Temporal profile of replication of human chromosomes

Yesse Jeon*,†‡, Stefan Bekiranov§, Neerja Karnani*, Philipp Kapranov§, Srinka Ghosh§, David MacAlpine¶, Charles Lee†, Deog Su Hwang†‡, Thomas R. Gingeras§, and Anindya Dutta*††

*Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA 22908; †Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115; ‡Institute of Molecular Biology and Genetics, School of Biological Sciences, Seoul National University, Seoul 151-742, Korea; §Affymetrix, Santa Clara, CA 95051; and ¶Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

Edited by Thomas J. Kelly, Sloan–Kettering Institute, New York, NY, and approved March 12, 2005 (received for review July 13, 2004)

Chromosomes in human cancer cells are expected to initiate replication from predictably localized origins, firing reproducibly at discrete times in S phase. Replication products obtained from HeLa cells at different stages of S phase were hybridized to cDNA and genome tiling oligonucleotide microarrays to determine the temporal profile of replication of human chromosomes on a genome-wide scale. About 1,000 genes and chromosomal segments were identified as sites containing efficient origins that fire reproducibly. Early replication was correlated with high gene density. An acute transition of gene density from early to late replicating areas suggests that discrete chromatin states dictate early versus late replication. Surprisingly, at least 60% of the interrogated chromosomal segments replicate equally in all quarters of S phase, suggesting that large stretches of chromosomes are replicated by inefficient, variably located and asynchronous origins and forks, producing a pan-S phase pattern of replication. Thus, at least for aneuploid cancer cells, a typical discrete time of replication in S phase is not seen for large segments of the chromosomes.

DNA replication | genome-wide | microarrays | chromatin | human cancer

B
gener consensus, origins of replication are expected to map at precise locations on chromosomes, fire consistently at the same time in S phase, and produce forks that proceed bidirectionally at predictable and reproducible speeds to eventually replicate entire chromosomes. Based on the average speed of fork movement (1), origins are expected to be distributed at an average spacing of 100 kb, and yet they have been surprisingly difficult to map for single-copy genes in mammalian cells in culture. For the handful of well studied origins, there is one report suggesting use of a single discrete initiation site (2) and a few suggesting use of multiple initiation sites distributed 10–50 kb, with different sites used in different cells with variable efficiency (3–10). In addition, origins are expected to fire at discrete times in S phase (11). For example, Giemsa R bands on metaphase chromosomes mostly replicate earlier in S phase compared with the Giemsa G bands (12). Heterochromatin regions near the centromeres and telomeres are usually replicated late in S phase (13, 14). Examination of a few genes suggest that housekeeping genes like actin and nuclear lamin replicate early in S phase, whereas differentiation-specific genes like immunoglobulin or β-globin replicate late in cells where they are not expressed and early in cells where they are expressed (7, 15, 16). It has been postulated that the open nucleosome structure associated with euchromatin and with expressed genes is conducive to early replication in S phase.

With the advent of complete genome sequences, we can now ask what fraction of replication origins behaves in the ideal fashion described above and whether the rules regarding time of origin firing apply on a genome-wide scale. In Saccharomyces cerevisiae, the time of replication of all 6,000 genes was determined, and the results were modeled to estimate the distribution of origins of replication along the yeast chromosomes (17). Although such studies revealed the locations and time of firing of origins, they produced the surprising result that expression of a yeast gene did not correlate with time of replication in S phase. A similar study on cultured cells in Drosophila, however, demonstrated that there was a correlation between the expression of a gene and its likelihood of being replicated early (18).

We have now carried out a study in human cancer cells in culture to estimate the time of replication of hundreds of genes. Thymidine-aphidicolin block was used to arrest cells at the G1/S boundary. After confirming that cells released from the block progress synchronously through S phase, we harvested timed replication pools of DNA and used these as probes first on cDNA arrays to validate this method for establishing time of replication. The pools were subsequently used on newly available high-density genome-tiling arrays covering all unique sequences on chromosomes 21 and 22. The high definition obtained by use of finer timed replication pools and high density genomic arrays shows that, whereas gene density and chromatin states predict time of replication of many segments, many other segments are replicated in a pan-S phase pattern suggestive of significant flexibility in origin usage, origin timing, and fork movement.

Methods

High-Density Genome-Tiling Array. The arrays comprise 1,020,643 probe pairs (i.e., Perfect Match, Mis-Match) interrogating the repeat masked sequence of chromosomes 21 and 22 (≈35 Mb) (Affymetrix, Santa Clara, CA). Thus, on average, there is a probe pair every 35 bp in the non-repeat regions of the two chromosomes. There is no bias regarding exonic/coding regions versus noncoding regions. Heavy/light (H/L) DNA from each time point was prepared as mentioned in Fig. 1, fragmented with DNaseI to ≈50 bp, and then end-labeled with biotin-dATP by using terminal transferase. The labeled DNA was hybridized to the genome-tiling arrays. The array results were normalized and analyzed by using g-TRANS software (Affymetrix). The graphs from these processed data were generated by using INTEGRATED GENOME BROWSER software (Affymetrix).

