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The Coordinate Replication of the Human β-Globin Gene Domain Reflects Its Transcriptional Activity and Nuclease Hypersensitivity

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The temporal order of replication of DNA sequences in the chromosomal domain containing the human β-globin gene cluster and its flanking sequences (140 kilobases) was measured and compared in two different human cell lines. In human erythroleukemia (K562) cells, in which embryonic and fetal globin genes are transcribed, all of the sequences we examined from the β-globin domain replicated early during S phase, while in HeLa cells, in which globin genes are transcriptionally silent, these sequences replicated late during S. Potential sites of initiation of DNA replication within this domain were identified. The β-globin gene domain was also found to differ with respect to the nuclease sensitivity of the chromatin in these two cell lines. In K562 cells, hypersensitive sites for endogenous nucleases and DNase I were present in the chromatin near the earliest-replicating segments in the β-globin domain.

In viruses and procaryotes, DNA replication initiates at well-defined sequences (origins of replication) and proceeds bidirectionally. The genome of higher eucaryotes replicates during a defined period (S phase) of the cell cycle by initiating at multiple sites and replicates only once during each DNA-synthetic phase (21). Although various techniques have been used to localize replication origins in eucaryotic cells, relatively little information is available concerning the molecular specificity of eucaryotic origins of replication. Electron microscopic analysis has indicated that replication forks in the rDNA of several eucaryotic species originate from specific sites (for review, see reference 36). In an amplified dihydrofolate reductase (*dhfr*) domain of a Chinese hamster cell line, a small subset of *Eco*RI segments have been shown to replicate first at the onset of the S phase, and it has been suggested that initiation sites are located within these early-replicating segments (18).

The temporal order of replication of the nontranscribed murine immunoglobulin heavy-chain gene locus containing the constant, joining (J), and diversity (D) region sequences corresponds to their linear order in the genome in murine cell lines (5). The most 3′ sequences near the Cα heavy-chain gene replicate during the first third of S, and the most-5′ sequences near the D region replicate during the middle of S. Thus, the replication of immunoglobulin and many other tissue-specific genes (17) proceeds in an organized manner suggestive of the presence of specific initiation sites. Furthermore, origin-containing sequences were reported to be essential for the replication of simian virus 40 (SV40) and polyomavirus DNA molecules following microinjection into mouse embryos (41). All of these data are consistent with the existence of sequence-specific replication origins in mammalian cells. In contrast to these results, SV40 DNA segments were found to replicate efficiently upon microinjection into Xenopus eggs regardless of whether they contained an origin sequence (16, 30). However, studies with *Drosophila melanogaster* and amphibian egg cells have suggested that more origins are active in these rapidly dividing cells than in somatic or differentiated cells (for review, see references 8, 15, and 36).

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Several investigators have suggested that the position of a replication origin may have a role in gene activity (e.g., see reference 34). These suggestions are compatible with the recent observations that in in vitro replication assays, the transcriptionally active histone H5 genes in chicken embryonic erythrocytes are replicated in the transcriptional direction from an origin located 5′ to the gene, whereas the inactive H5 genes are replicated in the opposite direction from a downstream origin (37). Furthermore, studies on the temporal order of replication of the immunoglobulin heavy-chain cluster (IgCH) in mouse cell lines is also consistent with replication proceeding from a presumptive origin downstream of the gene cluster when the locus is inactive (5). However, when one or more genes in the IgCH cluster are transcribed, the entire cluster replicates at the same time at the beginning of S with no apparent temporal directionality (5). Thus, a change in the timing and possibly a change in the origin and direction of replication is associated with the change in the transcriptional activity within this cluster.

To gain more information about the relationship between gene expression and the timing of DNA replication in mammalian cells, we have studied the human β-globin gene cluster. This gene cluster has been well characterized at the molecular level and consists of an embryonic (ε) gene expressed in the early embryo, two fetal genes ($^{G}\gamma$, $^{A}\gamma$) expressed in the fetal liver, two adult genes (δ, β) expressed in adult bone marrow, and a pseudogene (ψβ). These genes undergo a switch in their expression during erythroid cell development. These genes are clustered in a 50-kilobase (kb) region on chromosome 11 and occur in the order 5′-$\varepsilon^{G}\gamma^{A}\gamma\delta\beta$-3′ (11). Centrifugal elutriation was used to fractionate 5-bromodeoxyuridine (BUdR)-labeled human cells into various intervals of S phase, followed by detailed hybridization analysis of bromouracil (BU)-labeled, newly replicated DNA with DNA probes spanning the length of the human β-globin gene locus. This fractionation technique has a number of distinct advantages over other assays; it is more rapid, a large number of cells can be fractionated, and most importantly, it obviates the need for the toxic chemicals that are used in cell synchronization. In this report, we show that the timing of replication during the S phase of a contiguous stretch of chromosomal DNA (140 kb) containing the human

β-globin gene cluster and its flanking sequences is coordinately regulated. Early replication of the locus in erythroleukemia cells (K562) is associated with chromatin sensitivity to nucleases and transcription of the embryonic and fetal globin genes. The transcriptionally inactive locus in nonerythroid HeLa cells is resistant to limited nuclease digestion and late replicating. Furthermore, in K562 cells, two DNA segments are found to replicate earlier in the β-globin locus and are also associated with nuclease-hypersensitive sites.

MATERIALS AND METHODS

Cell culture. The human erythroleukemia (K562) cells were propagated in Eagle minimal essential medium supplemented with 15% heat-inactivated fetal bovine serum, nonessential amino acids, and glutamine. HeLa cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum.

