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Replication of α and β globin DNA sequences occurs during early S phase in murine erythroleukemia cells

(DNA replication/cell cycle/globin genes/ribosomal RNA genes)

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ABSTRACT **Murine erythroleukemia cells (MELC) can be induced to express the characteristics of erythroid differentiation by a variety of agents. Previous studies indicate that an action of inducer, occurring during early S phase, may be critical to the expression of differentiated characteristics such as initiation of accumulation of newly synthesized α and β globin mRNAs. In this investigation, the time of replication of globin genes in MELC was studied. DNA was isolated from synchronous populations of cells obtained by centrifugal elutriation. Newly replicated DNA sequences were prepared from synchronized cells cultured for 1½ hr with 5-bromodeoxyuridine; bromodeoxyuridine-containing DNA was isolated by CsCl gradient centrifugation. By employing cloned probes for hybridization to newly synthesized DNA, it was found that α and β globin gene sequences are replicated early in S phase, while ribosomal RNA gene sequences are replicated to about the same extent in early, middle, and late S phases.**

Murine erythroleukemia cells (MELC) are induced to express a program of erythroid differentiation by culture with one of several agents (for review, see ref. 1). This program includes many biochemical functions characteristic of normal erythroid differentiation. Previous studies in this and other laboratories have suggested that initiation of this program of differentiation is cell cycle dependent. For example, Levy *et al.* (2) and McClintock and Papaconstantinou (3) demonstrated that inducer must be present through at least one S phase of the cell cycle to induce MELC to differentiate. Harrison (4) observed that MELC arrested in the cell cycle in the presence of inducer fail to differentiate. Recently, Gambari *et al.* (5) provided evidence that inducer-mediated events during early S phase may be critical to the expression of certain characteristics of MELC differentiation, including α and β globin mRNAs. Other studies suggested that the portion of the MELC genome that replicates early in S phase may play a role in regulating cell growth as well as the expression of erythroid characteristics (6, 7). More generally, DNA replication has been implicated in changes in gene expression in prokaryotes (8), eukaryotes (5–7, 9–15), bacteriophages (16), and animal viruses (17); the mechanisms that govern these changes are unknown.

Previous studies suggest that the S phase in eukaryotic cells is temporally ordered with respect to replication of certain specific DNA sequences. For example, by using 5-bromodeoxyuridine (BrdUrd) density labeling to isolate newly synthesized DNA from synchronous cell populations and appropriate radioactive DNA probes, it was determined that ribosomal RNA genes replicate throughout S phase in HeLa and SV3T3 cells (9) and late in S phase in Chinese hamster cells (10). The replication of HeLa 5S RNA genes, studied by similar techniques, occurs primarily in early S phase (11). Heterochromatin and satellite DNA sequences (considered to be transcriptionally inactive) replicate late in S phase (12–15). The relationship between sequences replicated early in S phase and transcription is unknown.

The present studies were initiated to define the pattern of replication of specific genes in MELC. Synchronous populations of cells were obtained by centrifugal elutriation, which separates cells into relatively discrete populations with respect to selected phases of the cell cycle (5, 18). This technique avoids the use of cell cycle inhibiting agents to create synchrony; such agents alter cell metabolism and perturb the cell cycle. Newly synthesized DNA was isolated by incorporating BrdUrd into DNA and separating BrdUrd-DNA from nonsubstituted DNA by centrifugation to equilibrium in CsCl gradients (19). The results indicate that α and β globin gene sequences are predominantly located in regions of the genome that replicate early in S phase in logarithmically growing MELC cells. Ribosomal RNA genes are found to replicate in early, middle, and late S phase.