Measurement of Enrichment of Replication in a 2-hr Interval Relative to the Entirety of S Phase. Because the distribution of array intensities should be the same across all replicate arrays, all replicate arrays were quantile-normalized (19). For each genomic position to which a probe pair (PM, MM) mapped, i, a data set, Si, was generated consisting of all (PM, MM) pairs mapping within a window of ±5 kb. We test for the statistically significant enrichment of treatment (signal over the 2-hr labeling period) over control (signal over the 8-hr labeling period) by applying a Wilcoxon rank sum test (20) to these estimates of abundance. Genomic positions belonging to regions that were

1This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: H/L, heavy/light; TR50, time of 50% cumulative replication.

†To whom correspondence should be addressed. E-mail: ad8q@virginia.edu.

© 2005 by The National Academy of Sciences of the USA

www.pnas.org/cgi/doi/10.1073/pnas.0405088102

PNAS | May 3, 2005 | vol. 102 | no. 18 | 6419–6424
Two cycles of centrifugation were sufficient to purify the H/L double-stranded DNA separated from unlabeled light-oxuridine for 2-hr intervals and BrdUrd-incorporated Hsynchronously through S phase were labeled with bromode-normal progression through S phase (Fig. 1)

The sum total of cpm values was regarded as 100% of replication. Results were obtained in triplicate from two independent experiments. (C) Isolation of newly replicated DNA on CsCl density gradient. (Top) Genomic DNA labeled during 0–2 hr of S phase. Solid line is the anti-BrdUrd reactivity in an ELISA, and dashed line is the absorbance at 260 nm, which reflect distribution of H/L DNA and total DNA, respectively. (Middle) Second purification of H/L fractions obtained from Top. Complete overlap of two peaks indicates reliable purity of H/L DNA. (Bottom) First round of CsCl centrifugation of genomic DNA from cells labeled with BrdUrd for 0–10 hr of S phase. All genomic DNA was labeled into H/L DNA within 10 hr.

Probes for Interphase FISH. For interphase FISH, the pan-S phase pattern probes were RP1-5O6 and RP11-247I13 (representing chromosome 22 region 36,675,000–36,775,000 and 30,301,231–30,313,718 as per NCBI30 build). RP4-671O14 and RP11-91E22 (chromosome 22 region 42,550,000–42,725,000, and chromosome 11 gene 39987:Hs.167185) were from segments with monophasic replication, early and late replicating, respectively.

Results and Discussion

Synchronization of HeLa Cells and Harvesting of Timed Replication Products. Thymidine-aphidicolin block was used to arrest human cervical carcinoma cells (HeLa) at the G1/S boundary. Release of the cells from the block followed by FACS for DNA content showed that DNA content began to increase by 2 hr and eventually doubled after 8–10 hr (Fig. 1A). The bell-shaped distribution of DNA synthesis during S phase was consistent with normal progression through S phase (Fig. 1B). Cells progressing synchronously through S phase were labeled with bromodeoxyuridine for 2-hr intervals and BrdUrd-incorporated H/L double-stranded DNA separated from unlabeled light-light (L/L) DNA by CsCl density gradient centrifugation (Fig. 1C). Two cycles of centrifugation were sufficient to purify the H/L DNA, and labeling of cells for the entire duration of S phase converted all of the DNA in the population to H/L DNA.

Hybridization to cDNA Arrays. DNA replicated in each time interval was purified as in Fig. 1C and labeled with Cy5 whereas total genomic DNA (H/L DNA obtained by labeling for 0–10 hr) was labeled with Cy3 as a negative control. The two probes were mixed in equal molar ratios and hybridized to a human cDNA array containing 1,589 genes spotted in duplicate on glass slides. Only 625 of the 1,589 spotted genes (39%) showed reproducible patterns of replication that allowed them to be clustered according to their time of replication in S phase (Fig. 2A). Examples of genes replicated preferentially during a given time interval are shown in the boxes on the side.

Validation of Time of Replication. Because these methods have not been applied for these analyses before, we confirmed the classification of a subset of the genes by two approaches. First, the timed replication products were immobilized on membranes, and selected genes were used as radioactive probes. The bar graphs of the hybridization signals confirmed the classification of early replicating (Fig. 2B) and late-replicating (Fig. 2C) genes. Nine (five early and four late candidates) of 10 selected cDNAs were confirmed by dot blotting to be correctly classified (data not shown). The second approach, interphase FISH, will be discussed later.