Centrifugal elutriation and isolation of newly synthesized DNA. To obtain replicated DNA, we used centrifugal elutriation to fractionate K562 and HeLa cells into various stages of the S phase based on cell size. The rationale for this fractionation was based on our earlier observations that as cells enter and progress through the S phase, cell size increases linearly with DNA content (5, 6). Exponentially growing K562 or HeLa cells were pulse labeled with BUdR (20 μg/ml) for 2 to 2.5 h, which was the time necessary to recover enough newly replicated DNA (BU-DNA) from each S phase interval for single-copy gene analysis. Cells (1.5×10^9 to 2×10^9) were injected into the centrifugal elutriator chamber at a constant rotor (Beckman JE-10X) speed, which was maintained throughout the elutriation. The flow rate of the medium into the cell chamber was increased in a stepwise manner to elutriate 15 to 25 different fractions.

To examine the purity of these fractions and to determine the position in the cell cycle of the cells in each fraction, a sample containing 2×10^5 cells was stained with propidium iodide in sodium citrate as described previously (25), and the intensity of the DNA-bound fluorescence per cell was measured with the fluorescence-activated cell sorter (Fig. 1). The smallest cells from the first few fractions have a DNA content of 2C (C is the haploid DNA content of cells) and are in G1. As cell size increases, the DNA content of the fractions increases proportionately until it reaches the 4C value representing G2 cells. After discarding the first few fractions containing G1 cells and the last one or two fractions containing cell aggregates and multinuclear cells, the 10 to 20 remaining fractions were pooled to produce four cell classes (I to IV) representing four different temporal intervals of S (early through late). In independent elutriation experiments, alterations were made in the length of the BU pulse, the speed of the elutriator rotor, and the number of successive fractions that were pooled to produce cell fractions representing four different intervals of S.

To isolate the newly replicated, BU-substituted DNA (BU-DNA), which constitutes only a small proportion of the total DNA in each S phase fraction, high-molecular-weight DNA was digested with *Eco*RI and fractionated on cesium sulfate gradients as described before (3). BU-DNA, which has a higher buoyant density than the unsubstituted DNA, could be distinguished in the gradient with the aid of a UV absorptiometer. Since the smallest cell class contained significant numbers of G1 cells, the proportion of BU-DNA obtained was lower than for the other cell classes. To confirm the purity of the BU-DNA representing each interval, a sample (2 μg) of this DNA was recentrifuged on a cesium sulfate gradient in the model E analytical ultracentrifuge. A single band at the density of BU-DNA having 90% of its thymine replaced by BU was observed with less than 5% contamination by unsubstituted DNA. DNA concentrations were determined by optical density at 260 nm and by the DABA (3,5-diaminobenzoic acid dihydrochloride) assay (24), which agreed to within 5 to 20%.

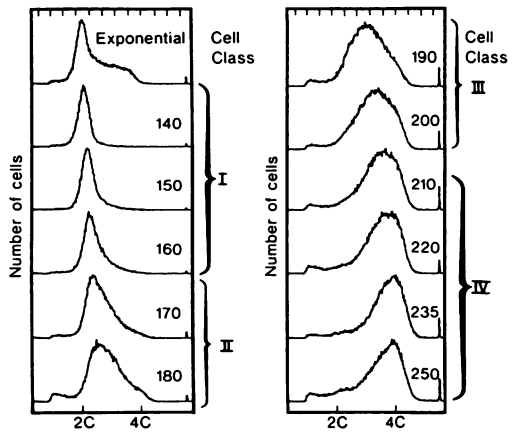

FIG. 1. Flow microfluorometric (FMF) analysis showing cellular DNA content of fractions obtained by centrifugal elutriation. The FMF profiles are histograms showing number of cells (ordinate), with the DNA content (measured by fluorescence) indicated on the abscissa. The first profile represents exponentially growing K562 cells labeled for 2 h with BUdR before fractionation. The numbers to the right of each profile indicate the flow rate (milliliters per minute) of the medium used for elutriation of that fraction. Successive fractions display a gradual increase in cellular DNA content from 2C (for cells in G1) to 4C (for cells in G2 and mitosis). C is the haploid DNA content of K562 cells in G1. These fractions were pooled to produce four classes of cells (I to IV) containing BU-DNA that replicated during each of four different intervals of the S phase, representing early through late S. BU-DNA from each interval of the S phase was isolated and analyzed by hybridization with probes from the human β-globin gene locus.

DBM transfer and hybridization. Equal amounts of the *Eco*RI-digested BU-DNA (5 μg) from each of the four intervals of S phase were electrophoresed for about 18 h in 0.8% agarose gels and transferred to diazobenzyloxymethyl (DBM) paper as described before (40). Unique DNA segments from the human β-globin gene locus (see Fig. 2) were purified of vector sequences, nick translated to a specific activity of about 1×10^8 to 2×10^8 cpm, and hybridized to DBM transfers for 2 to 3 days at 37°C as described before (40). The transfers were washed in 0.1 × SSPE (18 mM NaCl, 1 mM $NaH_2PO_4 \cdot H_2O$, 0.1 mM EDTA) buffer containing 0.1% sodium dodecyl sulfate (SDS) at 55°C repeatedly and exposed to X-ray films with intensifying screens for 1 to 3 days at −70°C. The hybridization signals were quantitated both by a computer-assisted Cambridge Instruments Quantimet 920 and by a Joyce Loebl double-beam recording microdensitometer.