METHODS

Preparation of Populations of Cells Synchronized with Respect to Cell Cycle Phase. MELC strain DS19 was isolated and maintained as described (20). At initiation of the culture (10^5 cells per ml), [^{3}H]thymidine (0.1 μCi/ml, 20 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was added to make the DNA radioactive. After 36 hr of culture, when the cell density was approximately 5×10^5 cells per ml, BrdUrd (10 μg/ml) and additional [^{3}H]thymidine (0.4 μCi/ml) were added to the medium and culture was continued for 1½ hr. In all subsequent steps, exposure to light was avoided. Approximately 5×10^8 cells were recovered from the culture media by centrifugation at 800 × *g*, washed twice with Hanks' buffered salt solution, resuspended in 20 ml of the same buffer, and separated into populations of cells corresponding to different phases of the cell cycle by centrifugal elutriation (5, 18). Ten to 15 fractions (containing 1–3 $\times 10^7$ cells) were obtained (Fig. 1). Aliquots (5×10^5 cells) from each fraction were removed, stained with propidium diiodide, and analyzed for DNA content by flow microfluorometry (5, 18). DNA was prepared from the remaining cells in fractions corresponding to early, middle, and late S phase as described below.

Isolation of Newly Synthesized DNA. Cells were pelleted by centrifugation for 10 min at 800 × *g* at 4°C and nuclei were prepared by lysing the cells in 0.2% Triton X-100/0.6 M sucrose/10 mM Tris·HCl, pH 7.5/4 mM $MgCl_2$ (21). Nuclei were recovered by centrifugation at 800 × *g* for 10 min and the nu-

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Abbreviations: MELC, murine erythroleukemia cells; H and L, heavy (bromodeoxyuridine-containing) and light chains of DNA.

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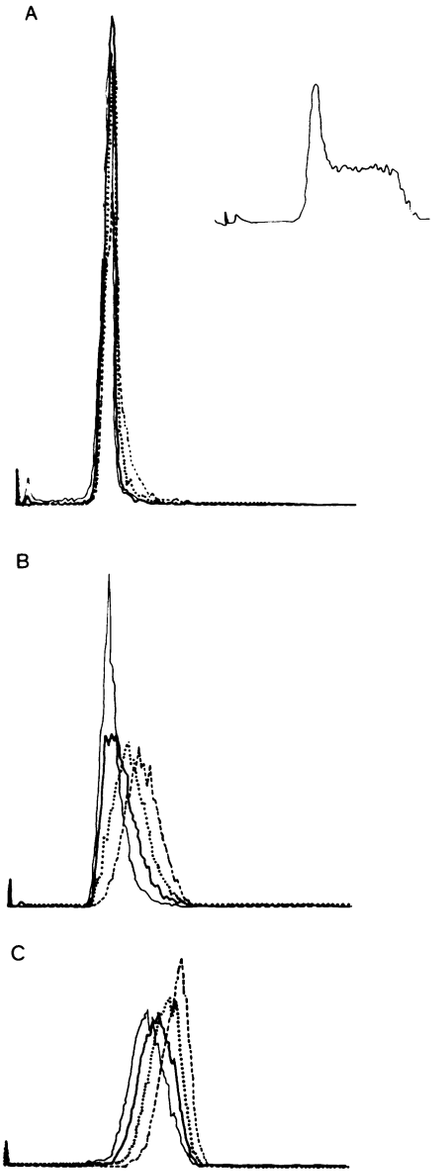

FIG. 1. Pattern of distribution of cellular DNA content of MELC fractionated according to size by centrifugal elutriation. Relative DNA content (abscissa) was determined as relative fluorescence intensity of 5×10^5 cells stained with propidium diiodide. The *Inset* represents the distribution of unfractionated MELC after 36 hr in culture plus 1½ hr in culture with BrdUrd (10 μg/ml) and [^{3}H]thymidine (0.4 μCi/ml). G_1 cells (*A*, peak of solid dark line) are characterized by a nominal 2C DNA content; G_2 cells are characterized by twice this DNA content (designated 4C, *C*, peak of far right broken line). The 12 fractions recovered represent fractions of cells predominately in G_1 progressing through early S (*A*), middle S (*B*), and late S–G_2 (*C*). The curves are displayed in three panels (*A*, *B*, and *C*) for convenience in illustrating these results.

clear pellet was suspended in 2 ml of 100 mM NaCl/50 mM EDTA and lysed by addition of Sarkosyl (CIBA–Geigy) to a final concentration of 0.5%. The DNA in the nuclear lysate was sheared by successive passages through 18-, 20-, and 23-gauge needles (three times each). RNase A (Worthington) was added to 50 μg/ml and the mixture was incubated for 30 min at 37°C, after which proteinase K (Boehringer Mannheim) was added to 100 μg/ml and the incubation was continued for an additional 4 hr. Newly synthesized DNA was separated from parental DNA in nuclear lysates by centrifugation of the nuclear lysate in CsCl to minimize loss of DNA.

