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Asynchronous DNA replication within the human β-globin gene locus

(cell cycle/termination/erythropoiesis)

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ABSTRACT **The timing of DNA replication of the human β-globin gene locus has been studied by blot hybridization of newly synthesized BrdUrd-substituted DNA from cells in different stages of the S phase. Using probes that span >120 kilobases across the human β-globin gene locus, we show that the majority of this domain replicates in early S phase in the human erythroleukemia cell line K562 and in middle-to-late S phase in the lymphoid cell line Manca. However, in K562 cells three small regions display a strikingly different replication pattern than adjacent sequences. These islands, located in the inter-γ-globin gene region and approximately 20 kilobases 5′ to the ε-globin gene and 20 kilobases 3′ to the β-globin gene, replicate later and throughout S phase. A similar area is also present in the α-globin gene region in K562 cells. We suggest that these regions may represent sites of termination of replication forks.**

Tissue-specific gene expression is associated with structural changes in chromatin that are made and propagated to progeny cells at the time of cell division (1, 2). Genes that are competent for or actively engaged in transcription replicate early in S phase (3–5), and it has been suggested that domains of chromatin (reviewed in ref. 6) correspond to individual or groups of replicons. Thus, the patterns of origins used during cell division may be important in determining chromatin structure and gene expression (7, 8).

Hematopoiesis and particularly erythropoiesis are systems where the relationships among gene activation, chromatin structure, and DNA replication can be addressed. The human β-globin gene locus consists of six linked genes, in the 5′-to 3′ order $\varepsilon\text{-}^{G}\gamma\text{-}^{A}\gamma\text{-}\psi\beta\text{-}\delta\text{-}\beta$, spanning approximately 50 kilobases (kb) of DNA on the short arm of chromosome 11 (11p15) (9). In human fetal liver, adult erythroid cells, and erythroleukemic cell lines, different subsets of the globin genes are transcribed, yet the entire β-globin-like gene locus, including flanking sequences, is preferentially sensitive to DNase I digestion (10, 11). Although the exact boundaries of the DNase I-sensitive domain have not been established, sequences adjacent to the $(\gamma\delta\beta)^0$-thalassemia breakpoint about 55 kb 5′ to the ε-globin gene are DNase I resistant (12), and sequences at the 3′ breakpoint of patients with the deletional form of hereditary persistence of fetal hemoglobin (HPFH) >100 kb 3′ to the β-globin gene (13) are DNase I sensitive (M.G., unpublished results; see Fig. 5 below). In addition to the domain of overall DNase I sensitivity, erythroid-specific and developmentally stable DNase I-hypersensitive sites have been mapped from 6 to 18 kb 5′ and 20 kb 3′ to the ε-globin and β-globin genes, respectively (11, 15, 16). Recent experiments using somatic cell hybrids (16) and transgenic mice (17) have provided evidence that these hypersensitive sites may be important in the activation of the β-globin locus. Whether these sites form the boundaries of the DNase I-sensitive domain has not been established.

Using density labeling of newly synthesized DNA with BrdUrd and isolation of cell cycle-specific fractions by centrifugal elutriation, we have determined the time of replication of the β-globin gene locus in the human erythroleukemia cell line K562 (18) and the B-cell lymphoma cell line Manca (19). K562 cells express the embryonic and fetal but not the adult β-globin genes (10), whereas in Manca cells, the α- and β-globin genes are transcriptionally silent and DNase I resistant (20). We show that, while the majority of the expressed and unexpressed sequences in the β-globin locus replicate early in S phase in K562 cells, three small dispersed regions, one in the inter-γ-globin gene region and two others about 20 kb 5′ to the ε-globin gene and 20 kb 3′ to the β-globin gene, replicate later in or throughout S phase. The potential significance of these islands of asynchronously replicating DNA is discussed. In contrast, the entire β-globin locus replicates in middle-to-late S phase in Manca cells. Our analysis of the replication timing of the unlinked α-globin gene locus as well as a number of other genes indicates that genes that are inactive and DNase I resistant can replicate in early as well as late S phase. These results suggest that factors in addition to replication in early S phase are necessary to establish an active chromatin structure and transcriptional activity.