Nuclease digestion of nuclei and DNA hypersensitivity. Exponentially growing K562 or HeLa cells were washed twice in phosphate-buffered saline. Nuclei were isolated by homogenizing cells in Tris buffer (10 mM Tris hydrochloride [pH 7.8], 4 mM $MgCl_2$, 1 mM EDTA) with 5 to 10 strokes of a B pestle in a glass Dounce homogenizer. Nuclei were purified from cellular debris by low-speed centrifugation and suspended in HEPES buffer (33 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.8], 50 mM NaCl, 6.7 mM $MgCl_2$) at 10^8 nuclei per ml. Alternatively,

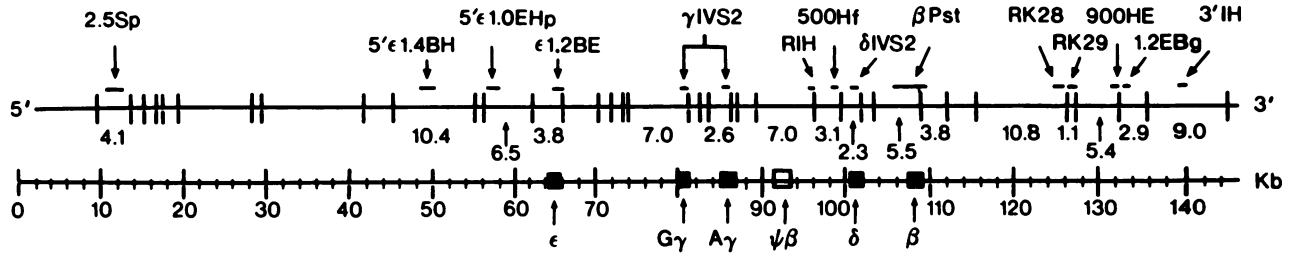

FIG. 2. *Eco*RI map of the human β-globin gene cluster and its flanking sequences. *Eco*RI sites within 140 kb of chromosomal DNA encompassing the β-like-globin genes are indicated by vertical lines on the map and are based on those determined by several investigators (e.g., see references 11 and 35). The locations of the five β-like-globin genes are indicated by solid bars, and a pseudogene is shown by an open square. The locations of the probes used here are shown as horizontal lines above map. The lengths of the *Eco*RI segments detected by the probes are also indicated.

nuclei were isolated by incubating cells in NP-40 lysis buffer (10 mM Tris hydrochloride [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% [vol/vol] Nonidet P-40 [NP-40]) for 5 min on ice. After pelleting, nuclei were resuspended in this buffer without NP-40 at 10^8 nuclei per ml.

For digestion with endogenous nucleases, nuclei were incubated for increasing lengths of time (0, 10, 30, 60, 120, and 150 min) at 37°C and for exogenous nucleases with increasing amounts of DNase I (Cooper Biomedical) (0, 1, 5, 10, 20, and 30 μg/ml) for 10 min at 37°C. In both instances, the reactions were terminated with EDTA (20 mM), and the nuclei were lysed with 0.1% SDS and deproteinized with proteinase K (100 μg/ml). DNA was isolated by standard procedures (3) and digested with appropriate restriction endonucleases. For the analysis of DNA hypersensitivity, 10 μg of DNA from various digests was electrophoresed in 0.8% agarose gels and transferred to nitrocellulose paper. The filters were then probed with β-globin gene locus-specific probes (see Fig. 2), washed in several changes of 0.1 × SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0) containing 0.1% SDS at 55°C, and exposed to X-ray film.

Probes. Probe 2.5Sp is a 2.5-kb *Sph*I fragment and was provided by E. Vanin (39). Probe 5'ε1.4BH is a 1.4-kb *Bam*HI-*Hin*dIII fragment provided by P. Powers (27). Probe 5'ε1.0EHp is a 1.0-kb *Eco*RI-*Hpa*I fragment which was derived from the plasmid p5'ε2 (27). Probe ε1.2BE is a 1.27-kb *Bam*HI-*Eco*RI fragment which includes part of the ε-globin gene. Probe γIVS2 is a 960-base-pair (bp) *Bam*HI-*Eco*RI fragment from the second intervening sequence of the ^{A}γ-globin gene. It detects both the ^{G}γ and ^{A}γ genes. Probe RIH is a 500-bp *Eco*RI fragment from phage HβGI (11). Probe 500Hf is a 500-bp *Hin*fI fragment provided by N. Maeda (28). Probe δIVS2 is a 950-bp *Bam*HI-*Eco*RI fragment from the second intervening sequence of the δ-globin gene. Probe βPst is a 4.4-kb *Pst*I fragment encompassing the β-globin gene. Probe RK28 is a 1.1-kb *Eco*RI-*Sal*I fragment from pRK28, and probe RK29 is a 1.1-kb *Eco*RI fragment from pRK29; both plasmids were provided by R. Kaufman. Probe 900HE is a 900-bp *Hin*dIII-*Eco*RI fragment isolated from the plasmid p15B5.6 provided by F. Grosveld. Probe 1.2EBg is a 1.2-kb *Eco*RI-*Bgl*II fragment isolated from p15B5.6. Probe 3'IH is a 680-bp *Fok*I-*Bgl*II fragment isolated from the plasmid pl.lBBg and provided by P. Henthorn (19). Probe H500 is a 900-bp *Hin*dIII fragment isolated from phage N3'β-1 (29). Probe 1.4ESt is a 1.4-kb *Eco*RI-*Stu*I fragment isolated from phage ChT-1 (29). Probe 1.08SB is a 1.08-kb *Sst*I-*Bam*HI fragment isolated from phage BLH2-1 (29, 39). The βIVS2 probe used in Table 2 is a 950-bp *Eco*RI-*Bam*HI fragment from the second intervening sequence of the β-globin gene. Probe C4AL1 is a 1.0-kb *Pst*I cDNA segment for the human complement (C4) protein gene (40a). A 500-bp *Nco*I/*Bst*EII segment (500NBE) from plasmid p191 served as a probe for the human immunoglobulin heavy-chain variable-region subgroup III (30a). Probe AT6.5 is a 6.5-kb *Bam*HI segment from the human α_1-antitrypsin gene sequences (26a). The cDNA probe pBFA28 used to identify the *Eco*RI segments containing human complement protein factor B was provided by D. Woods and H. Colten (42).