In experiments designed to separate newly replicated double-stranded DNA of which one strand has incorporated BrdUrd (HL-DNA) from unsubstituted DNA (LL-DNA) (19), neutral CsCl (Bethesda Research Laboratories, Rockville, MD, optical grade) dissolved in 15 mM NaCl/15 mM EDTA was added to the nuclear lysate to a final refractive index of 1.402. In experiments designed to separate newly replicated single-stranded DNA containing BrdUrd (H-DNA) from L-DNA (9), alkaline CsCl gradients were employed, prepared by adding NaOH to the nuclear lysate to 0.15 M, allowing the mixture to stand for 15 min at room temperature, and then adding CsCl (dissolved in 50 mM NaOH/3 mM EDTA) to a final refractive index of 1.408. The mixture was centrifuged at 35,000 rpm for 60–72 hr in a Beckman Ti 65 or Ti 70.1 rotor at 15°C (neutral) or 25°C (alkaline gradients). After centrifugation to equilibrium, approximately 50 fractions were recovered from the bottom of each centrifuge tube. An aliquot of every fourth fraction was taken for determination of refractive index, and an aliquot of every other fraction was assayed for radioactivity in 10 ml of Aquasol (New England Nuclear). Water (0.5 ml) was added to every other fraction and the absorbance at 260 nm (A_{260}) was determined. Gradient fractions containing BrdUrd-substituted DNA and those containing parental DNA were pooled.

DNA Probes Used for Hybridization. The α and β globin cDNA plasmids, pCR1αm10 and pCR1βM9, were a generous gift of B. Mach and were grown and prepared as described (22). A 1.8-kilobase fragment containing globin gene sequences was isolated after digestion with *Hha* I (New England BioLabs) and centrifugation at 4°C in a 5–20% neutral sucrose gradient in a Beckman SW 41 rotor at 35,000 rpm for 20–24 hr (21). A λ*WES* bacteriophage clone containing mouse ribosomal RNA gene sequences, the generous gift of P. Leder, was grown and DNA was isolated as described by Tiemeier *et al.* (23). Purified phage DNA was cleaved with *Eco*RI (Boehringer Mannheim) and the 6.6-kilobase fragment containing the ribosomal RNA genomic insert was isolated from flanking phage DNA sequences by sucrose density centrifugation. DNA fragments of enzyme-digested plasmids and phage were monitored by electrophoresis in 1% agarose gels (21). DNA probes were labeled with ^{32}P by nick-translation according to Weinstock *et al.* (24). All procedures employing recombinant DNA were performed according to National Institutes of Health guidelines.

Hybridization of DNA. DNA samples, consisting of equal amounts of BrdUrd-substituted or unsubstituted DNA (5–20 μg), were applied to nitrocellulose filters (Schleicher & Schuell BA85, 24 or 47 mm) in 900 mM NaCl/90 mM sodium citrate (25). Filters were dried, baked at 80°C for 4–8 hr, prehybridized, and hybridized in 50% (vol/vol) formamide and 10% dextran sulfate for 16–24 hr at 42°C with a ^{32}P radioactive DNA probe at 5–10 ng/ml (specific activity $0.4–1 \times 10^9$ cpm/μg). The following cloned DNA probes were used: α globin cDNA (22), β globin cDNA (22), and genomic ribosomal DNA (23) (see above).

Filters containing hybridized DNA were washed according to Wahl *et al.* (26) except that the final two 15-min washes were in 15 mM NaCl/1.5 μM sodium citrate at 52°C, then dried under a heat lamp, and their radioactivities were measured in 5 ml of a toluene-based scintillation fluor, using a ^{3}H/^{32}P setting. Blank values (usually less than 0.01% of input cpm) were subtracted from radioactivity on hybridization filters. Loss of DNA from the filters was monitored by measuring the ^{3}H cpm remaining after washing.