MATERIALS AND METHODS

K562 and Manca cells were maintained as described (16). Two to three liters of exponentially growing cells were incubated with BrdUrd (20 μg/ml; Sigma) for 1–2 hr, washed with cold phosphate-buffered saline (PBS), and separated into ≈10 fractions by centrifugal elutriation as described (21). Aliquots were assayed for cell size and number, and cell cycle position was determined by flow microfluorimetry (21, 22). Nuclei and DNA isolation were as described (16), with the addition of treatment with RNase A (25 μg/ml) for 1 hr at 37°C, and DNA was digested with *Eco*RI. Newly synthesized BrdUrd-containing DNA was separated by NaI gradient centrifugation containing ethidium bromide (23). DNA was dialyzed against 10 mM Tris, pH 7.0/1 mM EDTA, concentrated by butanol extraction, ether-extracted, and ethanol-precipitated. BrdUrd-DNA (5–10 μg) from fractions corresponding to early, middle, and late S-phase cells were redigested with *Eco*RI, electrophoresed in 1% agarose gels containing ethidium bromide, and base-transferred to nylon (Zeta-Probe, Bio-Rad) filters (16). Filters were baked, prehybridized, hybridized, and washed as described (16). Probes were labeled by random priming or nick-translation (11). Prior to rehybridization, filters were incubated at 65°C with prehybridization buffer for 1 hr, and completeness of probe

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Abbreviations: HPFH, hereditary persistence of fetal hemoglobin; IVS, intervening sequence; J_H, heavy chain joining region gene.

removal was monitored by autoradiography. For slot blotting, BrdUrd-DNA was isolated as above except that DNA was sheared to 5–10 kb by sequential passage through 18-, 20- and 23-gauge needles, three times each, prior to density gradient centrifugation (22), and slot blots were prepared as described (16). Quantitative analysis of autoradiograms was performed by using an LKB laser densitometer.

RESULTS

Exponentially growing K562 cells were labeled with BrdUrd for 1 hr and elutriated, and the DNA content of individual cell fractions was determined. Approximately 10 fractions corresponding in DNA content from 2N to 4N (N = haploid DNA content) were obtained (Fig. 1*A*). Nuclear DNA was isolated and digested with *Eco*RI, and BrdUrd-DNA was purified by NaI gradient centrifugation. Southern blots containing equal amounts of BrdUrd-DNA corresponding to DNA synthesized during early, middle, and late S phase were hybridized to probes spanning >120 kb of the β-globin gene locus. Virtually the entire β-globin gene locus replicated simultaneously in early S phase (Fig. 1*B*). For example, >80% of hybridization to the ε- and β-globin gene probes was found in the first four fractions containing BrdUrd-DNA synthesized during early S phase (Table 1). Sequences from regions flanking the β-globin gene locus also replicated during this time. These included a 3.3-kb *Eco*RI fragment ("5′ε 3.3") 20 kb 5′ to the ε-globin gene and a 2.5-kb *Sph* probe ("5′ε 2.5") which hybridized to a 4.1-kb *Eco*RI fragment located in a heavily methylated and DNase I-resistant region about 55 kb 5′ to the ε-globin gene (15). In addition, probes from the 3′ breakpoints of a patient with the Indian HPFH (25) and one with Black (13) HPFH located 25 and >100 kb 3′ to the β-globin gene, respectively, also hybridized to early replicating BrdUrd-DNA.

In contrast to the early replicating nature of the majority of the β-globin-like gene cluster, two probes within this locus detected DNA sequences whose time of replication differed markedly from adjacent sequences. Hybridization with a γ-globin intervening sequence (IVS) (10) gene probe revealed that the ^{A}γ-globin gene (2.7 kb) replicates slightly later than the ^{G}γ-globin gene (7.2 kb), even though these genes are only 4 kb apart. The use of two probes located between the γ-globin genes confirmed and extended this observation: a 0.7-kb *Eco*RI fragment was early replicating, whereas the adjacent 1.6-kb *Eco*RI fragment replicated much later and seemingly throughout S (Fig. 1*B*). A similar but less striking result was observed in a region located about 20 kb 3′ to the β-globin gene. A 1.1-kb *Eco*RI fragment ("3′β 1.1") replicated significantly later than a 2.9-kb *Eco*RI fragment ("3′β 1.3") probe located 5 kb 3′ to it, although not as late nor as extended throughout S phase as the inter-γ-globin gene 1.6-kb *Eco*RI fragment. Densitometry (Table 1) confirmed that >95% of the 2.9-kb *Eco*RI fragment hybridization compared to 75% of the 1.1-kb hybridization is found in the first four early replicating fractions.