RESULTS

Coordinate replication of the β-globin domain in an erythroid and a nonerythroid cell line. To determine the temporal order of DNA replication in the chromosomal domain containing the human β-globin gene cluster and its flanking sequences, equal amounts of *Eco*RI-digested BU-DNA, replicated during four different intervals of S, was fractionated by agarose gel electrophoresis, transferred to DBM paper, and subjected to detailed hybridization analysis with 14 different unique probes from the human β-globin gene locus (Fig. 2). Sixteen different *Eco*RI segments ranging in length from 1 to 10 kb were detected. In this manner, the temporal replication of DNA sequences spanning about 100 kb of the β-globin locus was determined.

In K562 cells, the 16 different *Eco*RI segments from the β-globin gene cluster replicate early in S. Typical examples of autoradiographs are shown for the *Eco*RI segments from the 3' region of the β-globin gene locus (Fig. 3). Results obtained from densitometric analysis of the band intensities revealed that these segments were more concentrated (80 to 85%) in the DNA that had replicated in the first half of S (intervals I and II). Similar results were obtained when additional *Eco*RI segments spanning most of the locus were analyzed. The most-3' segment for which results are presented in Fig. 3 was detected by the 3'IH probe. Recently, three segments have been linked to the β-globin cluster by pulsed-field mapping (7). These segments are located 3' to the adult β-globin gene at approximate distances of 65 kb for the 7-kb *Eco*RI segment detected by the H500 probe, 80 kb for the 5.0-kb segment detected by the 1.4ESt probe, and 88 kb for the 11-kb segment detected by the 1.08SB probe (7, 29). *Eco*RI segments detected by these probes all replicated in early S phase in K562 cells (Table 1). Thus, in this cell line the domain of early-replicating DNA encompassing the β-globin gene cluster spans at least 200 kb.

In contrast to the results for K562 cells, in the nonerythroid HeLa cell line, ten *Eco*RI segments that we examined were present in the highest concentration in BU-DNA that replicated during S phase intervals III and IV (Table 2). This result indicates that in HeLa cells, in which the genes of

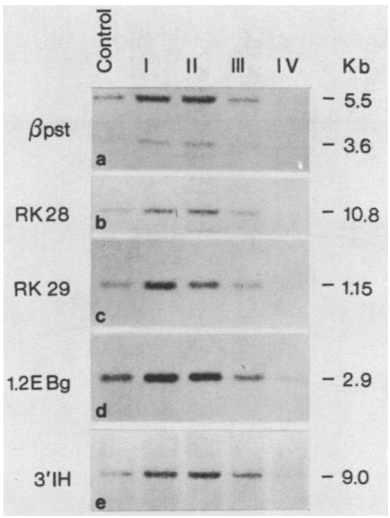

FIG. 3. Early replication of the DNA segments spanning the nontranscribed β-globin gene and its 3'-flanking region in K562 cells. K562 cells grown in the presence of BUdR were fractionated according to size by centrifugal elutriation. Fractions were pooled into four size classes representing four different intervals of the S phase. *Eco*RI-digested BU-DNA (5 μg) from each of the four intervals of S phase (lanes I to IV) was electrophoresed in an 0.8% agarose gel, transferred to DBM paper, and hybridized to the human β-globin locus-specific probes indicated at left (see Fig. 2 for locations of the probes). The sizes of the *Eco*RI segments are indicated to the right of each panel. The control lane represents *Eco*RI-digested DNA (5 μg) from exponentially growing cells.

the β-globin cluster are not expressed, the domain replicates during the second half of S phase.

Earliest-replicating segments in the β-globin gene locus in K562 cells. To determine the temporal order in which *Eco*RI segments replicated and to identify the earliest-replicating segments in this domain, the relative concentration of each *Eco*RI segment in the first interval of S phase was compared. Most of the segments appeared to replicate at similar times

TABLE 1. Early replication of the β-globin gene extreme 3'-flanking regions in K562 cells[a]

Probe	Size (kb) of *Eco*RI segment	Approximate distance (kb) from β-globin gene	Relative concn (%) of segments in BU-DNA isolated from K562 cell class[b]:			
			I	II	III	IV
H500	7.0	65	55.2	33.1	9.2	2.5
1.4ESt	5.0	80	52.9	35.3	8.3	3.6
1.08SB	11.0	88	49.3	35.3	10.1	5.2
Controls						
C4AL1 (C4 complement)	15.0		53.9	32.5	11.1	2.6
500NBE (immunoglobulin heavy-chain variable region)	1.5		8.7	16.0	30.6	44.8
AT6.5 (α_1-antitrypsin)	9.5		5.4	16.4	34.6	43.6

[a] Data represent the results from elutriation experiment ii shown in Fig. 4A.
[b] Four classes of cells (see Materials and Methods) that had synthesized BU-DNA during four different intervals of the S phase were chosen. The relative concentrations (expressed as a percentage of the total concentration in all four intervals) of the *Eco*RI segments indicate their temporal order of replication.

