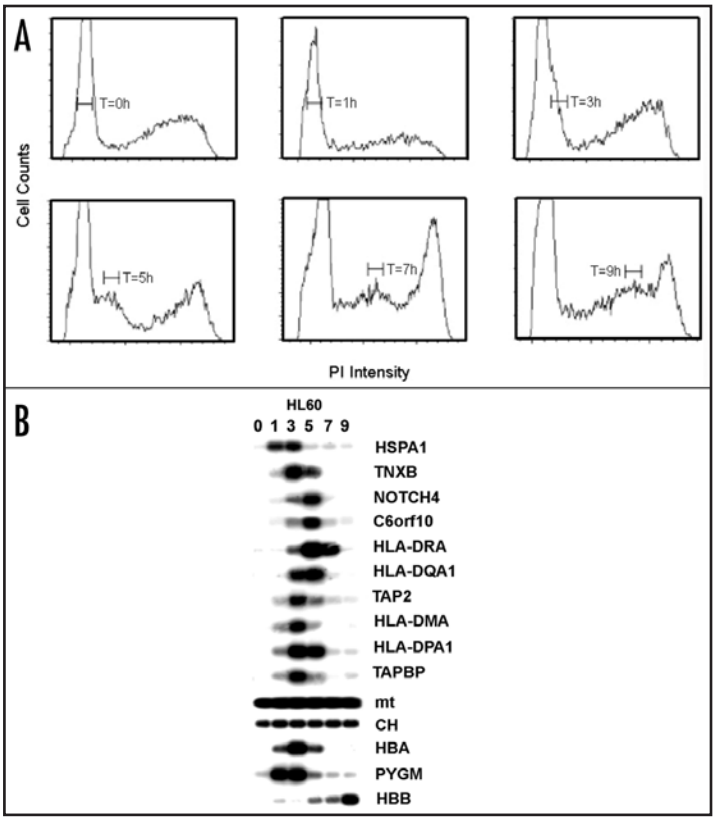

Figure 3. Biochemical analysis of replication timing of the MHC in synchronized HL60 cells. HL60 cells were synchronized, released, pulse-labelled with BrdU, and fractions containing equal numbers of cells collected by flow-sorting at 0, 1, 3, 5, 7 and 9 hours. The relative abundance of PCR products in each fraction was then tested after immunoprecipitation. (A) Typical FACS profiles of cell populations at different fixation time-points post-release. Cell counts are shown across varying propidium iodide (PI) intensities, corresponding to DNA content. Cells were sorted using the appropriate gates around the peaks of synchronized cells. (B) Relative abundance of nascent DNA from MHC loci and controls in different time-point fractions. The IP controls, mitochondrial and Chinese hamster DNA, showed even intensities across the different fractions. Early replicating controls *HBA* and *PYGM* were most enriched within the first 3 hours post-release from block. The late replicating *HBB*, was enriched at fraction t = 9 hours. HSPA1L was the earliest replicating of loci tested. Results are typical of at least two independent experiments.

from the FISH assay showing a biphasic replication programme in this region.

To identify the timing of replication in MHC class II and III with greater precision, a second strategy was employed (adapted from refs. 18 and 19). Using HL60 cells, which are amenable to aphidicolin-induced replication block, a cell population was synchronized at the G_1/S boundary with double aphidicolin block-and-release. Fractions containing equal numbers of cells were collected by FACS at various timepoints after release from the block (Fig. 3, panel A). Simultaneously, each sorted fraction was analysed by flow cytometry in order to quantitate the proportion of cells that have incorporated BrdU, as an indication of the number of cells that would contribute to the observed signal. The earliest and latest fractions, near to G_1 and G_2/M cell cycle stages, contained a high percentage of cells that were not BrdU-labelled (Suppl. Table S2). The relative abundance of the PCR products in each fraction was then determined. The loci from *HSPA1* in the class III region to *HLA-DRA* in the class II region were found to replicate progressively later, while those from *HLA-DQA* to *TAPBP* in the class II region were found to replicate earlier (Fig. 3, panel B). Once again, the MHC class II region has a zone of later replication, in agreement with the FISH studies. The early control loci *HBA* and *PYGM* showed the greatest enrichment in the 1 and 3 hour fractions, while the late replicating control *HBB* was enriched in the nine hour fraction (Fig. 3, panel B).