These results were confirmed and extended by analysis of slot blots of sheared (5–10 kb) BrdUrd-DNA from elutriated fractions of K562 cells. The use of sheared DNA permits determination of the timing of replication of sequences adjacent to defined restriction fragments in regions where single-copy probes are not available. The γ-globin gene IVS-2 probe (10) hybridized to BrdUrd-DNA that is later replicating than the β-globin gene (Fig. 2); densitometry (Table 2) confirmed that 65% of the β-globin compared to 29% of the γ-globin gene hybridization is found in the early (first) fraction. The earlier replication pattern of DNA homologous to the β-globin gene IVS-2 probe observed by slot-blot compared to Southern analysis most likely reflects the presence of very early replicating DNA adjacent to this

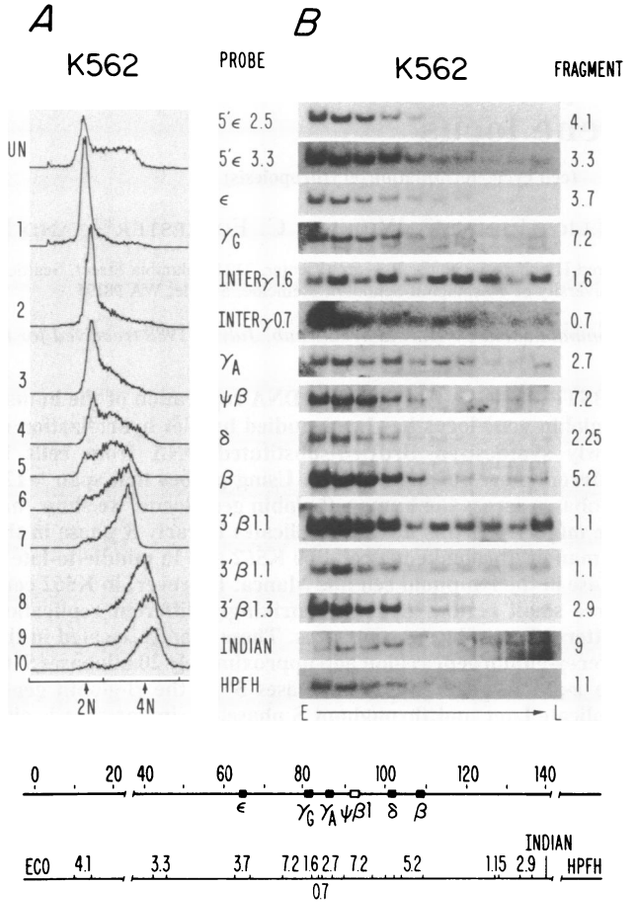

FIG. 1. (*A*) Distribution pattern of cellular DNA content of K562 cells growing exponentially in the presence of BrdUrd prior to fractionation according to size by centrifugal elutriation. An aliquot from each fraction was prepared, stained with propidium iodide, and analyzed by flow cytometry as described (21, 22). The top trace shows the unfractionated population and subsequent traces correspond to early, middle, and late S-phase cells. (*B*) Blot hybridization of newly synthesized BrdUrd-DNA from elutriated fractions of K562 cells to probes spanning the human β-globin-like gene locus. BrdUrd-DNA (10 μg) was cleaved with *Eco*RI, Southern-blotted, and hybridized as described. Each lane represents DNA from an elutriated fraction from early (E) S phase (left-most lane) to late (L) S phase (right-most lane). The location of each probe used and the size in kb of the *Eco*RI fragment hybridized are shown. The map is adapted from ref. 24. Two exposures of the 3′β 1.1 *Eco*RI fragment are shown to emphasize its different pattern of replication. The signal seen in lane 1 using the inter-γ-globin gene 0.7-kb probe has a superimposed background artefact. Probes used are described in refs. 10, 11, and 16 except for the following: 5′ε 2.5, a 2.5-kb *Sph* fragment (12, 24); 5′ε 3.3, a 3.3-kb *Eco*RI fragment (see the map in ref. 17); 3′β 1.3, a 1.3-kb *Eco*RI–*Bgl* II fragment (see the map in ref. 17); Indian, a 3′ breakpoint 0.7-kb *Hin*fIII–*Bgl* II fragment (25); and HPFH, a 3′ breakpoint 2.4-kb *Bam*HI–*Eco*RI fragment (13).