TABLE 2. Late replication of the β-globin domain in HeLa cells

Probes (5'→3')	Size (kb) of *Eco*RI segment	Relative concn (%) of segments in BU-DNA isolated from HeLa cell class:			
		I	II	III	IV
2.5Sp	4.1	0.8	2.6	42.9	53.7
5'ε1.4BH	10.4	1.3	3.6	34.6	60.5
ε1.2BE	3.8	1.5	2.4	36.7	55.5
γIVS2	2.6	5.0	5.5	46.9	42.7
	7.0	4.2	7.2	41.5	47.2
βIVS2	5.5	3.2	4.7	28.5	63.4
RK28	10.8	1.8	3.2	38.5	56.4
3'IH	9.0	1.5	4.9	41.4	52.3
H500	7.0	5.5	4.1	39.4	51.0
1.08SB	11.0	3.2	3.5	52.1	41.2
Control					
pBFA28 (factor B complement)	6.0	78.3	16.6	2.9	2.2

during the first third of S (Fig. 3 and 4). There were, however, significant differences in the temporal replication of some of the segments. For example, the DNA segment detected by probe 3'IH replicated at a significantly later time than any of the other segments. For other segments there were smaller but statistically significant differences. Furthermore, the relative concentrations of the segments followed a similar pattern in the results from both elutriation experiments (Fig. 4A) and also after the two sets of results were normalized (Fig. 4B). In both experiments, replication appeared to be earlier for two specific DNA segments than for the surrounding segments. The 2.7-kb *Eco*RI segment (hybridizing to the γIVS2 probe) including part of the ^{A}γ-globin gene was more enriched than the surrounding segments in the BU-DNA that replicated during the first interval of S. Similarly, closer to the 3' end of the locus, the 1.15-kb *Eco*RI segment hybridizing to the probe RK29 was more concentrated relative to the adjoining segments. Thus, DNA segments located approximately 42 kb apart, one encompassing most of the ^{A}γ-globin gene and one found 18 kb 3' to the β-globin gene, appear to be the earliest-replicating segments in this cluster.

Nuclease sensitivity in the β-globin gene locus. Early replication of a contiguous stretch of chromosomal DNA may require an altered or active chromatin structure to render the domain more accessible to proteins involved in DNA replication. To investigate any structural changes in the chromatin around the earliest-replicating segments or in the chromatin around the β-globin domain that could be related to their role in the timing of replication, we examined the chromatin in the β-globin locus for its sensitivity to nucleases. Isolated nuclei from K562 and HeLa cells were subjected to limited digestion with endogenous endonucleases or with exogenously added DNase I. To map nuclease cleavage sites on the 3' end of the β-globin locus in K562 cells, purified nuclease-digested DNA from each time point was further digested with *Bam*HI, transferred to nitrocellulose paper after size fractionation by gel electrophoresis, and probed with the 1.15-kb *Eco*RI segment from pRK29, which hybridized to a 16-kb *Bam*HI DNA segment. The results of this analysis are shown in Fig. 5A and demonstrate that in the time zero control in the absence of endonucleolytic cleavage (lane 1), *Bam*HI digestion of the purified genomic DNA from these cells produced a prominent 16-kb band and, because of a DNA polymorphism at the 5' *Bam*HI site in the second allele (23), a second band about 25 kb in size. As the

