Journal of Cellular Biochemistry 92:755–769 (2004)

Transitions in Replication Timing in a 340 kb Region of Human Chromosomal R-Band 1p36.1

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Abstract DNA replication is initiated within a few chromosomal bands as normal human fibroblasts enter the S phase (Cohen et al. [1998]: Exp Cell Res. 245:321–329). In the present study, we determined the timing of replication of sequences along a 340 kb region in one of these bands, 1p36.13, an R band on chromosome 1. Within this region, we identified a segment of DNA (approximately 140 kb) that is replicated in the first hour of the S phase and is flanked by segments replicated 1–2 h later. Using a quantitative PCR-based assay to measure sequence abundance in size-fractionated (900–1,700 nt) nascent DNA (Giacca et al. [1994]: Proc Natl Acad Sci USA. 91:7119), we mapped two functional origins of replication separated by 54 kb and firing 1 h apart. One origin was found to be functional during the first hour of S and was located within a CpG island associated with a predicted gene of unknown function (Genscan NT_004610.2). The second origin was activated in the second hour of S and was mapped to a CpG island near the promoter of the aldehyde dehydrogenase 4A1 (*ALDH4A1*) gene. At the opposite end of the early replicating segment, a more gradual change in replication timing was observed within the span of approximately 100 kb. These data suggest that DNA replication in adjacent segments of band 1p36.13 is organized differently, perhaps in terms of replicon number and length, or rate of fork progression. In the transition areas that mark the boundaries between different temporal domains, the replication forks initiated in the early replicated region are likely to pause or delay progression before replication of the 340 kb contig is completed. J. Cell. Biochem. 92: 755–769, 2004.

Key words: replication origins; replicons; quantitative PCR; chromosome 1; early S phase

The duplication of the genome of eukaryotic cells is a complex process that is temporally and spatially regulated and highly reproducible from cell generation to cell generation. DNA replication starts at multiple sites called origins, operationally defined as sites where replication forks are established through the unwinding of the parental DNA strands and the synthesis of the first primers for nascent DNA elongation [Ritzi and Knippers, 2000]. Mapping of specific origins of DNA replication has been greatly advanced by the development of functional assays based on the structure of replication intermediates or the dynamics of DNA replication. In one of these assays [Giacca et al., 1994], the chromosomal location of functional origins was inferred from the relative abundance of several neighboring genetic markers in a preparation of short nascent DNA. This PCR-based assay has been used to identify an increasing number of origins of replication, including those near the following human genes: *lamin B2* [Giacca et al., 1994], *β-globin* [Aladjem et al., 1995], *c-myc* [Tao et al., 2000], *HPRT* [Cohen et al., 2002], and *G6PD* [Cohen et al., 2003]. There is not, to date, a consensus on whether specific DNA sequences are needed for origin function in mammalian cells, or epigenetic mechanisms are sufficient to direct the assembly of the replication machinery to different sites throughout the genome. Among the few mammalian origins reported to date, there are examples of both precisely defined origin sequences, which also confer replication activity

Grant sponsor: NIH, PHS; Grant numbers: ES09112, CA84493.

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Received 10 November 2003; Accepted 6 February 2004

DOI 10.1002/jcb.20101

when inserted into ectopic sites, as in the human β-globin locus [Aladjem et al., 1998], and of broad initiation zones, as in the hamster DHFR locus, where several different sites are used to initiate DNA synthesis, albeit with variable degrees of efficiency [Dijkwel et al., 2002; Mesner et al., 2003].

The activation of an origin of replication leads to the formation of two replication forks that move away from the origin in opposite directions. The segment of DNA replicated under the control of a single origin represents a unit of DNA replication (replicon). Units of bidirectional DNA replication have been long visualized by DNA fiber autoradiography after labeling of the nascent strands with a radioactive tracer [Huberman and Riggs, 1966] and, more recently, with bromodeoxyuridine (BrdU) [Jackson and Pombo, 1998] and biotinylated nucleotide analogs [Takebayashi et al., 2001]. These approaches are very useful for visualizing a number of units of DNA replication and obtaining statistical information on replicon size and distribution. Replicons are organized into clusters that are activated at different times in the S phase for complete duplication of the 3×10^9 bp human genome in approximately 8 h [Hand, 1978]. It has been known for a long time that in mammalian cells replicons are heterogeneous in size. They appear to vary from about 30 to 450 kb [Edenberg et al., 1976; Hand, 1978], with the majority of replicons falling in the range of 75–150 kb [reviewed in Berezney et al., 2000]. In a study of foci of BrdU incorporation at the onset of S phase in CHO cells, it was estimated that about 1,000 foci (replicon clusters) are active at any time in early S phase and that each Mb of DNA contains at least six replicons that are activated in a synchronous manner [Ma et al., 1998]. Fewer and larger foci were observed in mid- and late-S phase [Berezney et al., 2000]. The timing of replication of a given DNA sequence has been correlated to gene transcription activity and the general consensus is that expressed genes replicate early, while inactive ones tend to replicate later in the S phase [Goldman et al., 1984; Holmquist, 1987; Hatton et al., 1988]. Whole-genome analyses found a strong correlation between replication in early S phase and transcriptional activity in *Drosophila* [Schübeler et al., 2002] but not in yeast [Raghuraman et al., 2001]. It has been suggested that this finding reflects an evolutionary trend towards achieving and maintaining spatial order in the much more complex metazoan nucleus [Gilbert, 2002].

An average human chromosome contains about 120 Mb of DNA in a continuous chromatin fiber. When this fiber coils and condenses during mitosis, staining of the chromosome reveals approximately 50 discrete bands, which can be distinguished as Giemsa-positive, or G-bands (late replicating), and Giemsa-negative, reverse or R-bands (replicating early). R-bands are GC-rich and contain expressed genes, while G-bands are relatively gene poor and enriched in repetitive sequences. It has been suggested that a topographical boundary exists between R and G bands that possibly determines the transition in replication timing [Strehl et al., 1997; Tenzen et al., 1997]. Replication barriers have been observed in *S. cerevisiae* [Brewer and Fangman, 1988] and in human and mouse rDNA. A site-specific termination of replication (replication fork barrier, or RFB) was found near transcriptional terminator elements, known as Sal boxes, close to the end of the rDNA transcription unit. In mouse, this RFB was shown by 2D gel analysis to act in a polar manner, arresting only replication forks moving in the direction opposite to transcription [Lopez-Estraño et al., 1998], while in human rDNA the RFBs seem to arrest forks moving in both directions [Little et al., 1993; Wallisch et al., 2002]. In *S. pombe*, an imprinting-dependent terminator of replication, RTS1, is believed to stall the leading-strand replication complex, causing a break in one of the sister chromatids and leading to mating-type switching in one of the daughter cells [reviewed in Vengrova et al., 2002].

We showed previously in synchronized normal human fibroblasts entering the S phase that initiation of DNA synthesis occurs at discrete loci in R-bands of only a few chromosomes. One of these locations is on the p arm of chromosome 1 (band 1p36.1) [Cohen et al., 1998]. In the process of searching a cosmid library of early replicated DNA from normal human fibroblasts [Brylawski et al., 2000b] for clones mapping to 1p36.1, we were surprised to find that for one of these clones (approximately 50 kb in length) the sequence at one end replicated in the first hour of the S phase, while the sequence at the opposite end replicated at least 1 h later. Chromosome 1 sequence, including and surrounding this clone, was retrieved from the Genome Bioinformatic Site of the University of

California at Santa Cruz (http://genome.ucsc.edu/, April 2003 freeze). In this study, we report the use of quantitative PCR for the study of the replication timing of DNA sequence markers along this contig of approximately 340 kb. We defined a region that is replicated during the first hour of the S phase and is flanked by DNA segments that are replicated 1 or 2 h later. In addition, we report the finding of two adjacent origins of replication that are activated more than 1 h apart during the early S phase. This difference in timing of origin activation suggests that each of these two origins could be a member of distinct banks of replicons that are activated sequentially in the S phase. The region between them may contain a pause site that prevents the replication fork emanating from the origin that is activated first from advancing and replicating the domain containing replicons activated 1 h later.