RESULTS

In preliminary experiments, increasing amounts of MELC DNA were immobilized on nitrocellulose filters and hybridized

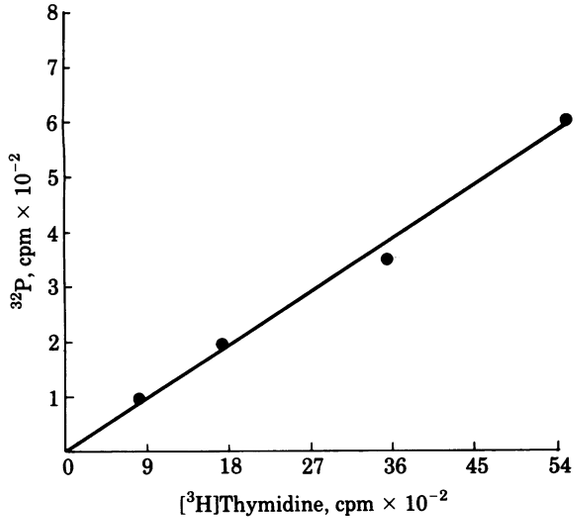

FIG. 2. Hybridization of increasing amounts of [^{3}H]thymidine-labeled MELC DNA (4–24 μg) to β globin [^{32}P]cDNA. Nitrocellulose filters were prepared, prehybridized, hybridized, and washed as described in the text.

to α and β globin [^{32}P]DNA probes. The radioactivity bound to the filters increased linearly with increasing amounts of MELC DNA applied to the filter (Fig. 2). This indicates that the hybridization method can be used to quantitate the amount of globin gene in newly synthesized DNA obtained from cells in different phases of the cell cycle.

MELC cultured with BrdUrd for 1½ hr (approximately one-fourth of the S phase) were separated by elutriation into fractions corresponding to different phases of the cell cycle; 10–15 fractions, each containing approximately 10^{7} cells, were recovered (Fig. 1). Newly synthesized, BrdUrd-DNA (either HL-DNA on neutral CsCl gradients or H-DNA alkaline gradients) was separated from the parental DNA of cells in elutriation fractions corresponding to early, middle, and late S DNA (Figs. 3 and 4). With double-stranded DNA isolated by neutral CsCl gradients, α globin [^{32}P]DNA preferentially hybridized to BrdUrd DNA (HL-DNA) prepared from early S phase cells (Table 1). α globin [^{32}P]DNA preferentially hybridized to LL-DNA (without BrdUrd) from middle and late S phase cells. These data suggest that the DNA sequences hybridizing to α globin [^{32}P]DNA are among the DNA regions replicating in early S phase of the cell cycle.

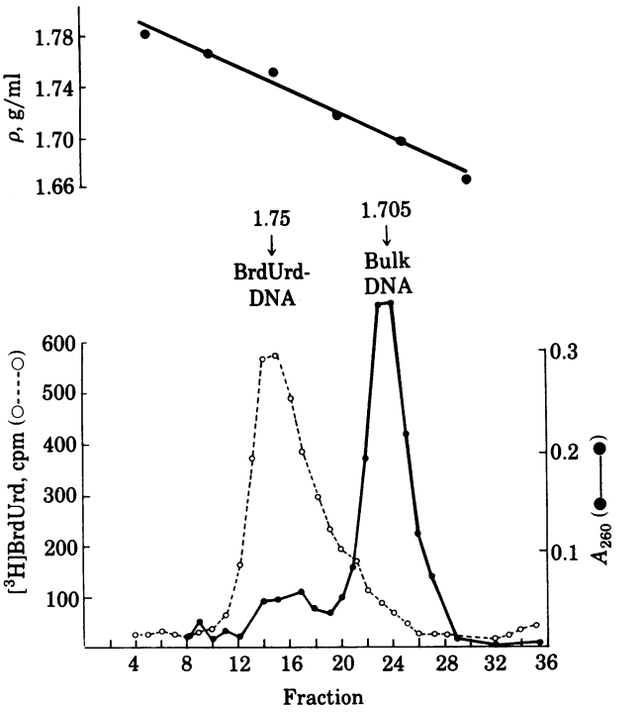

FIG. 3. Separation of BrdUrd-substituted MELC DNA from non-substituted DNA by neutral CsCl gradient centrifugation. Exponentially growing MELC cells were incubated with BrdUrd (10 μg/ml) and [^{3}H]BrdUrd (0.4 μCi/ml) for 1½ hr and nuclear lysates were prepared and centrifuged in neutral CsCl to separate double-stranded DNA containing BrdUrd-DNA (HL) from unsubstituted bulk DNA (LL).