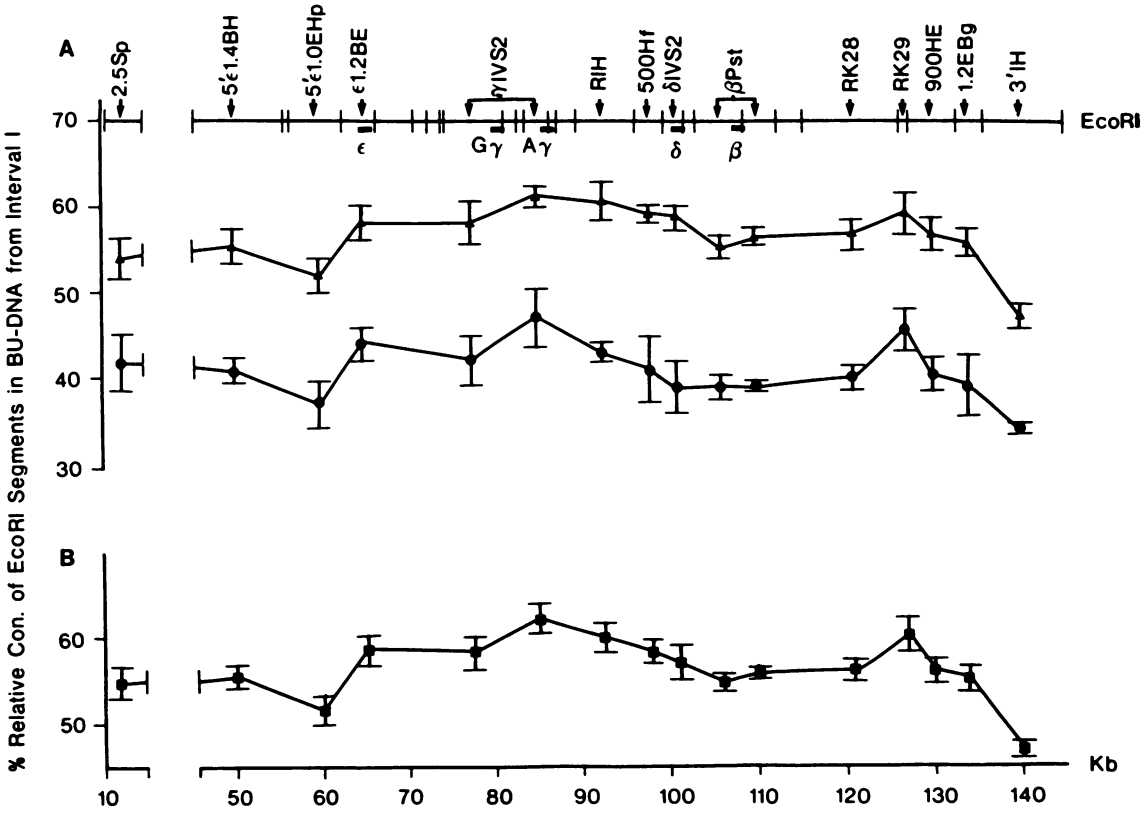

FIG. 4. Comparison of the percent relative concentration of the *Eco*RI segments of the human β-globin gene locus in BU-DNA synthesized during the first interval of the S phase in K562 cells. *Eco*RI digests of BU-DNA from four intervals of S phase were electrophoresed in agarose gels and transferred to DBM paper. Each DBM transfer was hybridized to 14 different probes (shown in Fig. 2) one at a time, and the hybridization signal was quantitated from autoradiographs by densitometry. The relative concentration of each *Eco*RI segment in the first interval of S (expressed as a percentage of the total concentration in all four intervals) was plotted versus distance in kilobases. The positions of the points on the abscissa represent the midpoints of the *Eco*RI segments detected by the probes listed above. (A) Data for two independent experiments or elutriations (●, i; ▲, ii) represent the mean values obtained from two and five transfers, respectively. Vertical bars represent ± 1 standard deviation of the mean. (B) Normalization of the two experiments. The experimental data points shown for experiment i (●) in part A were normalized to those for experiment ii (▲) by multiplying each point by a constant (1.37). The normalized points (■) represent the mean ± 1 standard deviation of the mean.

extent of nuclease digestion was increased, a discrete 11-kb subband was produced (arrow in Fig. 5A). Since this segment was not observed in the *Bam*HI-digested DNA from the initial time point, it must have been generated from a single cut produced on either the 5′ or 3′ side of the RK29 segment in the 16-kb or 25-kb *Bam*HI segment. To map the location of this nuclease-hypersensitive site, a duplicate transfer was hybridized with a second probe, a 900-bp *Hin*dIII-*Eco*RI segment (900HE in Fig. 2). The results of such an analysis revealed the appearance of a 4.8-kb subband in the nuclease-treated, *Bam*HI-digested DNA (Fig. 5B). Since the 3′ end of the 1.15-kb *Eco*RI segment (RK29) was about 5.3 kb away from the 3′ *Bam*HI site, the nuclease cleavage site (shown by a solid vertical arrow) would then be less than 0.5 kb from the 3′ end of the RK29 segment, or about 20 kb downstream from the adult β-globin gene. We estimate that these size determinations are accurate to within 500 bp. Both types of nuclease treatment (data for DNase I not shown) produced identical sizes of nuclease-sensitive bands, indicating that the preferential nuclease cleavage site in the chromatin is position specific. The presence of this nuclease-hypersensitive site, also reported by Tuan et al. (38), near the earliest-replicating segment (1.15 kb) detected by probe RK29 in the present studies demonstrates the presence of an altered chromatin configuration near the earliest-replicating segment. Similarly, chromatin around the second-earliest-replicating segment (2.7-kb *Eco*RI, $^{A}\gamma$IVS2) has also been reported to be hypersensitive to nucleases in K562 cells (2, 14, 26, 38).

In addition to the major hypersensitive site detected by the RK29 probe, several hypersensitive sites have been reported in the β-globin domain in K562 cells (14, 38). These are shown in the top portion of Fig. 6. Four of the major hypersensitive sites were located about 21.5, 18.4, 10.5, and 6.1 kb upstream of the ε-globin gene, and the fifth one was located about 20 kb downstream from the 3′ end of the adult β-globin gene. We independently examined (Fig. 6) the chromatin of K562 cells and HeLa cells for the presence of all of these major sites and the minor sites (at the 5′ end of the embryonic globin gene). We confirmed the presence of these sites in K562 cells and found that all but one of these sites was absent in HeLa cells (Fig. 6). This one site in HeLa cells located 10.5 kb upstream of the ε-globin gene was less hypersensitive to nuclease digestion than in K562 cells. In addition, in the nonerythroid HeLa cells, the β-globin locus as a whole was less sensitive to nucleases than that in K562 cells. Together, these results indicate that the early replication of the human β-globin domain in K562 cells is associated