METHODS

Cell Cultures

The normal human fibroblasts used in these studies were: NHF1, obtained in this laboratory from neonatal foreskin [Boyer et al., 1991], the immortalized cell line derived from them (NHF1-hTERT) by ectopic expression of the catalytic subunit of telomerase [Heffernan et al., 2002], and GM1604-hTERT, a human fetal lung fibroblast cell line also immortalized by telomerase expression [Ouellette et al., 2000]. NHF1 cultures were maintained in Eagle's minimal essential medium and the immortalized cell lines were grown in Dulbecco's modified essential medium (GIBCO-BRL, Grand Island, NY) containing 2× the concentration of MEM non-essential amino acids (GIBCO-BRL). These growth media were supplemented with 2 mM L-glutamine (GIBCO-BRL) and 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT).

Cell Synchronization and Determination of Replication Timing in Newly Replicated, BrdU-Labeled DNA

The protocol used to synchronize cultured human fibroblasts [Cordeiro-Stone et al., 1986] relies on arresting cell proliferation at confluence, re-plating the cells at lower densities in medium containing aphidicolin, and incubating the cultures for 24 h. During this time, cells re-entering the cell cycle traverse G_1 and start the S phase, but at a reduced rate of DNA synthesis, equal to approximately 2% of control [Tribioli et al., 1987; Brylawski et al., 2000a]. Once cells are released from the aphidicolin inhibition, they resume DNA synthesis at normal rate and transit through the S phase as a parasynchronous cohort. In the present studies, synchronized human fibroblasts released from the aphidicolin block were pulse-labeled with BrdU during successive 1-h time intervals as they progressed through the S phase (Fig. 1). The cells were harvested every hour and the replicated, hybrid-density DNA from the nuclear lysate was fractionated by CsCl gradient centrifugation (Doggett et al., 1988). After dialysis and concentration, the total amount of replicated DNA in the samples from each 1-h window

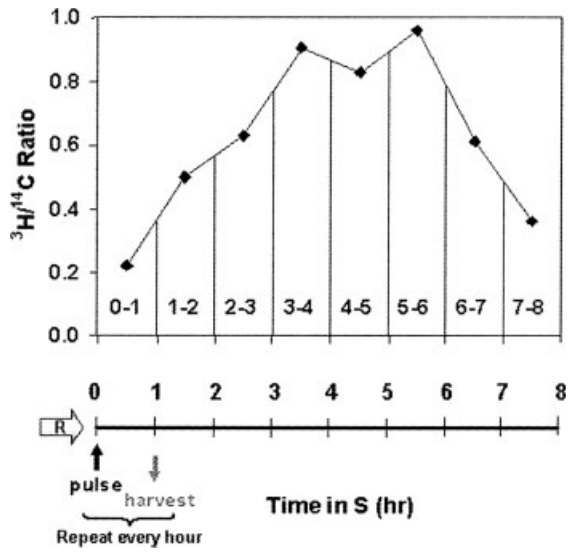

Fig. 1. Rate of DNA synthesis during S phase progression of synchronized human fibroblasts. Cultures of human fibroblasts (NHF1-hTERT) were incubated with 5 nCi/ml of ^{14}C-thymidine for 3 days to label nuclear DNA uniformly, and grown to confluence. Arrested cultures were re-plated at 1×10^6 cells per 100 mm plate in the presence of 2 μg/ml aphidicolin. After 24-h incubation, cells were washed to release them from the aphidicolin block (gray arrow labeled with R), and pulsed with bromodeoxyuridine (BrdU) and ^{3}H-thymidine for 1 h at every hour of the 8-h S phase. The graph illustrates the change with time of the specific activity of incorporation of DNA precursors in replicated DNA ($^3H/^{14}C$) after each 1-h pulse (◆), which reflects the rate of DNA synthesis at the indicated windows of the S phase. The plotted $^3H/^{14}C$ values are the average of two separate synchronization experiments using the same cell line. Fluorescent immunostaining of BrdU-containing DNA showed that 60% of the cells participated in the synchronous transition through the S phase (results not shown). DNA replicated during these 1-h periods of the S phase was isolated by CsCl centrifugation and used for PCR testing of the timing of replication of selected markers along the DNA contig. Only DNA from the first four 1-h periods was used in this study.

of the S phase was determined from the absorbance at 260 nm or from the specific activity of ^{14}C-labeled DNA, when the human fibroblasts were uniformly labeled with ^{14}C-thymidine prior to synchronization [Cordeiro-Stone et al., 1990].

Samples containing DNA replicated in different windows of the S phase were used for testing the timing of replication of selected genetic markers by PCR, as illustrated in Figure 2. For each primer set (Table I), an equal amount of replicating DNA (1–4 ng) from samples representing each of the first 4 h of the S phase was tested in duplicate. In order to obtain quantitative results, it was necessary to carry out the PCR reactions under non-saturating conditions, and to construct a standard curve with genomic DNA of the same size as the tested DNA [Cohen et al., 2002]. DNA used for these PCR standard curves consisted of purified human genomic DNA, which was sheared 10 times through a 21-gauge needle, in the same way as the BrdU-labeled DNA prior to CsCl gradient centrifugation [Doggett et al., 1988]. Only when the correlation coefficient for the standard curve was equal to or greater than 0.9 were the PCR results taken into consideration.

Preparation of Nascent DNA and Mapping of Origins of Replication

Size-fractionated single-stranded nascent DNA (900–1,700 nt) was prepared from logarithmically growing cells by heat denaturation of genomic DNA and size fractionation on sucrose gradients, as described previously [Cohen et al., 2002]. Equal volumes of a given nascent DNA preparation were used for quantification of the relative copy number of a series of genetic markers in the region of interest. DNA for standard curves used to determine relative abundance in short nascent DNA strands consisted of human genomic DNA sonicated to an average length of 1,500 nt [Cohen et al., 2002]. Each preparation of short nascent DNA was also tested by measuring the relative abundance of sequences at the lamin B2 origin using previously described primers L5 [Cohen et al., 2002] and B13 [Giacca et al., 1994].

PCR

Primer3 (www.genome.mit.edu/cgi-bin/primer/primer3.cgi) was used to design PCR primers for the amplification of DNA sequences within a length of DNA from nt 18355231 to nt 18692083 of chromosome band 1p36.1 in the April 2003 release of genomic sequences at the UCSC Genome Bioinformatics site (www.genome.ucsc.edu). The primers are listed in Table I with their location expressed as the nt number from the beginning of the sequence being analyzed (nt 1 in the contig corresponds to nt 18355231 in the database sequence). PCR was performed in OmnE, TouchDown, and PCR-Express thermocyclers (Hybaid Ltd., Ashford, Middlesex, UK). PCR reaction conditions were as described [Cohen et al., 2002], with the exception of two primer sets (marked with asterisks in Table I) for which a two-step PCR program was used: 95°C for 12 min (once, for the activation of the polymerase), 30 cycles of 94°C for 30 s and annealing/elongation temperature (see Table I) for 1 min. In the case of primer set 73.4 k, 2% DMSO was also added to the reaction mixture. A genomic standard curve was amplified together with each primer set, so that differences in amplification efficiency would not affect the determination of relative abundance (Fig. 1). An equal amount of DNA was used for every test sample. Each standard curve was constructed such that the ng-equivalent value for the DNA test samples would fall in the linear range of amplification. PCR products were separated by gel electrophoresis on 2% agarose and stained with ethidium bromide. Gel images were recorded with a digital imaging system (Alpha-Innotech, San Leandro, CA) and the intensity of the bands was determined with AlphaEase software from the same manufacturer. Microsoft Excel was used to graph the standard curves, determine the equations of the linear regression lines, and calculate the genomic ng-equivalent value of the unknown samples.