sequence (see *Discussion*). The inter-γ-globin gene 1.6- and 0.7-kb *Eco*RI fragment probes hybridized to DNA sequences that replicated slightly later than those hybridizing to the γ-globin IVS-2 probe. However, in contrast to the results obtained by Southern analysis of *Eco*RI-digested DNA (Fig. 1*B* and Table 1), slot-blot assay of sheared DNA indicated that the 1.6- and 0.7-kb *Eco*RI fragments hybridize to DNA sequences that replicate at similar times. This is due to the physical proximity of the two fragments and the relatively large size of the sheared slotted DNA.

These same slots were also hybridized to probes representing sequences located about 20 kb 5′ to the ε-globin gene and 20 kb 3′ to the β-globin gene, adjacent to the respective

Table 1. Densitometric analysis of blot hybridization to BrdUrd-DNA

Probe	Fraction 1	2	3	4	5	6	7	8	9	10
5′ε 2.5	31	25	21	13	7	2	1	—	—	—
5′ε 3.3	28	18	18	17	6	4	4	2	—	—
ε	27	19	18	11	10	6	4	1	2	2
$^{G}\gamma$	41	19	13	6	4	3	2	2	6	3
$^{A}\gamma$	22	21	12	14	7	9	6	2	2	4
Inter-γ 1.6	9	13	8	13	6	11	14	12	7	9
Inter-γ 0.7	38	26	10	7	6	6	4	1	1	1
β	30	18	18	11	6	2	4	2	5	5
ψβ	28	28	24	11	5	1	1	1	1	—
δ	39	37	15	7	—	1	1	—	—	—
3′β 1.1	19	28	16	13	3	4	4	4	2	8
3′β 1.3	35	31	16	15	—	2	1	—	—	—
HPFH	33	24	18	9	3	2	5	2	2	2

Autoradiograms were scanned on an LKB laser densitometer and normalized to determine the percent hybridization within a given cell cycle fraction. Usually three readings were averaged, and more than one film exposure was scanned to ensure linearity of response.

developmentally stable DNase I-hypersensitive sites (see maps in ref. 17; Fig. 5). A 1.3-kb *Hin*dIII–*Bam*HI probe ("5′ε 1.3") corresponding to DNA sequences about 15 kb 5′ to the ε-globin gene hybridized to early replicating sequences (70% of hybridization to fractions 1 and 2), whereas the 3.3-kb *Eco*RI fragment (5′ε 3.3) located about 20 kb 5′ to the ε-globin gene replicated significantly later in S phase (50% of hybridization to fractions 1 and 2). The later replication of the 3.3-kb *Eco*RI fragment by slot-blot compared to Southern blot analysis suggests the presence of later replicating sequences just 5′ to it. This was confirmed by the later replication (30% of hybridization to fractions 1 and 2) of BrdUrd-DNA hybridized to a 0.5-kb *Eco*RI–*Bgl* II fragment ("5′ε 0.5") located within and at the 5′ end of the 3.3-kb *Eco*RI fragment. Thus, there appears to be a replication domain boundary located just 5′ to the 3.3-kb *Eco*RI fragment (see *Discussion*). This region

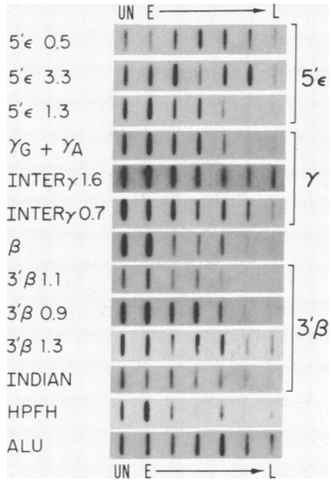

FIG. 2. Slot-blot hybridization of sheared BrdUrd-DNA to β-globin-like gene probes. Slot-blots were prepared by using DNA from elutriated cell fractions and hybridized to β-globin-like gene probes; filters were rehybridized as detailed. Unfractionated BrdUrd-DNA was loaded on the first slot with early-to-late S-phase BrdUrd-DNA in individual slots from left to right as shown. The probes used are indicated on the left and are arranged in a 5′-to-3′ order (see the map in Fig. 1). Slots were hybridized to a repetitive sequence *Alu*-containing probe to control for equal loading of DNA. Probes were identical to those used in Fig. 1*B* and refs. 10, 11, and 16 except for 5′ε 0.5, a 0.5-kb *Bgl* II–*Eco*RI fragment, and 3′β 0.9, a 0.9-kb *Eco*RI–*Hin*dIII fragment (see the maps in ref. 17).