Hybridization to Genome-Tiling Arrays Reveals Segments That Replicate in a Defined Part of S Phase and Other Segments That Replicate in a Pan-S Phase Pattern. Despite the success in classifying time of replication for 40% of the genes, the failure to classify the time of replication for 60% of the genes was surprising and could be because we were investigating genomic events with spliced cDNAs. We therefore turned to a high-density genome-tiling Affymetrix array comprising all unique oligonucleotides in chromosomes 21 and 22 representing ~35 Mb of DNA (21, 22). These
on chromosome 21, and 70, 87, 28, and 45 regions on chromosomes and cells.

### Supporting Methods

Supporting Methods

### Early Replication Correlates with High Gene Density.

We next compared the time of replication of a chromosomal segment with the exon density in that segment (Supporting Methods). For each mapped ideally replicating region, the exon density was estimated by computing the median of the exon density values that were mapped high confidence ($P < 10^{-5}$) regions of replication for each quarter of S phase applying the Wilcoxon rank sum test in a sliding window of 10 kb. We found 40, 39, 17, and 77 regions on chromosome 21, and 70, 87, 28, and 45 regions on chromosomes and cells.

### FISH Analyses to Confirm the Monophasic and Pan-S Pattern of Replication.

We confirmed the time of replication of regions selected from the cDNA and genome-tiling arrays by interphase FISH (23). Synchronously progressing HeLa cells were harvested at indicated time points during S phase, and genomic clones were labeled and hybridized to the denatured interphase chromosomes. A single hybridization signal indicates that the targeted DNA region is unreplicated, and a doublet hybridization signal indicates that the targeted DNA region is replicated. The replication of each probe during a time interval is estimated by the increase in the number of dots during that time interval. For all of the regions shown in Fig. 4, cells at the G1/S boundary (0 h) contained three single dots due to the aneuploidy of HeLa cells. After entry into S phase, however, cells showed hybridization signals ranging from three single dots to three doublets (six dots) (Fig. 8, which is published as supporting information on the PNAS web site). The appearance of intermediate numbers of dots/cell (four, five, and six) indicate that all three copies of the gene do not replicate at exactly the same time in a cell. The early replicating pattern of RP4-671O14 is clearly evident with a robust increase in the number of dots in the first 4 hr of S phase. For the probes representing pan-S pattern of replication, RP1-5O6 showed a biphasic pattern of replication as the increment in dots showed two peaks separated by one interval relative to the entirety of S phase (another example in Fig. 6, box B, which is published as supporting information on the PNAS web site) are indicative of replication from well defined origins and uniformly migrating forks that are used in most of the cells in the culture at a similar time in S phase. In contrast to the 35.1% of the sequences that replicate in a temporally monophasic pattern, many regions gave a robust replication signal over the entire 0–8 hr interval but were not selectively replicated in one particular quarter of S phase (Figs. 3C and 6 and Supporting Methods; see also Fig. 7, which is published as supporting information on the PNAS web site). We suggest that this pan-S phase pattern of replication is due to variably located replication initiation sites that fire asynchronously and due to replication forks that migrate at variable speeds over the same genomic segments in different chromosomes and cells.

### Early Replication Correlates with High Gene Density.

We next compared the time of replication of a chromosomal segment with the exon density in that segment (Supporting Methods). For each mapped ideally replicating region, the exon density was estimated by computing the median of the exon density values that fall within the region. The exon densities are high ($\approx 0.033$) for regions replicated during the first 4 hr of S phase compared with the areas replicated in the last 4 hr (0.009 for 4–6 hr; 0 for 6–8 hr replication periods) (Fig. 5A). The drop in exon density from either of the two early replicating regions to the later replicating regions is significant ($P < 10^{-15}$).
Time of 50% Cumulative Replication (TR50) Profile of Chromosomal Segments. The replication time at each interrogated position on the array was estimated more precisely by calculating the increase in the signal for each of the four time periods and then calculating the time that corresponds to 50% cumulative replication, TR50 (Supporting Methods). Plotting the TR50 against the distance along the chromosome produces a temporal profile of replication that identifies sites that are replicated earlier than their adjoining segments (Fig. 5B). These sites will be useful places to search for origins of replication. Plotting of the exon density and TR50 shows a striking anticorrelation between exon density and replication timing on a chromosomal scale (Fig. 5B). The Spearman correlation coefficient between the two profiles was \(-0.34\) and \(-0.58\) for chromosomes 21 and 22, respectively (Supporting Methods). The profile also suggests that centromeric regions replicate late in S phase (Fig. 9, which is published as supporting information on the PNAS web site), consistent with earlier cytogenetic studies and with the paucity of genes in these areas. Although a clear transition from low to high A+T content is evident for regions that replicate early versus late in S phase (Fig. 10, which is published as supporting information on the PNAS web site), this correlation could be explained by the association of high exon density with early replicating regions and the fact that exon-dense regions tend to have low A+T content compared with the genome average.