RESULTS

We tested the replication timing of the region from nt 18355231 to nt 18692083 of chromosome band 1p36.1 (for a total of 336,852 bp) using 31 markers distributed along the length of this sequence (Table I). Figure 3 illustrates representative results from quantitative PCR analyses for nine of these markers in one synchronization experiment. These data show clearly that timing of replication changed from one end to the other of the 340 kb contig. Seeking to illustrate the aggregate results from three independent experiments with synchronized

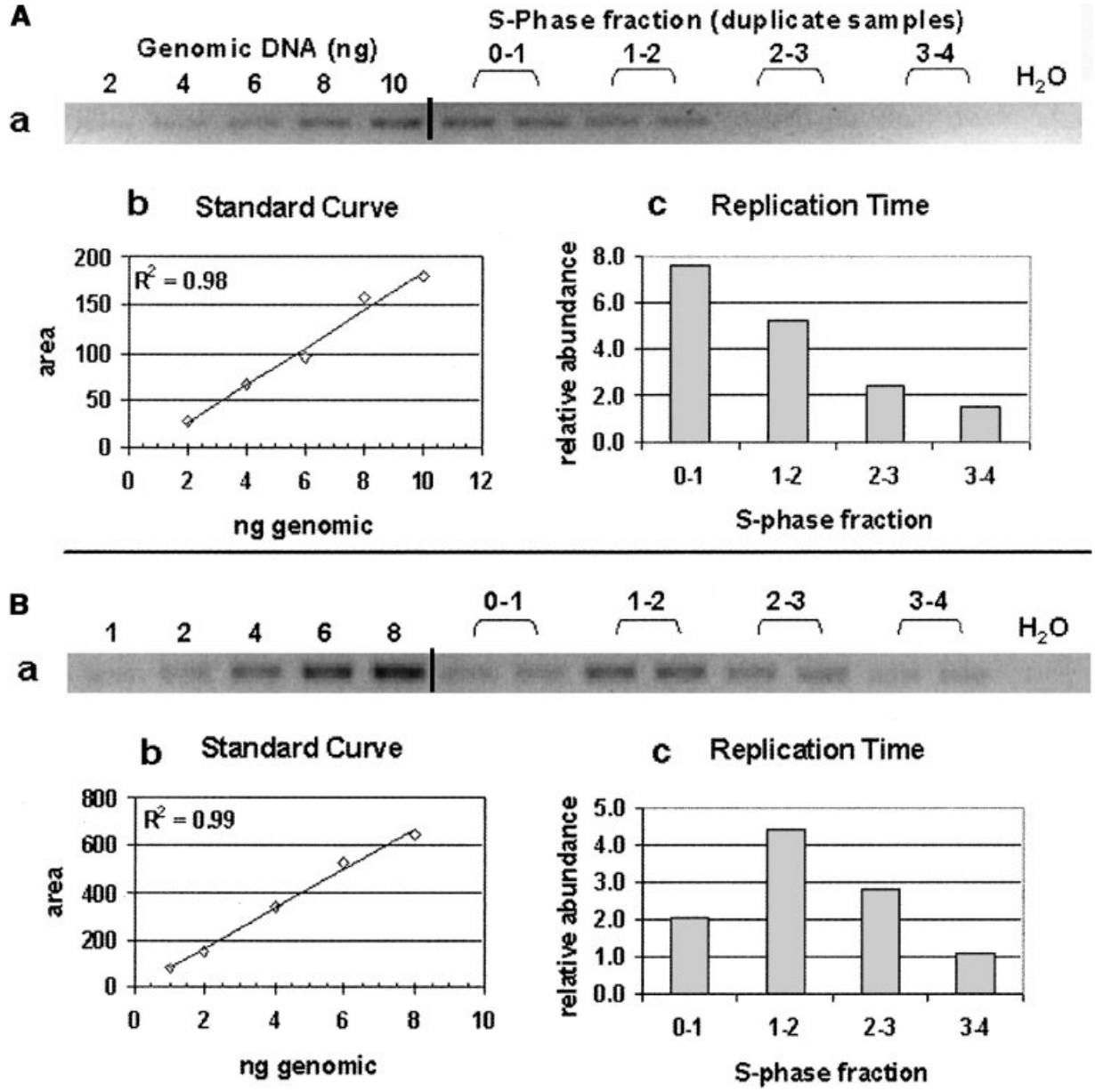

Fig. 2. Determination of the timing of replication of genetic markers by PCR. Both composite panels illustrate the procedure used for determining which of four samples of DNA, each synthesized at a different 1-h period of the S phase, was enriched for copies of a particular marker. DNA replicated at the indicated 1-h intervals of the S phase of synchronized fibroblasts was labeled with BrdU as outlined in Figure 1 and isolated by CsCl centrifugation. **Panel A** illustrates the results obtained with a marker that replicates in the first hour of the S phase (primer set 74.5 k in Fig. 3); **panel B** shows the results for a marker that replicates during the second hour (primer set 18 k in Fig. 3); the results illustrated in both panels were obtained in the same synchronization experiment. In each panel, **a**: Inverted image of PCR products stained with ethidium bromide after gel electrophoresis. The same primer set was used to amplify increasing amounts of genomic DNA and duplicate samples of DNA replicated during each of the first 4 h of the S phase; a control with no template DNA was included in every PCR experiment. **b**: Standard curve obtained by plotting the signal intensity of the bands of PCR products in the gel above versus the amount of genomic DNA. **c**: Bar graph illustrating the abundance of the marker in each of the four 1-h samples representing the first half of the S phase. Relative abundance was calculated from the linear regression equation of the standard curve and expressed in ng-equivalent of genomic DNA. The bar with the highest value identifies the time interval in which a certain marker is replicated.

TABLE I. Primer Sets Used for the Analysis of a 340 kb Contig in Chromosomal Band 1p36.13