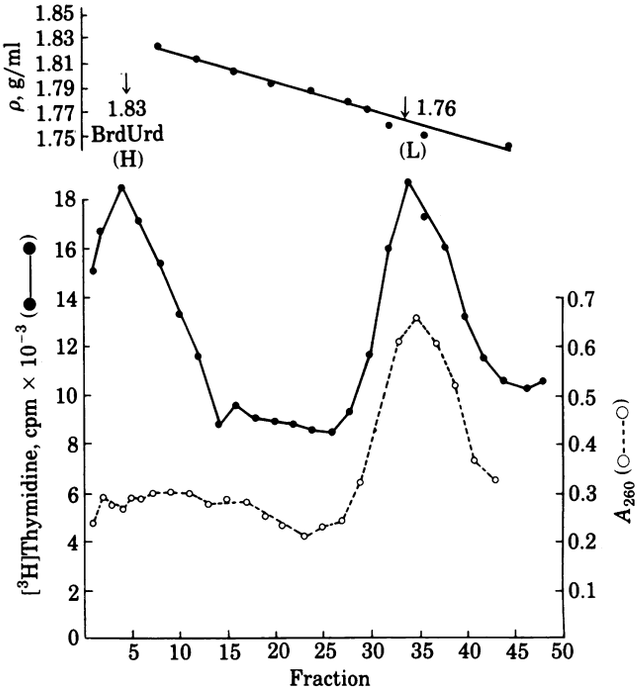

FIG. 4. Separation of single-stranded BrdUrd-containing DNA (H) from unsubstituted DNA (L) by centrifugation in alkaline CsCl gradients.

In subsequent experiments, the replication of DNA sequences complementary to α and β globin [^{32}P]DNA and to DNA sequences for ribosomal RNA was examined in preparations of single-stranded DNA. Alkaline CsCl gradients were used to separate newly synthesized single-stranded BrdUrd-DNA (H-DNA) from the non-BrdUrd-DNA (L-DNA) (Fig. 4). The α and β globin structural gene sequences were enriched in regions of the genome that replicate early in S phase (Table 2). Ribosomal RNA gene sequences were approximately equally enriched in BrdUrd DNA prepared from cells in early, middle, and late S phase.

DISCUSSION

The present study shows that α and β globin sequences are replicated during early S phase in exponentially growing MELC. This was observed in experiments in which newly replicated DNA was separated from total DNA by employing BrdUrd incorporation into newly synthesized DNA and separation of the denser BrdUrd-DNA by density gradient centrifugation in CsCl. While α and β globin gene sequences were associated with DNA replicating during early S phase, ribosomal RNA gene sequences were associated with DNA replicating in the

Table 1. Hybridization of α globin [^{32}P]cDNA with BrdUrd-DNA (HL-DNA) and BrdUrd-free DNA (LL-DNA) prepared from cells predominately in early, middle, or late S phase of the cell cycle

S phase	^{32}P, cpm/10 μg* HL-DNA	LL-DNA	Ratio HL/LL†
Early	1607	645	2.5
Middle	799	1338	0.6
Late	505	1265	0.4

HL-DNA and LL-DNA were separated on neutral CsCl gradients.
* For each DNA sample, 10 μg was immobilized on nitrocellulose filters, prehybridized, hybridized, and washed.
† Ratio HL/LL is the ratio of (cpm/10 μg of HL-DNA)/(cpm/10 μg of LL-DNA).

early, middle, and late portions of the S phase.