DISCUSSION

Taken together, these data show that the entire MHC replicates within the first half of S phase in all cell populations tested. A large portion of MHC class II replicates later than the adjacent regions, and there are steep transitions between the earlier and later replicating regions. However, replication timing does not correlate strictly with gene expression, as we do not see very early replication in the class II region in AHB and IFNγ treated MRC5 cells, or late replication in untreated MRC5 and in HL60 cells. Rather, the class II region replicates in the early-to-middle period of S phase, and the FISH assay shows small differences between expressing and non-expressing cells.

Replication timing in the MHC is thus likely to be influenced by other features, such as gene density or sequence composition. As a gene-rich region, the MHC would be expected to have early replication. Indeed, the class III region has the highest gene density in the genome, containing an expressed gene every 15 Kb or less, and we find the earliest replication here in agreement with a previous study.[7] Several lines of evidence suggest that replication timing is also strongly associated with local epigenetic features such as acetylation or methylation of specific histone residues and the presence or absence of certain regulatory protein(s) (reviewed in ref. 28). Therefore, if chromatin in the MHC is "poised" for rapid transcriptional activation, as suggested by the massive chromatin decondensation that occurs within minutes of exposure to IFNγ,[14] an "open" chromatin architecture might predispose to relatively early replication even in non-expressing cells. This is consistent with a report that replication timing in the human β-globin locus correlates with an open chromatin architecture rather than expression per se in certain cell types.[29]

The classical class II region contains clusters of coordinately controlled, immune-related genes that are likely to have arisen during evolution by segmental duplications.[12] It thus behaves as a distinct genomic region in terms of its expression. Why does such a large part of the class II region replicate in the early-to-middle period of S phase, considerably later than the rest of the MHC? The AT-richness is unusual for a gene rich region, but it may have a role in determining the replication timing here. Differentiation-induced early-to-late and late-to-early shifts in replication timing are reported to occur predominantly in genes that are present in AT/LINE-rich regions.[30] The FISH analysis shows slightly earlier replication in B-lymphoblastoid cells and IFNγ treated fibroblasts than in untreated fibroblasts in sections of the class II and III region, but these sections have a relatively low LINE density.

This study highlights the advantages of combining FISH and biochemical assays for replication timing. The FISH analysis suggested that the class II region replicates late in the three cell populations studied, but the biochemical analysis showed that in fact it replicates during the first half of S phase. The following explanation can be offered for the apparent disagreement between the two sets of data. The resolution of replication timing between the MHC probes according to FISH is very detailed with a section of the MHC class II region replicating apparently "late" in S phase or, more precisely, later than its neighbours. In terms of absolute replication timing, however, the evidence from the biochemical assay indicates that in fact, the loci tested replicate within the first half of S phase. The FISH data offer an insight into the relative replication time for each probe within each cell type examined. The biochemical data on the other hand are more informative for the absolute timing of replication for each cell type, but the resolution does not enable detection of details to the levels as seen by FISH. The FISH analysis which has given a good temporal resolution, has revealed a consistent pattern of peaks and dips in all three cell populations. Since replication timing is a function of both the time that an origin fires and the spacing between origins, these data suggest overall similarities in the replication dynamics in the cell types examined. The report of a replication origin close to the *HSPA1* cluster[24] where we find the earliest replication time suggests that the dips may be close to the positions of preferred origins. Furthermore, the borders of the regions showing a slight shift to earlier replication timing in IFNγ treated fibroblasts are likely to demarcate the boundaries of different replicons - those whose timing is influenced by transcriptional activation and those that are not.

In conclusion, we have shown that replication timing in the entire MHC occurs in the first half of S phase, and that the class II region replicates slightly later than the adjacent regions irrespective of transcription. Therefore, replication timing in the MHC does not correlate strictly with either the expression status or the intrinsic features of the DNA sequence alone. Instead, both factors combined contribute to the early replication across the MHC and the distinct profile of the class II region. Our findings suggest that replication timing in the MHC correlates with chromatin in the region being poised for transcriptional activation as an essential component of the cell's defence against pathogens.

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Note

Supplemental material can be found at:
http://www.landesbioscience.com/supplement/TakousisCC6-19-Suppl.pdf

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