Primer set		Sequence	Position in contig	T[a]	O[b]	Annealing temp (°C)
6.1 k	F	AGGGCTGACCTTTTATTCTC	6153–6173	x		52
	R	AGCTAATGACAAAAAGCTGG	6296–6316			
12 k	F	TTCAAAGTTCAGCCTGAGTC	12024–12043	x		52
	R	CCAAGTCCTGTGACTGATTC	12197–12216			
18.0 k	F	CGTTTCCCATGATAATACAG	18081–18100	x	x	52
	R	GAGAAATGCATAGGAAAACC	18221–18240			
18.9 k	F	AATAATGACCTGCCTCACAG	18918–18937		x	52
	R	TGAGTTAATACATGGGGAGTG	19028–19048			
19.4 k	F	ACAGAGGCTACTTTCCGAG	19400–19418		x	52
	R	TCCCACTGAGATCCCTTAG	19518–19536			
19.7 k	F	CAGCATCTCGGGTTAGAAG	19762–19780		x	54
	R	AATATAGTGGGACTTTGGCTG	19900–19921			
19.9 k	F	CCACTATATTTAGCTTCGGC	19912–19931		x	52
	R	TAAAGAGAGGAGGGGAAAAC	20018–20037			
20 k	F	GAACTCTGTCGACCAGTCAG	20063–20082		x	52
	R	CCATTTCGGTTATCTCATTG	20169–20187			
20.6 k	F	TCTTCTCTGATGACGAGGTC	20668–20688		x	52
	R	AACTGACTCCTAAGCAGGTG	20778–20797			
32.3 k	F	TCAGCCTAGAATAGGGACAG	32312–32331	x		52
	R	CCATAGTCCAGAGCTCACTC	32453–32472			
48.2 k	F	TCCCCCTACCCCTAAAAAAC	48238–48257	x		53
	R	AATTCACAGCCCCTGGATG	48486–48504			
57.8 k	F	CAAAAGCCATGAGGTTTATC	57838–57857	x		52
	R	AGCTAGAGAGTGGCAGAGTG	57986–58005			
59.0 k	F	GCGTTTTCCTTTTGTATGAG	59056–59075	x		52
	R	CTCCAACATTCTAGGCAAAG	59183–59202			
62.1 k	F	CAGGAAACCTTTCTTGACTG	62096–62115	x		52
	R	GTGGAAGATTTAGGGAAAGC	62227–62246			
63.1 k	F	CGGGTATCATTTTAGACAGC	63160–63179	x		52
	R	GTTCCAACCTTAAACCTGTG	63290–63309			
64.3 k	F	GCATTAATTGAGAGCCTGAG	64312–64331	x		52
	R	CAGATCCAACCTTTATCCTG	64440–64459			
65.5 k	F	ATCACAAACCTTCAGCAGTC	65577–65596	x		52
	R	AGAACCAAGCTGGAAACTG	65701–65719			
66.1 k	F	TGTTGTCCGTCTTCTGTCC	66087–66105	x		52
	R	AACTACCTGGCATGTGTCTG	66274–66293			
67.2 k	F	GAAATGGACTTGTTAGGCAG	67219–67238	x		52
	R	GAAGGAACAAATGAGGTCTG	67359–67378			
70.3 k	F	GCATGTTAGTAGAACCCAGTG	70337–70358		x	50
	R	AGAGATGCTTTAGGGTTTCC	70455–70474			
71.2 k	F	CAACTGTGGGCAGATTTTAT	71256–71275		x	52
	R	AGGTCTCCTTCCGTATTCTC	71385–71404			
71.7 k	F	TCTAGCCAGAGATTGTGGTC	71760–71779		x	52
	R	CTCCTTTCATCCCTCTTCTC	71885–71904			
72.3 kA	F	ATACGCTGTATTGTGCTGAG	72303–72322		x	52
	R	TTATCTAGATCCCAGGATGG	72432–72451			
72.3 kB	F	CCTAGAAAACAATGTGCCTC	72365–72384		x	48.1
	R	TGTTTGCAGAGCGTCTTAC	72471–72489			
73.4 k*	F	AGACGGCAGGACGGATG	73431–73437		x	63
	R	CCACAGCGTCTCTGACAGC	73559–73577			
73.9 k*	F	TCGCTTTCCACTTTTTAGG	73991–74009		x	60
	R	ACCTTCGGATACTGTGGTC	74127–74155			
74.3 k	F	TGACACCCACAAACTCG	74377–74393		x	52
	R	CCATGTCTGTGAAATGAATC	74481–74500			
74.5 k	F	GTTTTCACACCTACCACGTT	74574–74593	x	x	50
	R	TGTGGCTACTTAACGTCTCA	74701–74720			
74.7 k	F	TCCAAAGTGCATGCTCTAAC	74725–74744		x	54
	R	CATACAATGCAAGCCACAG	74855–74873			
77.4 k	F	AACCTTCTCTTCTCGGAAAC	77465–77484	x		52
	R	GACAAGAGCGTATACTTGGG	77576–77595			
94.3 k	F	CATGGAGTCATACTGTGTTAGTC	94327–94349	x		53
	R	CTCTCACTCCAGCTGTTACC	94527–94546			
102.0 k	F	AAAGGAGGAGAATTGAGGAC	102087–102106	x		52
	R	ATGAACCAAAAAGGTGACAG	102209–102228			
111.9 k	F	TCCATTTGGGTTATAACTGG	111997–112016	x		52
	R	AGATTCACCTATCACATGGC	112101–112120			
119.2 k	F	TGTCAGTATAGCAGCACCAG	119196–119215	x		52
	R	CAAGGCTCAATCAGAGAAAC	119328–119347			
124.1 k	F	CTGTTGAGAAAAGTTCCTGG	124101–124120	x		52
	R	ATCTAGCACATGGTGAGTCC	124221–124240			
162.8 k	F	TCCATGAGAATTCAGAGGAC	162833–162852	x		52
	R	CTGGGCACTTCCAGTAATAG	162985–163004			
172.8 k	F	CAACAGGAGACTATTCAGCC	172833–172852	x		49
	R	TGTGAGTGCGACTTTTGTAG	172995–173014			

(*Continued*)

TABLE I. (*Continued*)

Primer set		Sequence	Position in contig	T[a]	O[b]	Annealing temp (°C)
185.2 k	F	CAGATGCTGAAGGTCACTG	185214–185232	x		51
	R	GAGCTGGTGCTCTGTTTTAG	185359–185378			
191.2 k	F	CTCATGGGCAAAGAGATTAG	191230–191249	x		52
	R	CATTTGACATTCTCATGCTG	191382–191401			
195.2 k	F	GTCCTTCACTGCCTTATCTG	195285–195304	x		56
	R	GATGCATACACCACTGTGAC	195398–195417			
201.9 k	F	TGTTGTGTCTAGAGGGAAGC	201953–201972	x		54
	R	GTATTCTTGTCTCCGTTTGC	201072–201091			
211.3 k	F	ATCACCAGTTGATCCTTGTC	211342–211361	x		52
	R	ATTCCTGATGACTCTGATGG	211469–211488			
229.6 k	F	AGGGAGAGTTTCCTCAAGAC	229612–229631	x		52
	R	AGAGACACAGGGTGTAGGTG	229717–229736			
266.8 k	F	GAGTGCACTTACGATCTTCC	266815–266834	x		54
	R	ACGAGTGAGTGTGTGTCAGA	266958–266977			
301.2 k	F	TACTGACCCTTGAGTCCATC	301252–301271	x		52
	R	CTCTTCCATGCTGAACTCTC	301362–301381			
336.9 k	F	GAGATCCCTTTACAGCAGTG	336975–336994	x		52
	R	GGCTCTACTCACCAAACAAG	337150–337169			

The location of primers is listed as their distance from nt 18355231 in chromosome 1p36.13, as available at the Genome Bioinformatic site of UCSC, April 2003 release. The primers marked with an asterisk required a two-step PCR amplification protocol (see "Methods").
[a]Primers used for the determination of replication timing.
[b]Primers used for the identification of origins.