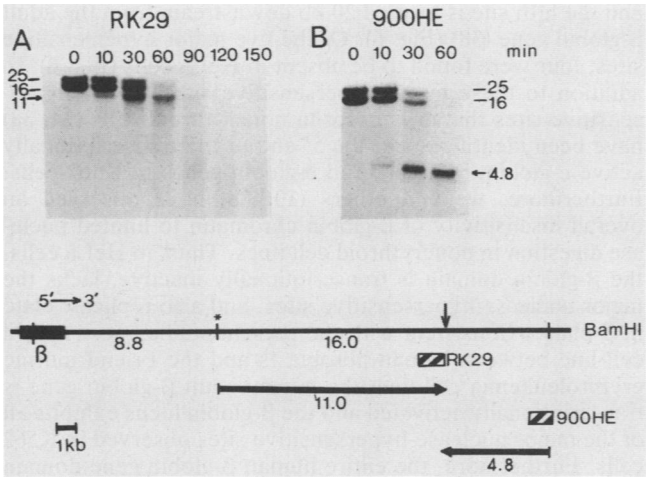

FIG. 5. Mapping of a nuclease-hypersensitive site in the 3'-flanking region of the β-globin gene locus in K562 cells. Nuclei isolated by an NP-40 lysis procedure from K562 cells were incubated at 37°C for the times indicated. The purified DNA was digested with *Bam*HI, electrophoresed (10 μg per lane) in a 0.7% agarose gel, and transferred to nitrocellulose. Duplicate filters were hybridized with probes (A) RK29, 1.1-kb *Eco*RI segment from pRK29, and (B) 900HE, 0.9-kb *Hin*dIII-*Eco*RI segment from p15B5.6. The sizes of the original *Bam*HI segments (approximately 25 kb and 16 kb) are indicated next to the panels, and nuclease digestion-derived fragments of 11 kb in panel A and 4.8 kb in panel B are marked with arrows. The black square in the map indicates the location of the β-globin gene; the asterisk indicates the site of a *Bam*HI polymorphism in one of the two alleles and is described in the text. The location of a nuclease-hypersensitive site is shown by a solid vertical arrow.

with both gene expression and nuclease-sensitive chromatin and late replication in HeLa cells is associated with transcriptional inactivity and nuclease-resistant chromatin conformation.

DISCUSSION

The data presented here demonstrate, first, that a contiguous stretch of chromosomal DNA (140 kb) containing the β-globin gene cluster and its flanking sequences replicates during the first half of the S phase in a human erythroleukemia (K562) cell line and during the second half of S in HeLa cells. Second, in the early-replicating DNA of K562 cells, two *Eco*RI segments are relatively more abundant than the other segments in this locus and therefore appear to be the earliest-replicating segments. Third, the chromatin near these earliest-replicating segments, one of which (RK29) is about 40 kb from a transcribed gene, is associated with a nuclease-hypersensitive site. Fourth, replication occurs at later times for sequences surrounding these two earliest-replicating segments, consistent with the presence of at least two bidirectional replicons in the β-globin gene locus in K562 cells.

In higher eucaryotes, transcriptionally active, tissue specific genes generally replicate early in the S phase (4, 12, 13, 17, 20 and references therein). Detailed studies on the relationship between the replication time and transcriptional activity of tissue-specific genes in several mouse cell lines have demonstrated that transcriptionally inactive tissue-specific genes can replicate during any interval of the S phase (17). These studies also showed that genes located in a cluster ranging in length from 15 to 250 kb tend to replicate during the same quarter of S phase. The present studies further support those observations by demonstrating that all of the sequences we have examined in the 140-kb human β-globin gene locus replicate during the same interval of S in two human cell lines. In K562 cells, of the five genes in the 140-kb domain, only the embryonic and fetal globin genes, which constitute less than 10 kb, are transcriptionally active (1, 32). Replication of the nontranscribed adult globin genes (δ and β) and flanking sequences at the same time as the embryonic and fetal globin genes in early S suggests coordinate regulation of the temporal order of replication of this entire domain in these cells. Furthermore, when all the globin genes in this cluster are transcriptionally inactive in the nonerythroid HeLa cell line, the entire domain is found to replicate late in S. Together, these results suggest that early replication of a chromosomal domain of at least 200 kb is correlated with the transcriptional activity within the locus.

Further dissection of the temporal order of replication of various DNA segments was carried out in order to localize potential replication initiation sites in the β-globin locus in K562 cells. The results showed small differences in the concentrations of adjacent DNA segments in early-replicating DNA. One interpretation of these data is that various DNA segments within this domain replicate simultaneously, possibly from many origins. A second interpretation, based on the consistency in the pattern of the subtle concentration gradient obtained from independent sets of experiments (Fig. 4A), is that the replication initiates within or near two specific DNA segments that are most enriched in early-replicated DNA. These observations are consistent with the presence of at least two 40-kb bidirectional replicons, with replication initiation sites being located either on the earliest-replicating segments (2.7-kb $^{A}\gamma$IVS2, 1.15-kb RK29) shown in Fig. 4 or on an adjacent segment. For example, the 10.8-kb RK28 segment might contain a replication origin but

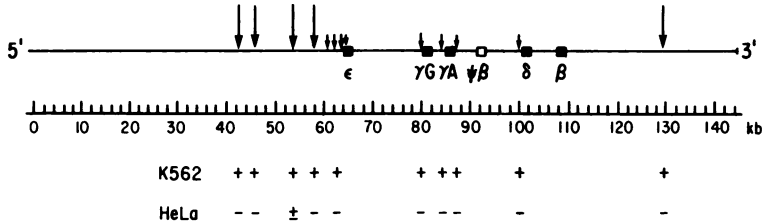

FIG. 6. Locations of nuclease-hypersensitive sites in the β-globin gene domain. The positions of the hypersensitive sites are indicated by the long (major) and short (minor) vertical arrows. These sites are present (+) in K562 cells and absent (−) in HeLa cells. The signal (±) observed for one site in HeLa cells was less sensitive to nuclease digestion than in K562 cells. In K562, data for the 5' and 3' hypersensitive sites near $^{G}\gamma$, $^{A}\gamma$, and δ-globin genes were obtained from previous studies (2, 14, 26, 38).

still replicate slightly after the earliest-replicating 1.15-kb (RK29) segment if the origin is located close to RK29 (see Fig. 4). In this hypothetical instance, replication from the 3′ end of the RK28 segment would allow the RK29 segment to become completely labeled with BUdR before the longer RK28 segment. Hence, RK29 would appear to replicate earlier than RK28, even if the origin of replication is at the 3′ end of the RK28 segment.