Table 2. Densitometric analysis of slot-blot hybridization to elutriated sheared BrdUrd-DNA

Probe	Fraction 1	2	3	4	5	6
5′ε 0.5	10	20	28	21	15	7
5′ε 3.3	22	31	9	13	20	5
5′ε 1.3	48	22	22	7	1	0
$^{G}\gamma$ + $^{A}\gamma$	29	24	14	17	12	4
Inter-γ 1.6	35	21	17	12	7	9
Inter-γ 0.7	37	20	16	11	7	9
β	65	10	9	13	1	1
3′β 1.1	51	18	22	8	—	—
3′β 0.9	40	20	26	13	1	1
3′β 1.3	25	14	21	21	10	10
HPFH	53	18	6	20	2	1

contains highly repetitive DNA sequences for which single-copy probes are not presently available (24). A replication boundary region in the region 20 kb 3′ to the β-globin gene was also apparent. The 1.1-kb *Eco*RI fragment (3′β 1.1) was later replicating than the β-globin gene, whereas probes located about 5 kb 3′ (3′β 0.9 and 3′β 1.3) were even later replicating. Since this region is also filled with repetitive sequences, the exact location of this late-replicating region cannot be mapped with certainty. However, a probe for the 3′ breakpoint of a patient with the Indian HPFH phenotype located about 10 kb further 3′ (25) also hybridized to slots containing predominantly later replicating BrdUrd-DNA, whereas the HPFH breakpoint probe located >100 kb further 3′ hybridized to early replicating BrdUrd-DNA.

We also analyzed the timing of replication of sequences within the α-globin-like gene locus. The α-globin gene domain occupies about 30 kb on chromosome 16 and contains five genes ζ-ψζ-α_2-α_1-θ in 5′-to-3′ order (9). Both α- and ζ-globin genes are expressed and are DNase I-sensitive in K562 cells (20). The 22-kb *Eco*RI fragment containing both α-globin genes and the ζ-pseudogene replicated early in S phase, similar to the majority of the β-globin-like gene locus (Fig. 3). In contrast, the 4.4-kb *Eco*RI fragment containing the embryonic ζ-globin gene replicated much later in S phase and

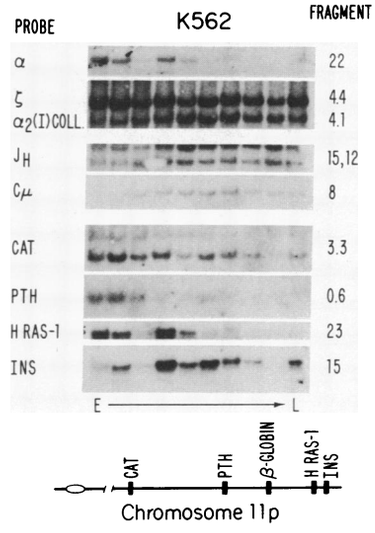

FIG. 3. Blot hybridization of K562 elutriated BrdUrd-DNA to probes for α-globin-like genes and nonglobin probes. The identical filter used in Fig. 1 was washed and rehybridized with the probes shown. The size in kb of the *Eco*RI fragment is indicated. The reduced signal seen in the third lane for high molecular weight fragments (α-globin gene, *HRAS1*, and *INS*) is due to a transfer artefact. Probes used are from refs. 10, 11, 20, and 26–30.

apparently throughout S phase, similar to the pattern observed with the 1.6-kb inter-γ-globin gene *Eco*RI fragment.