normal human fibroblasts, we chose to assign to each primary data (see histograms in Fig. 3) a numerical value that approximated the time of replication for each of the sequence markers under consideration. In principle, each marker would be expected to replicate within a specific time in S phase and our protocol would capture all Brdu-labeled DNA that was synthesized during sequential 1-h periods of the S phase (Fig. 1). Thus, using a perfectly synchronized population of S phase cells, our PCR analyses would return for each marker a very high signal with the DNA sample from a particular 1-h window of the S phase and little or no signal would be detectable in the other samples. However, the best that cell synchronization protocols can achieve is to force a cohort of cells to progress through the cell cycle, with the broadness of such a cohort determining the degree of synchrony in the population. Consequently, our primary results indicate the sample of replicated DNA in which a given marker is present at the greatest abundance (highest bar in the histogram) and reduced enrichment in the other samples. In addition, sequences that are replicated at the boundaries of the discrete time periods used to pulse newly replicated DNA with BrdU are likely to be represented at similar levels in two different samples (e.g., first panel in Fig. 3). With these considerations in mind, we adopted the following procedure to estimate the weighted average replication timing for each marker in a given experiment. First, we normalized the primary PCR data (ng-equivalent of genomic DNA, Fig. 2) by assigning the value of 1 to the highest sample and scaling all the other measurements to their corresponding fraction, as illustrated in the histograms shown in Figure 3. Then, we simply selected the time intervals for which the relative abundance of the marker in the correspondent replicated DNA samples was at least one half of the highest value (i.e., represented by the histogram bars with relative heights equal to or above 0.5 in Fig. 3). We multiplied the relative heights of the selected histogram bars by the midpoints of the respective time intervals (i.e., 0.5, 1.5, 2.5 h, etc.) and divided the sum of these products by the sum of the relative heights of the histogram bars to obtain the weighted average replication timing of each marker. For instance, the replication timing for the 6.1 k marker illustrated in the first panel of Figure 3 was estimated to be 2.4 h into the S phase because the three samples displaying relative abundance values at or above 0.5 were those from the 1–2, 2–3, and 3–4 windows in S phase. Therefore, according to our formula, $[(0.77 \times 1.5) + (1.0 \times 2.5) + (0.61 \times 3.5)]$ divided by $(0.77 + 1 + 0.61)$ returns the value of 2.43 h. We recognize that this method of estimation may not yield a precise time for the replication of the DNA segments in question because of systematic errors introduced by our arbitrary assumptions and data simplification. Nonetheless, the method allowed us to compare the relative time of repli-

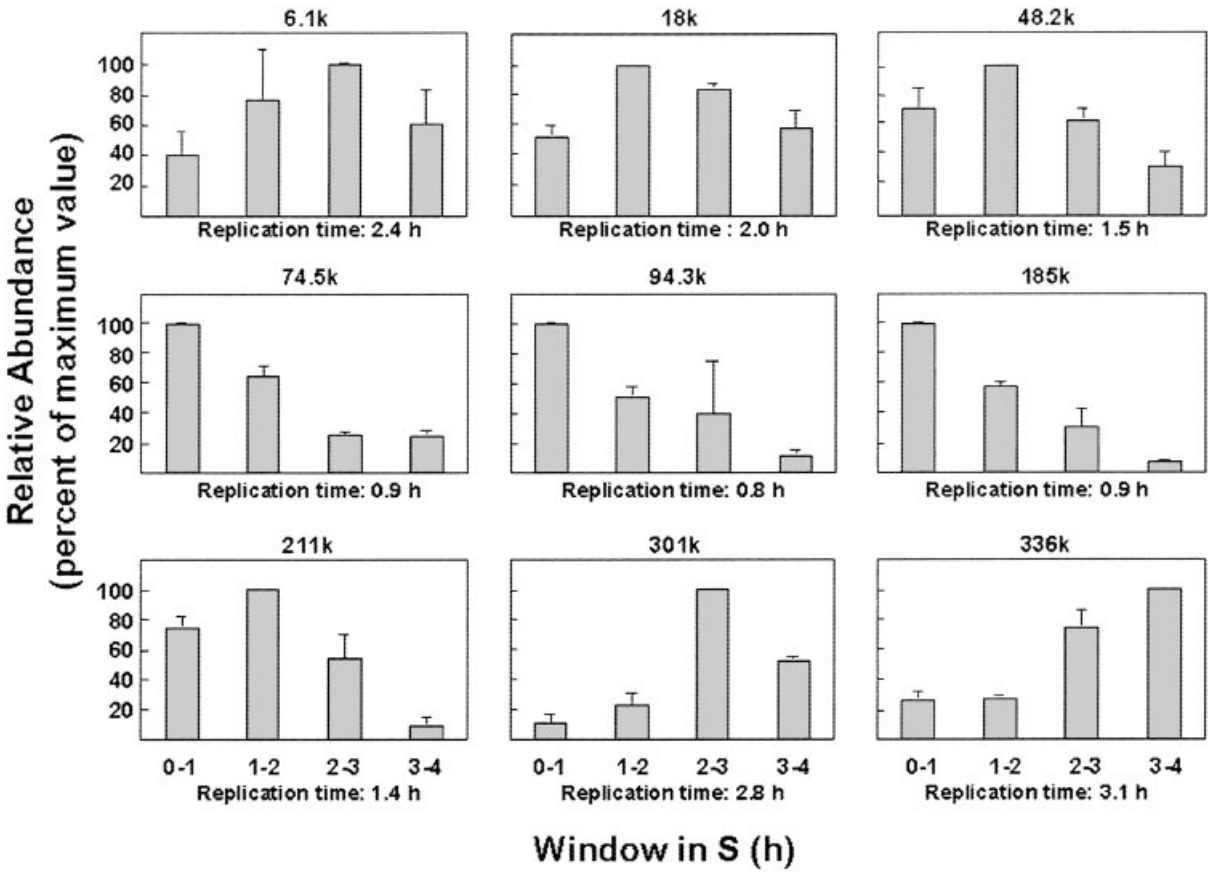

Fig. 3. Representative data of replication timing analysis. Each bar graph shows the relative abundance of a different marker along the contig in DNA replicated during the first four 1-h intervals of the S phase. Data were obtained by the method illustrated in Figure 2 and normalized by expressing the relative abundance of each marker in the different samples as a fraction of the highest value. The marker tested (identified by its distance from the beginning of the contig) is listed at the top of each panel. These results are the average of two or three separate PCR tests of samples from the same synchronization experiment. These and similar data from other experiments were used to estimate the replication time for each marker (Fig. 4). At the bottom of each bar graph the corresponding estimated replication timing is shown.

cation of different DNA segments (Fig. 4A). For the purposes of this report, it is the relative time of replication of the individual segments that is of greatest importance.

The data points in Figure 4A represent the mean of two or three PCR tests in each of three independent synchronization experiment. Some of the scatter in the results may reflect variations in synchronization among different experiments and different cell lines. Keeping this point in mind, inspection of the data in Figure 4A reveals three changes in replication timing along the DNA contig. There is a 1-h difference between the replication times of markers around 10 k (peak of replication at 2.5 h) and 50 k (peak of replication at 1.5 h). There is also a 30-min difference between the replication times of markers around 50 k and those at 70 k (peak of replication at 1 h). These distances (40 and 20 kb) could have been covered in about 13 and 6 min, respectively, if the region had been replicated by a replication fork moving at a rate of 50 nt/s [Tribioli et al., 1987]. Even a more conservative estimate of 1.5 kb/min for the rate of fork advancement [Ermakova et al., 1999] would not account for the observed differences in replication timing. In addition, Figure 4A shows three apparent steps or plateaus at replication timing of 1, 1.5, and 2.5 h in S phase, with several markers from the first 120 kb of the contig clustered in each step. This distribution pattern seems to argue against the interpretation that this region would be replicated by a single replication fork moving from the 60–70 k markers toward the 5–10 k markers at the low average rate of ~0.7 kb/min. Therefore, it is possible that in this region there are sequences where the replication fork advances at a much