The possibility that two replicons are activated in the β-globin locus during the same interval of S in K562 cells would be consistent with earlier autoradiographic results. With DNA fiber autoradiography, evidence has been presented for the coordinate activation of adjacent replicons (21). Subsequently, several lines of evidence have indicated that clustered origins tend to initiate replication synchronously (for review, see reference 15). It remains to be determined whether a similar pattern of replication occurs in the β-globin domain in other cell lines in which either these genes are inactive or an adult globin gene is active.

Gene expression may not be a requirement for the activation of all the initiation sites in an early-replicating domain. In K562 cells, the transcriptionally inactive δ- and β-globin genes are located near RK29, which appears to be one of the earliest-replicating segments in the β-globin domain. Very little is known about the mechanism by which compartments of early- and late-replicating chromosomal domains in eucaryotic cells are regulated. However, a number of mechanisms can be envisaged. For example, a transcriptionally active or potentially active domain could have an altered chromatin structure that might facilitate early replication by allowing an increased interaction with factors involved in DNA replication. Several studies have shown that DNase I-hypersensitive sites are present in the chromatin at viral origins of replication (for review, see reference 9). Furthermore, the chromatin in these hypersensitive sites was observed to be devoid of nucleosomes (22, 33). It has been reported that nuclease-hypersensitive sites are located near replication origins of the rRNA genes of *Tetrahymena*, and it is suggested that such open structures may act as recognition signals during the initiation of DNA replication (31).

In K562 cells, analysis of 25 kb of the chromatin region 3′ to the adult β-globin gene confirmed the presence of a major nuclease-hypersensitive site mapped by Tuan et al. (38) and located about 20 kb downstream from the transcriptionally inactive adult β-globin gene (Fig. 5). This nuclease-hypersensitive region is not directly associated with the transcription of any of the globin genes. However, we have shown that this hypersensitive site is located within or near the 3′ end (within 1 kb) of the earliest-replicating segment (1.15 kb, RK29) in the β-globin locus. Furthermore, nuclease-hypersensitive sites have also been mapped on both the 5′ and 3′ ends (within 1 kb) of the transcriptionally active $^{A}\gamma$-globin gene in K562 cells (2, 14), and we have identified a second-earliest-replicating segment (2.7 kb, $^{A}\gamma$IVS2) between these hypersensitive sites. Based on the presence of the hypersensitive sites in the chromatin near the earliest-replicating DNA segments (2.7 kb $^{A}\gamma$IVS2, 1.15 kb RK29), we speculate that an altered or open chromatin configuration may be present at the site in which DNA replication initiates in the β-globin gene locus in K562 cells.

Comparison of the early- and late-replicating human β-globin domain with respect to nuclease sensitivity of the chromatin reveals the presence of a number of major and minor hypersensitive sites in K562 cells that are absent in HeLa cells. In K562, four major nuclease-hypersensitive sites are located within 25 kb upstream of the ε-globin gene and the fifth site is located 20 kb downstream from the adult β-globin gene (38) (Fig. 6). Of the five major hypersensitive sites, four were found to be absent in HeLa cells (Fig. 6). In addition to these major hypersensitive sites, minor hypersensitive sites that are absent in nonerythroid cells (14, 38) have been identified near the 5′ end of the transcriptionally active ε-globin, γ-globin, and δ-globin genes in K562 cells. Furthermore, we and others (10, 38) have observed an overall insensitivity of β-globin chromatin to limited nuclease digestion in nonerythroid cell lines. Thus, in HeLa cells, the β-globin domain is transcriptionally inactive, lacks the major nuclease-hypersensitive sites, and also replicates late in S phase. Consistent with the present studies, in a hybrid cell line between human fibroblasts and the Friend murine erythroleukemia cell line, the human adult β-globin gene is transcriptionally activated and the β-globin locus exhibits all of the major nuclease-hypersensitive sites observed in K562 cells. Furthermore, the entire human β-globin gene domain in these hybrid cells is early replicating (V. Dhar, A. Skoultchi, and C. L. Schildkraut, manuscript in preparation). Our data thus indicate that early replication of the transcriptionally active β-globin gene locus is associated with the presence of major nuclease-hypersensitive sites at the distal ends of the β-globin gene cluster and general nuclease sensitivity in K562 cells. It remains to be determined whether all the initiation sites responsible for the replication of DNA early in the S phase in mammalian cells are located in active chromatin domains associated with gene expression.

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ADDENDUM IN PROOF

In K562 cells, the 1.6-kb *Eco*RI segment located between the two γ-globin genes detected using a probe (inter γ 1.6) provided by M. Groudine, replicated during the first half of S in a manner similar to that of the majority of sequences within the β-globin locus.

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