A variety of probes for genes presumably unexpressed and DNase I-resistant in these cells were hybridized to the Southern blot shown in Fig. 1*B* (Fig. 3). The immunoglobulin μ chain constant region gene probe (ref. 26) and the α_2(I) collagen gene probe (10) hybridized to fragments replicating in middle S phase, whereas the J_H immunoglobulin heavy chain joining region probe (26) detected sequences that replicated slightly later in S phase. The time of replication of the insulin (*INS*) (27), parathyroid hormone (*PTH*) (28), catalase (*CAT*) (29), and *HRAS1* genes (30), which are all located on the short arm of chromosome 11 containing the β-globin gene locus (31), was also determined. The *HRAS1* and *PTH* probes hybridized to early replicating DNA, the *CAT* probe hybridized to early-to-middle S-phase BrdUrd-DNA, and *INS* probe hybridized to middle S-phase BrdUrd-DNA.

The Burkitt lymphoma B-cell line Manca was similarly labeled with BrdUrd-DNA for 2 hr and elutriated, and BrdUrd-DNA was isolated and analyzed. The flow microfluorimetry data obtained of the cell fractions is shown in Fig. 4*A*. Hybridization of Southern blots containing BrdUrd-DNA to β-globin locus probes (Fig. 4*B*) shows that the entire locus replicates in mid-to-late S phase in Manca cells where they are not expressed and are DNase I-resistant (20). However, the ξ-globin (Fig. 4*B*) and α-globin (not shown) genes gave hybridization patterns consistent with an early replication pattern even though these genes are also DNase I-resistant and not expressed (20). Probes for the *MYC* and immunoglobulin J_H genes hybridized to early replicating DNA, consistent with these genes being expressed and DNase I sensitive in Manca cells. As shown previously, both the germ-line and rearranged *MYC* alleles are transcribed in Manca cells (32), and both replicate early in S phase (Fig. 4*B*).

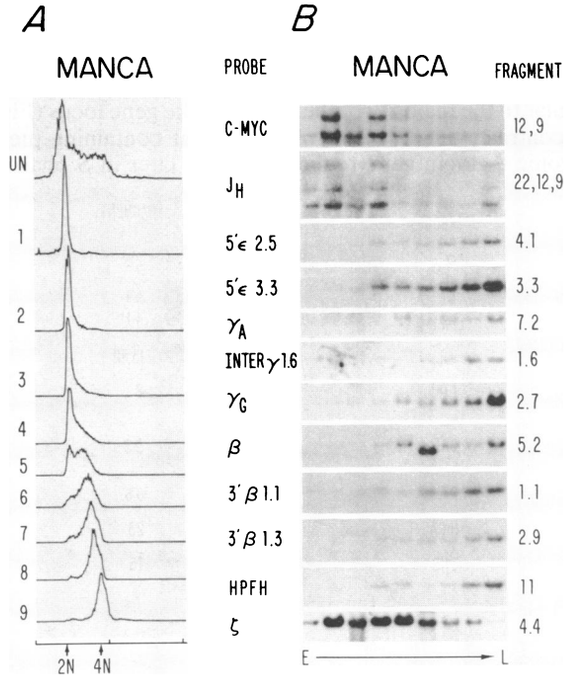

FIG. 4. (*A*) Distribution pattern of the cellular DNA content of exponentially growing Manca cells fractionated according to size by centrifugal elutriation. (*B*) Blot hybridization of BrdUrd-DNA from elutriated Manca cells. Filters prepared as in Fig. 1 were hybridized to probes as in Figs. 1 and 3. The increased hybridization seen in the sixth lane of the hybridization to the β-globin gene probe and in the last lane for many probes is due to plasmid contamination and overloading of DNA, respectively. Probes used were described in Figs. 1*B*, 2, and 3, and ref. 32 (for the *MYC* probe).

DISCUSSION

Our analysis of the temporal ordering of DNA replication in the human β-globin-like gene locus has revealed that in the lymphoid cell line Manca, in which the β-globin-like genes are transcriptionally silent, the entire locus replicates in mid-to-late S phase. In contrast, the majority of the β-globin gene domain, including genes that are both transcribed (e.g., ε-globin) and not transcribed (β-globin) and flanking regions that are both DNase I-sensitive and insensitive (summarized in Fig. 5), replicates synchronously in early S phase in K562 cells. In addition, our investigation of the replication timing of a number of other linked and unlinked genes has shown that genes that are active replicate early in S phase in cells where they are expressed, confirming previous work using similar techniques (22, 23, 33, 34). However, inactive genes replicate in a heterogeneous fashion—in some cases early in S phase (α- and ξ-globin genes in Manca cells) and in other cases in middle or late S phase. Thus, early replication seems to be associated with but not sufficient for transcriptional activity (3–5).