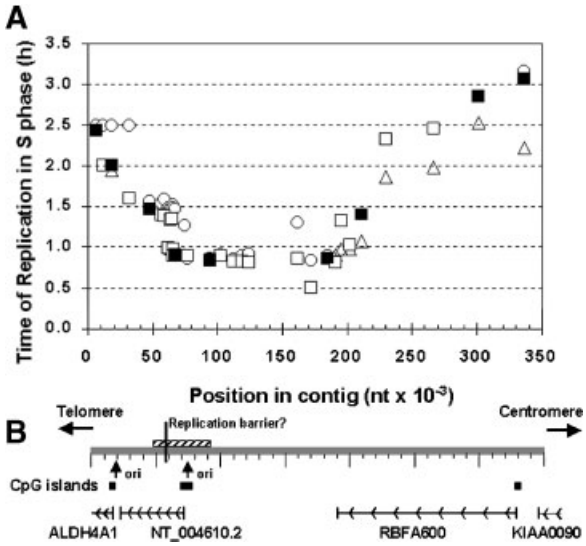

Fig. 4. Replication timing map of the chromosomal region under study. **Panel A**: The timing of replication of each marker (listed in Table I) was determined in two or three independent synchronization experiments with NHF1 (○) and NHF1-hTERT (□ and △) fibroblasts, using the method illustrated in Figure 2. The replication timing for each marker was then calculated as described in the text (see ''Results''). Each data point represents the mean of two or three separate determinations for each marker. The filled squares denote the data points derived from the histograms in Figure 3. **Panel B**: Schematic representation of the contig in chromosome 1p36.13 from nt 18355231 to nt 18705231 (www.genome.ucsc.edu, April 2003 release). The cosmid clone from our library that directed our attention to this region is represented by the striped bar. CpG islands and genes mapped to the region are also illustrated (arrowheads represent the direction of transcription). Small arrows in panel B point to the location of functional origins of replication (ori) mapped in this contig (see Figs. 5 and 6). The vertical bar across the contig marks the position of the large tandem repeat (see ''Results''), which could represent a replication fork barrier.

reduced rate or even pauses for about 20–40 min. Interestingly, we noticed a large inverted repeat at the 59–60 k position in this contig; starting at nt 59057 there is a 264 bp sequence, 92% of which is duplicated in reverse orientation approximately 1 kb away. If these sequences were to contribute to the formation of a hairpin structure, this might represent a barrier for the advancement of the replication fork.

The region replicating during the first hour of S in the contig illustrated in Figure 4A extends from around 60 to about 200 k, while the marker tested at 229 k is replicated 1 h later. This rather sharp transition in replication time is followed by a more gradual change towards later replication in the remainder of the contig. The distance of 108 kb between marker 229 k and marker 339 k (replicated 3 h into the S phase) could be covered in 60 min by a single replication fork moving at the rate of 1.8 kb/min.

This region of band 1p36.13 contains four genes, as reported by the UCSC Genome Bioinformatic site, April 2003 release (Fig. 4B). At the beginning of the sequence, the gene coding for aldehyde dehydrogenase 4A1 (ALDH4A1) has been mapped [Vasiliou and Pappa, 2000]. Another gene has been predicted from Genescan (NT_004610.2) to be between 24.6 and 74.6 kb in the sequence contig. The gene coding for retinoblastoma-associated factor 600 (RBAF600) has been provisionally mapped in the region 192 to 327 kb from the beginning of the sequence, and another gene, *KIA0090*, has been predicted at about 336 kb. The last three genes were confirmed also by the presence of spliced ESTs. Three CpG islands have been mapped in this region, one of which is located in the early replicating zone, coinciding with the transcriptional promoter of the gene NT_7004610.2 (Fig. 4B). Since a number of origins reported to date have been found in association with CpG islands or near transcriptional promoters, we searched this region for the presence of a functional origin of replication. Using the quantitative PCR method described previously [Cohen et al., 2002, 2003], we determined the relative abundance of markers spanning this region in short nascent DNA preparations from logarithmically growing normal human fibroblasts. PCR amplification within the CpG island (several primer sets were tested) proved to be difficult, probably because of the very high annealing temperature of primers and products in the whole region. However, by using a two-step PCR cycle and with the addition of 2% DMSO to the PCR reaction, it was possible to amplify at least one marker within the island (primer set 73.4 k in Table I). Control PCR reactions with another primer set in the presence or absence of DMSO yielded similar values for the relative abundance of the marker in short nascent DNA, indicating that the results obtained with primer set 73.4 k were not due to amplification artifacts (data not shown). Figure 5, panel A, illustrates the relative abundance of marker sequences in short nascent DNA, which were amplified by the 10 primer sets (Table I) used for the determination of origin activity in GM1604-hTERT cells. Results are plotted according to the location of these markers, relative to the sequence motifs

illustrated in the diagram below the histograms. These motifs include a large CpG island, encompassing approximately 2,000 nt, the expected position of exon 1 in the NT_004610.2 gene, and predicted binding sites for the transcription factor Sp1 (obtained at the site http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html, TFMATRIX transcription factor binding site profile database). A DNA unwinding element (DUE), defined as a sequence of 100 bp or more with a 70% or higher AT content, was also found near this origin. Figure 5, panel B illustrates the relative abundance of the same genetic markers in nascent DNA from NHF1-hTERT cells. Although the relative abundance values (in ng-equivalent) are different between the two preparations tested, in both of them the highest values were found with primer set 73.4 k. The ratio between the abundance values obtained with primer set 73.4 k (peak of abundance) and primer set 74.7 k (baseline value) was 11.8 in GM1604-hTERT cells, and 12.9 in NHF1-hTERT cells. For comparison, the ratio obtained between markers with the highest and lowest abundance values for the lamin B2 origin (see "Methods") was 3.0 in the same nascent DNA preparation from GM1604-hTERT cells and 3.9 in the NHF1-hTERT sample. Primer set 74.5 k was also used to determine the timing of replication of this origin, which was in the first hour of S in all three synchronization experiments tested.

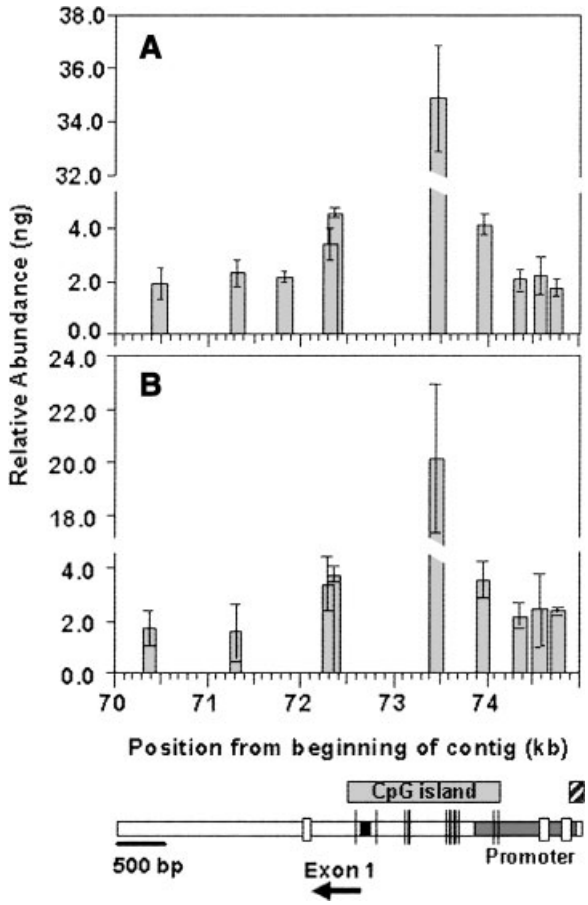

Fig. 5. Quantitative PCR mapping of an origin of replication in human chromosome 1p36.1. **Panel A**: Relative abundance of markers in size-fractionated nascent DNA from logarithmically growing GM1604-hTERT fibroblasts. The markers are distributed along the X-axis with each bar starting at the position of the forward primer in the contig (Table I) and relative to the diagram below the histogram, which shows a few relevant features of the region. These include a large CpG island, and the promoter region and exon 1 (black box) of the predicted gene NT_004610.2. Vertical bars represent predicted Sp1 binding sites (obtained by using the program http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH/html); white boxes represent ARS consensus sequences, and the hatched box represents a DNA-unwinding element (DUE). The block arrow below exon 1 indicates the direction of transcription. The relative abundance of each marker in nascent DNA is expressed in ng-equivalent of genomic DNA, as determined from amplification standard curves prepared with sonicated DNA and run at the same time as the test samples. **Panel B**: Relative abundance of the same markers tested with short nascent DNA from NHF1-hTERT fibroblasts. Each primer set was tested at least three times and the error bars indicate the standard deviation of the mean.