At Least Two Discrete Chromatin Environments Could Account for the Replication Times. High exon density could correlate with a more open chromatin structure. Assuming there are a finite number of chromatin structures on a genomic scale (e.g., euchromatin and heterochromatin), if chromatin structure drives replication time, the exon density versus replication time profile might be ex-
expected to assume a staircase profile where relatively open chromatin with high exon density is replicated first followed by sharp decreases (or steps) in exon density for every subsequent state of chromatin. Indeed, we find a relatively strong staircase-like profile in the running average of exon density versus replication time scatter plot (Fig. 5C). For regions replicated in 0–2.8 hr, the average exon density is flat around 0.028. For regions replicated at 2.8–3.5 hr, the exon density drops 64% to 0.01 where it remains flat until we reach regions replicated about the fifth hour of replication. This transition in exon density occurs across regions that differ in replication time by ~40 min and is very sharp given cell synchronization errors that are on the order of tens of minutes. The inflexion point (i.e., where the second derivative in the averaged exon density versus replication timing profile is zero) in the transition occurs at ~3.1 hr at an averaged exon density of 0.0174. This time likely corresponds to a first wave of replication over the most accessible states of chromatin and not necessarily all of euchromatin. Regions of much more compact chromatin are likely beginning to be replicated after 3.1 hr.

Early Replication Corresponds to Higher Gene Expression. Given that we expect gene expression to be suppressed in heterochromatin relative to euchromatin, we further tested the hypothesis that replication timing is determined by chromatin structure by comparing the gene expression levels against replication times for the efficient origins (Supporting Methods). The trend is clearly toward decreasing exon expression level with increasing repli-
cation time (Fig. 5D). The median exon expression level (in arbitrary intensity units) for the regions replicated in the first quarter of S phase is 515, which is very close to the cutoff that defines expressed exons. The median expression levels in loci replicating in the remaining three quarters of S phase are 388, 315, and 18. The drop in expression levels from loci replicated in the first two quarters of S phase to the last is significant ($P < 10^{-15}$).

**Conclusions**

DNA microarrays are being developed into a powerful tool for genome-wide assay of patterns of DNA replication in yeasts and *Drosophila* (17, 18). Experiments on cosmid-based genomic arrays to assay DNA abundance of human cells sorted into G1 and S phases also find that high gene density is correlated with early replication (24). Here, we used cDNA arrays to demonstrate that the finer timed replication pools from synchronized cells give valid results regarding time of replication of genes. The pools were then applied to very high-density DNA microarrays to assay DNA abundance of human cells sorted into G1 and S by flow cytometry also find that high gene density is correlated with early replication (24). Here, we used cDNA arrays to demonstrate that the finer timed replication pools from synchronized cells give valid results regarding time of replication of genes. The pools were then applied to very high-density genome-tilling DNA arrays. Forty percent of the genomic sequences in chromosomes 21 and 22, as well as the genes distributed throughout the genome, replicate reproducibly in a precise part of S phase. Even with a higher stringency cutoff ($P < 10^{-3}$), the results identify at least 400 segments where we can fruitfully search for ideal origins of replication. In addition, we identify hundreds of early and late replicating segments that can be studied molecularly to elucidate the mechanisms that control time of origin firing in human cells. The time of replication in S phase of these segments is correlated with gene density in a manner that suggests that two to three discrete chromatin environments contribute to the time of DNA replication.

A surprise from the study is the discovery that 60% of the interrogated genomic segments with a good replication signal replicate through the four quarters of S phase without any enrichment in one particular time period. Replication at several origins has been reported to initiate from delocalized initiation zones controlled by redundant replicator elements (25–28), and the initiator protein, human origin recognition complex (ORC), does not have detectable sequence specificity (29). In view of this finding, the simplest explanation for the pan-S phase pattern of replication is that large stretches of chromosomes in HeLa cells are replicated by distributed and inefficient origins (or replication forks) that fire (or move) variably in space and time during S phase. Although some of this pan-S phase replication might be due to aneuploidy, malignant transformation, mono-allelic gene expression, and imprinting, our results suggest significant flexibility in origin selection and firing and replication fork movement in mammalian cancer cells. It will be worthwhile to examine whether the pan-S phase pattern of replication is as widespread in diploid cells synchronized by other methods.

This work was supported by National Institutes of Health Grants RO1 CA60499 and UO1 HG003157 (to A.D.) and an award from the Brain Korea program of the Korean Ministry of Education (to Y.J. to work with A.D.). D.S.H. and Y.J. were partly supported by Systems Biology, Ministry of Science and Technology, Republic of Korea. This project was also funded in part by Federal funds from the National Cancer Institute, National Institutes of Health, under Contract N01-CO-12400, and by Affymetrix, Inc. (to T.R.G.).