Hybridization of cloned α and β globin cDNA to newly synthesized BrdUrd-DNA prepared from fractions of cells predominately in middle or late S phase was observed at a level 15–25% of that for BrdUrd-DNA isolated from cells predominately in early S phase. Among possible explanations for the low but distinct hybridization to middle and late S phase DNA is contamination of these cell fractions by cells in early S phase. Also, BrdUrd DNA could be contaminated with unsubstituted DNA, although this possibility should have been minimized under the denaturing conditions of the alkaline CsCl gradients. Alternatively, while both globin cDNA probes react primarily with the gene sequences active in mRNA transcription, namely, β major and β minor structural gene sequences with β cDNA probe and α_1 and α_2 structural gene sequences with α cDNA probe, there are DNA sequences in the region of these structural genes that hybridize with these probes and are apparently not transcribed (27–31). Under the present experimental conditions, over 95% of the hybridization of the cloned α and β cDNA probes was with DNA fragments corresponding in size to those containing globin structural sequences that are transcribed (data not shown).

Evidence that replication is an ordered event with respect to specific DNA gene sequences has been obtained in prokaryotes (such as *Escherichia coli*) in which it has been demonstrated that there is a unique origin of replication (32). Studies with yeast have provided evidence for a temporally ordered sequence of gene replication by pulsed mutagenesis with *N*-methylnitrosoguanidine (33). In eukaryotic cells in culture that were synchronized and labeled during one replication cycle with radioactive thymidine and in the subsequent replication cycle with BrdUrd, it was shown that sequences replicated early in one S phase tend to replicate early in the subsequent S phase (19, 34). Ribosomal RNA, 5S RNA, and satellite DNA sequences have been analyzed for time of replication during S phase, using BrdUrd substitution and CsCl gradients to separate newly synthesized DNA. Ribosomal RNA gene sequences replicate in late S phase in Chinese hamster cells (10), whereas in HeLa and 3T3 cells ribosomal RNA genes are reported to replicate throughout the S phase (9). In HeLa cells, 5S RNA gene sequences replicate early in S phase (11), whereas satellite DNA sequences replicate in late S phase in several types of cells (12, 13).

Table 2. Hybridization of α globin [^{32}P]cDNA, β globin [^{32}P]cDNA, and ribosomal RNA [^{32}P]DNA with single-stranded H-DNA (BrdUrd-DNA) prepared from cells predominately in early, middle, or late S phase of the cell cycle

S phase	Normalized DNA hybridization* α globin	β globin	Ribosomal
Early	100	100	100
Middle	58	40	95
Late	39	14	108

BrdUrd-DNA was separated from nonsubstituted single-stranded DNA (L-DNA) on alkaline (α and β globin) or neutral (ribosomal) CsCl gradients.
* Values represent the average of three determinations for β globin, two for α globin, and three for ribosomal RNA DNA. The cpm/μg of DNA was normalized to 100 for early S phase values, for each experiment, and the results are expressed as the average value. Actual cpm/μg of DNA for early S phase were: α, 140 and 170; β, 255, 160, and 97; and ribosomal, 1034, 298, and 1476.

Recently, the replication of single-copy DNA sequences in MELC has been investigated. Lo *et al.* (35) reported that globin gene sequences replicate predominately in middle S phase. Possible reasons for the discrepancy between our results and those obtained by Lo *et al.* (35) could include the use of different MELC strains, different conditions for culture of cells, and the use by Lo *et al.* of a chemical method to achieve cycle synchrony, which may perturb the pattern of DNA replication (36, 37).

Previous studies in this laboratory have suggested that inducer-mediated events occurring during G_1 or early S phase of the cell cycle are critical to the subsequent expression of globin genes and other characteristics of terminal erythroid cell differentiation (6). The present studies demonstrate that α and β globin genes, whose expressions are inducible in MELC, are replicated early in S phase. The significance of this correlation between replication of globin gene sequences and inducer effects during early S phase is not clear. Nontranscribed heterochromatic regions, such as satellite DNA and the inactive X chromosome, replicate late in S phase (12–15). It is possible that unique genes that are transcribed or have the potential of being transcribed are preferentially replicated early in S phase. There are several reports that suggest that genes controlling terminal cell division and expression of differentiated characteristics are replicated early in S phase in MELC (7, 35), HeLa (19), hamster (BHK) (38), chicken myoblast (39), and mouse L cells (40).

While this manuscript was in preparation, C. Schildkraut kindly forwarded to us a copy of his paper reporting results similar to those presented here, concerning the replication of α globin gene sequences in MELC (41).

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