The differences in timing of replication along the contig (Fig. 4A) suggested that this region should be under the control of more than one origin of replication, activated at different times in S. Therefore, we searched for another origin of replication within the first 60 kb of the contig. Based on our previous experience, we reasoned that a likely location for an origin of replication would be the CpG island at the transcriptional promoter of the aldehyde dehydrogenase 4A1 (*ALDH4A1*) gene mapped at this location [Hu et al., 1996]. The same short nascent DNA preparation from GM1604-hTERT cells was used for the PCR analysis of the abundance of markers located in this region (Table I). Figure 6A illustrates the relative abundance of the markers tested in GM1604-hTERT cells, and a diagram of the promoter region of the *ALDH4A1* gene. One predicted Sp1 binding site and two DUEs were found near this origin. As observed in the 73.4 k origin, the marker with the highest abundance was found in the gene promoter region, an area of very high CG content. The ratio between the highest (primer set 19.7 k) and lowest (primer set 18 k) abundance values was 6.2. Two more preparations of short nascent DNA were tested in this

functional origin assay: the same sample from NHF1-hTERT used to test for the NT_004610.2 origin and one additional preparation from GM1604-hTERT. We tested the marker giving the highest value (primer set 19.7 k) and the two flanking markers giving the lowest values (primer sets 18 and 20.6 k) in at least three separate reactions for each primer set. As found for the previous origin, the nascent DNA abundance values in ng-equivalents of genomic DNA were different in different nascent DNA preparations. Therefore, the data were normalized to the highest value in each preparation so that the two additional preparations could be illustrated in the same graph. The results are shown in Figure 6, panel B. The timing of activation of this origin was determined using marker 18 k. In one of the synchronization experiments (NHF1 hTERT), this origin fired between 2 and 3 h from the beginning of S phase (peak of replication 2.5 h in Fig. 4A), while in the other two (GM1604-hTERT) it was activated close to 2 h from the beginning of S (peak of replication 2.01 and 1.94 h in Fig. 4A).

DISCUSSION

While determining the timing of replication of a cosmid clone mapped to 1p36.13 from a library of early replicated DNA, we established that one end of the clone was replicated in the first hour of the S phase, while the other end was replicated 1–2 h later. This finding prompted us to determine the timing of replication along a DNA contig in chromosome 1p36.1, using closely spaced markers covering approximately 340 kb of sequence. This region is located entirely within an R-band, but the timing of replication of specific markers changes from the first to the third hour of the S phase. There appear to be three discrete changes in replication time along this rather small DNA region: one between 10 and 50 kb from the start of the contig, another between 50 and 65 kb, and a third between 210 and 230 kb (Fig. 4).

Several authors have been interested in determining whether specific DNA sequences mark areas of transition in replication timing. In the human major histocompatibility complex, it was

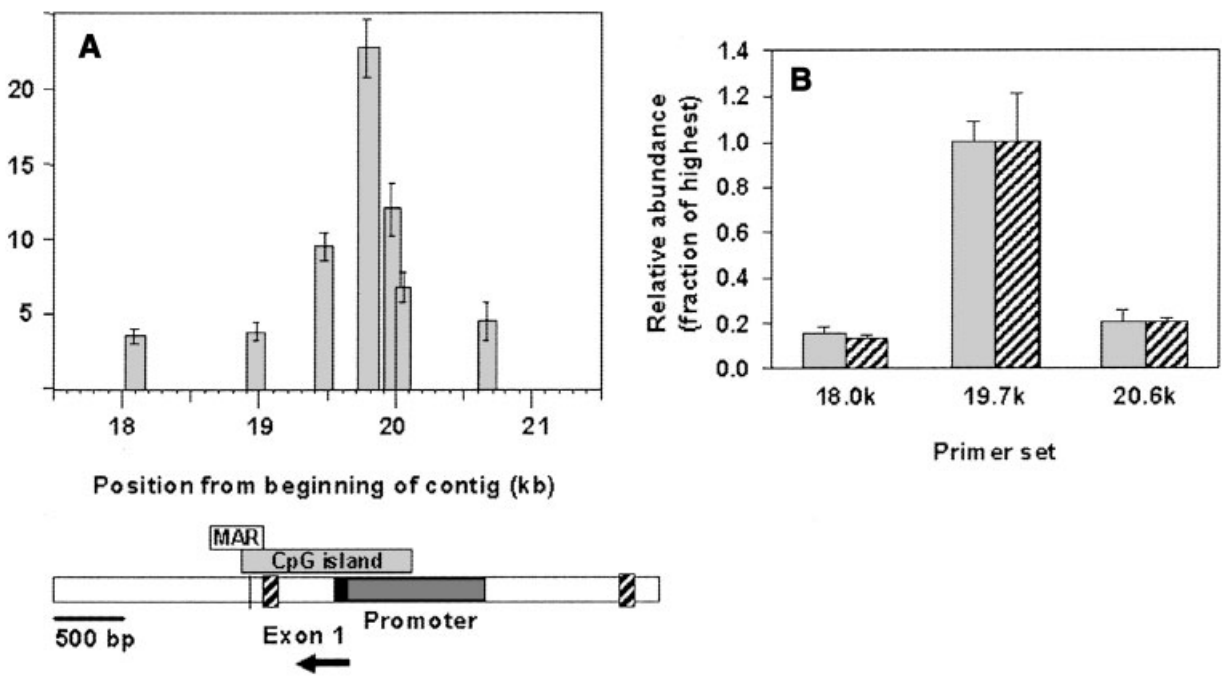

Fig. 6. Quantitative PCR mapping of another origin of replication in the same contig near the transcriptional promoter of the aldehyde dehydrogenase 4A1 gene. **Panel A**: Relative abundance of markers in a short nascent DNA preparation from GM1604-hTERT cells. Under the histogram is a diagram of the region containing the *ALDH4* gene, and some distinctive features (see legend to Fig. 5). A potential matrix attachment region (MAR) was determined using the marfinder feature at the site www.futuresoft.org. The single Sp1 binding site and DUEs are indicated as in Figure 5. **Panel B**: Relative abundance of three markers (selected among the original seven in panel A) in two additional nascent DNA preparations from NHF1-hTERT (shaded bars) and GM1604-hTERT (hatched bars).

found that time switching from early to late replication in a region spanning 500 kb between G and R bands was correlated to a sharp change in GC content. This structural feature was within a 40 kb region, where a transition of 2 h in replication time was observed. Possible signals involved in modulating the rate of progression of the replication fork were considered to be high-density clustering of Alu and LINE elements, polypurine/polypyrimidine tracts, and scaffold or matrix attachment regions [Tenzen et al., 1997]. Another transition zone displaying similar characteristics was found in human chromosome Xq13.2, near the X-inactivation center region [Watanabe et al., 2000]. This transition zone was about 170 kb and the difference in replication timing of adjacent sequences was 2 h. On the other hand, in a large chromosomal region at the boundary between chromosomal G- and R-bands of human chromosome 13 [Strehl et al., 1997], the timing of replication was found to shift gradually from early to late, without any particular features marking a transition point. In mouse erythroleukemia cells [Ermakova et al., 1999], a replication fork from an early-replicating cluster of replicons in the IgH-C locus appeared to travel at a rate of about 1.5 kb/min and merge with a replicon in a late-replicating cluster. This resulted in a gradual replication timing transition between the early and late replicating regions, which were separated by about 400 kb. In this case, the difference in replication timing was explained by the large size of the replicon. The authors concluded that transitions in timing of replication observed in other studies [Strehl et al., 1997; Tenzen et al., 1997; Bilyeu and Chinault, 1998] could also be explained by this model of a single replication fork traveling a long distance, rather than by the presence of special DNA sequence features interspersed among several replicons.