The most surprising result of our analysis of the timing of DNA replication in K562 cells is the identification of several areas that replicate throughout S phase. In the β-globin-like gene locus on chromosome 11, these islands are located in the inter-γ-globin gene region, about 20 kb 3′ to the β-globin gene near the site of an erythroid-specific DNase I-hypersensitive site and just 5′ to the DNase I-hypersensitive sites 6–20 kb 5′ to the ε-globin gene (see Fig. 5). The 5′ and 3′ areas of asynchronous replication do not define transition zones of early replicating DNase I-sensitive chromatin from outlying, late-replicating, and DNase I-resistant chromatin because both DNase I-sensitive and -resistant sequences outside the 5′ and 3′ boundary regions are early replicating by Southern blot analysis (Fig. 5). A similar region of continuously replicating DNA resides within the α-globin-like gene locus (Fig. 3). However, the probe and restriction enzyme combination used in this analysis does not permit us to define whether the active ξ-globin gene is within this continuously replicating region or adjacent to it.

At least two models can explain these islands of asynchronously replicating DNA. In the first, these islands represent regions of termination when two adjacent replication forks moving bidirectionally meet. Here, the rate of chain elongation might be slowed, resulting in areas that appear to replicate throughout the S phase. Alternatively, the replication of this region might be delayed if decatenation preceded the completion of DNA synthesis (35). This colliding replication fork phenomenon has been observed in replicating simian virus 40 (SV40) molecules (35), where the termination points for DNA replication are located at the convergence of

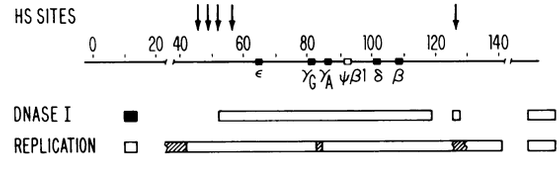

FIG. 5. Summary of the replication behavior, DNase I sensitivity, and location of DNase I-hypersensitive (HS) sites in the human β-globin gene locus. Black boxes represent DNase I-resistant chromatin, and white areas represent DNase I-sensitive chromatin. Hatched regions represent regions of aberrantly replicating DNA (jagged edges represent approximate boundaries) and white regions in the bottom line represent early replicating DNA sequences. Only the locations of erythroid-specific, developmentally stable DNase I-hypersensitive sites are shown. See the text for details.

the two replication forks. Although it remains possible that specific sequences define the sites of termination of replication forks (36), experiments using SV40 deletion mutants suggest that specific DNA termination sequences are not required (35). Thus, it will be interesting to analyze the replication timing of the β-globin locus in naturally occurring mutants that contain deletions in and adjacent to the inter-γ-globin gene region (reviewed in ref. 9).

Another possible explanation is that these islands represent termination regions but that the replication fork moves unidirectionally, as suggested for ≈10% of replication bubbles (14). This would imply that a replication origin is located adjacent to a termination region. Thus far, however, we have not observed sequences adjacent to the asynchronously replicating regions that replicate significantly earlier than the majority of DNA in the locus. Differentiation between these models will require the determination of the directionality of fork movement.

It is also possible that these regions represent origins of replication. In this model, multiple abortive initiation events, due perhaps to an excess of an initiator-like protein (37), would result in continuous replication of these regions. If these sequences were replicating many times during a single S phase, one might expect both DNA strands to contain BrdUrd. However, if the time between reinitiations is greater than the length of the BrdUrd pulse, only one strand would contain BrdUrd.

The distance between these regions of continuously replicating DNA in the human β-globin-like gene cluster (about 45–50 kb apart) is in agreement with estimates of potential interorigin distances (8, 14, 34). If these regions do represent termination sites and the replication forks are moving bidirectionally, origins of DNA replication should be located approximately midway between adjacent islands, near the β-globin and ε-globin genes. Although the very early replication behavior of the β-globin gene region by slot-blot analysis (Fig. 2 and Table 2) supports this idea, we have not yet detected a similar region near the ε-globin gene (data not shown). It will be important to map precisely the location of origins and termination points for DNA replication in the α- and β-globin gene loci and to determine if changes in the patterns of origins used can influence the developmental regulation of these loci (7, 8).

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