In the present study of a 340 kb region in chromosome 1p36.1, we identified a 140 kb zone replicating in the first hour of the S phase flanked on each side by areas replicated later (Fig. 4A). Given the short span of this region, this finding suggested that the replication of the contig could be under the control of different origins, which were activated at a different time during the S phase. Many authors have reported that replication origins in mammalian cells are associated with regions of high GC content, such as transcriptional promoters and CpG islands. The lamin B2 origin is flanked by CpG islands [Biamonti et al., 1992], while the origins associated with *c-myc* [Tao et al., 2000], and *Trk A* genes [Delgado et al., 1998], adenine phosphoribosyl transferase (*APRT*), growth arrest and DNA damage inducible (*GADD*), thymidine kinase (*TK*) [Delgado et al., 1998] and, more recently, hypoxantine phosphoribosyl transferase (*HPRT*) [Cohen et al., 2002], and glucose-6-phosphate dehydrogenase (*G6PD*) genes [Cohen et al., 2003] were mapped to sites of high GC content. Short nascent DNA from synchronized Chinese hamster ovary cells was found to be enriched in CpG islands by PCR amplification; regions flanking the CpG islands as close as 1.5 kb were not detected [Delgado et al., 1998]. This led the authors to conclude that CpG islands coincide with early replication origins in mammalian chromosomes. CpG islands span approximately 1 kb and have a high (65%) GC content. Except for the inactive X chromosome, CpG islands are hypomethylated; the rest of the mammalian genome is relatively GC poor (<40%) and heavily methylated at CpG nucleotides [Delgado et al., 1998].

Sequence analysis of the region of chromosome 1p36.13 as reported at the UCSC bioinformatics site revealed the presence of CpG islands, some in proximity of or coincidental with transcriptional promoters (Fig. 4B). The area near the promoter of the *ALDH4A1* gene contains a CpG island spanning 805 bp, and a large CpG island, extending over 1,934 bp, is associated with the transcriptional promoter of the predicted gene NT_004610.2. These areas were chosen for the testing of origin function. Both areas showed the typical profile of a functional origin of replication when tested by the abundance of PCR markers in size-fractionated (900–1,700 nt), single-stranded nascent DNA (Figs. 5 and 6). These origins were equally active in both fibroblast lines tested. The ratio between the PCR signal for markers with the highest and lowest abundance in nascent DNA was found to be higher for these origins than the ratio found for the lamin B2 origin in the same nascent DNA preparations. This finding might imply that these origins are utilized in a larger percentage of human fibroblasts entering the S phase, as compared to the lamin B2 origin. Although this method alone does not allow the determination at the nucleotide level of where the origin of bidirectional replication is located in the

sequence, the results of the present study strongly indicate that active origins of replication are present in both loci tested. These two origins are located within CpG islands (Figs. 5 and 6) and CG-box binding sites for transcription factor Sp1 were detected in both regions. Interestingly, the origin activated during the first hour of S seems to contain many more predicted Sp1 binding sites than the region replicated 1 h later. The distance between these two origins is about 54 kb, and it appears that 75% of this inter-origin region (between 73.4 and 32 kb in the contig) is duplicated before the ALDH4A1 origin fires (Fig. 4A). Two inverted copies of a 264 bp sequence, separated by about 1 kb, are present in the inter-origin zone (at 59–60 kb in the contig), and might function as a fork barrier or pause site, analogously to the role of transcriptional insulators. It is interesting to note that one function of replication fork barriers and pause sites has been proposed to be preventing the replication fork from entering an actively transcribed zone and colliding with the transcription complex. In this case, however, the transition in timing of replication seems to happen in the middle of a gene that is transcribed in the direction of replication fork progression. In the opposite direction, the whole region is replicated in the first hour of the S phase up to nt 211288 in the contig (marker 211 k, Table I). If this early area is replicated in its entirety by the fork starting off at the 73.4 kb origin, one has to envision an asymmetrical replicon with one fork stopping at some barrier, or proceeding extremely slowly, and the other fork advancing unhindered in the opposite direction. If the two forks were to proceed at the same rate (3 kb/min), it would take about 5 min for the left fork to reach a potential barrier at 60 kb in the contig and 45 min for the right fork to replicate the approximately 138 kb in the early replicating region before reaching the next transition zone. It is also possible that the early replicating region consists of a bank of small replicons, all activated simultaneously at the beginning of S. Our replication timing assay does not allow discrimination between these two possibilities. The maximum abundance signal would still be detected in the DNA replicated within the first hour of S, whether replication of the 138 kb region was accomplished by a single fork starting from the 73.4 kb early origin or from the synchronous activation of multiple ones. It is noted, however, that the early-replicating segment corresponds to a large intergenic region, where no promoters or CpG islands have been detected. Therefore, if one or more additional origins are present in this region, their structure will have to be different from the ones described in this study. Another timing transition zone seems to be present between markers 211 and 229 k, since the distance of 18 kb could be replicated by a single fork in 10–20 min, while the difference in replication timing is almost 1 h. It is possible that a pause site is present in this zone as well. Between 228 and 336 kb, the observed change in replication timing would be consistent with the displacement of a single replication fork traveling at a rate of about 1.8 kb/min.

A relationship between early replication and transcriptional activity has been long established [Goldman et al., 1984; Holmquist, 1987], with active genes typically replicated early in the S phase, and inactive, heterochromatic DNA replicated later. As mentioned in the introduction of this article, more recent studies confirmed this correlation on a genomic scale in *Drosophila* [Schübeler et al., 2002]. It is understandable how chromatin, assuming a relaxed, more open conformation at the time of transcription, might also favor the assembly of pre-replication complexes. Origin utilization, however, does not seem to change with the transcriptional status and altered replication time in the human β-globin locus [Aladjem et al., 1998] and in the *HPRT* and *G6PD* genes in active and inactive X chromosomes [Cohen et al., 2003]. Conversely, insertion of an orientation-dependent transcriptional silencing sequence from human β-globin into a mouse locus replicating late caused the same locus to replicate early when the orientation of the insert was transcription-permissive. However, the timing of replication remained late when the orientation of the insert favored gene silencing. No active promoter or replication origin needed to be inserted for the timing switch to take place [Lin et al., 2003]. These findings suggest that the DNA sequence at the origin is not responsible for the determination of the time in S phase at which such origin is fired, but that some other cis- or trans-acting accessory elements determine the timing of origin activation.

In conclusion, it appears that DNA replication can be organized very differently even in neighboring DNA sequences. Timing of replication of areas of the genome very close to one

another might be regulated and coordinated with other DNA metabolic processes.

ACKNOWLEDGMENTS

We are thankful to Dr. Lisa D. McDaniel and Dr. Roger A. Schultz (McDermott Center for Human Growth and Development, University of Texas Southwestern Medical Center) for the gift of the hTERT-GM01604 cell line created with telomerase reagents from Geron Corporation (Menlo Park, CA). We also wish to thank for the contributions of Dr. Xu Lin, who started the study of this chromosomal band, Ms. Franchesca Meacham, whose summer project confirmed clone results, and Ms. Miriam Bryant, who expertly prepared DNA.

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