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Coordinate replication of members of the multigene family of core and H1 human histone genes

(cell cycle/centrifugal elutriation/human erythroleukemia cell)

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ABSTRACT **Cells of the K562 human erythroleukemia cell line were obtained in different stages of the cell cycle by centrifugal elutriation. The cells had been previously labeled for 2 hr with BrdUrd so that BrdUrd–DNA synthesized during four different selected intervals of the S phase could be isolated. This DNA was used to determine the temporal replication during S phase of *Eco*RI segments containing histone genes. Cloned human genomic segments containing the core histone genes (*H2A*, *H2B*, *H3*, and *H4*), *H2A* and *H2B* pseudogenes, and the *H1* gene were prepared. The genomic inserts were excised from these plasmids, nick-translated, and used as hybridization probes. The results with different probes compared on the same and on independently prepared DBM-paper transfers indicate that all of these histone genes replicate during the first half of the S phase. These genes were not among the earliest to replicate in the K562 cell line. Similar studies were carried out with HeLa cells in which *Eco*RI segments containing the *H4* histone and *H2A* and *H2B* pseudogenes were found to replicate during the first half of the S phase. These histone genes replicate during the interval of the S phase when histone mRNA appears in the cytoplasm at the maximal rate. The possible relationship between these events is discussed.**

It has been well established that expression of human histone genes occurs during the S phase of the cell cycle. The synthesis of histone proteins has been shown to be temporally and functionally related (coordinated with) to DNA replication (for review, see ref. 1). The rate of appearance of core and H1 histone mRNAs in the cytoplasm is at least 10-fold higher in S phase than in G1 and G2 phases. Several recent reports indicate that there may be post-transcriptional as well as transcriptional components to the regulation of cytoplasmic histone mRNA levels in eukaryotic cells (2–6). The stability of histone mRNAs throughout S phase and the selective destabilization of histone mRNAs as cells exit S phase appear to be mediated almost entirely post-transcriptionally, perhaps by autogenous control (7). These observations support the interpretation that the cellular levels of histone mRNAs determine the extent of histone protein synthesis.

To address possible mechanisms operative in the onset of histone gene expression, we have determined the period during S phase when both core and *H1* genes are replicated. Our rationale for pursuing this approach is described briefly. In mammalian cells, the order of replication during the S phase of a large number of DNA sequences is genetically determined and has been measured. These include sequences present in multiple copies (e.g., see ref. 8) as well as DNA segments including and flanking single copy genes (e.g., see refs. 9–12 and references included therein). In yeast (3) and in HeLa cells (4), the rate of appearance of histone mRNA exhibits a periodic increase in the cell cycle and the rate is highest during the first half of the S phase. A model has been presented (3) in which changes in chromatin structure activate mRNA synthesis. It was postulated that the return of chromatin to its original structure after DNA replication would result in the cessation of mRNA synthesis.

We report here that the seven human histone genes we have examined are replicated during the first half of the S phase in both K562 human erythroleukemia cells studied by centrifugal elutriation and HeLa S3 cells synchronized by a double thymidine block. The functional implications of the observed coordinate replication and periodic expression of human histone genes are discussed.

MATERIALS AND METHODS

Cell Culture and Fractionation of S Phase Cells by Centrifugal Elutriation. The human erythroleukemia cell line K562, clone-4 (kindly provided by D. Mager and O. Smithies) was grown in 175-cm^2 tissue culture flasks (Falcon 3028) in minimum essential medium, supplemented with nonessential amino acids, glutamine, and 15% fetal calf serum. Under these conditions, the cell line had a doubling time of 16–18 hr. For the present study, exponentially growing cells were pulsed with BrdUrd (20 μg/ml) for 2.25 hr and cells were harvested as described (10). The technique of centrifugal elutriation, which separates cells on the basis of size and DNA content, was used to fractionate S phase cells as described (13). The cellular DNA content distributions of aliquots from each elutriated fraction were determined by flow microfluorometric (FMF) analysis. Fractions with modal per cell DNA contents in the approximate ranges of 2.0–2.1 C, 2.1–2.7 C, 2.9–3.4 C, and 3.7–4.0 C (where C is haploid DNA content) were pooled separately, giving rise to four different cell classes (I–IV). The cells of each class synthesized DNA during different but somewhat overlapping intervals of the S phase.

Growth and Synchronization of HeLa 53 Cells. HeLa S3 cells were grown in suspension culture in Joklik's modified Eagle's minimal essential medium supplemented with 7% calf serum. Under these conditions, the cells have a doubling time of 18–20 hr. Exponentially growing cells were synchronized by two cycles of 2 mM thymidine block as described (14). Synchronized cells were pulse-labeled with BrdUrd (10 μg/ml) for 2-hr intervals at various times throughout S phase (0–2, 2–4, 4–6, or 6–8 hr after release from the second thymidine block). Prior to hourly intervals after release from the second thymidine block, duplicate 2-ml cell aliquots at a concentration of 5×10^5 cells per ml were incubated for 30 min with 0.2 μCi (1 Ci = 37 GBq) of [^{14}C]thymidine at 37°C. Thymidine-labeled cells were harvested by centrifugation at 600 $\times$ *g* for 3 min and washed with Earle's balanced salt solution. Cells were resuspended in 10% trichloroacetic acid and after 15 min at 4°C, acid-precipitable material was collected on Millipore HA filters that were washed with 30 ml of 10%

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Abbreviation: FMF, flow microfluorometric.

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trichloroacetic acid; incorporated radioactivity was assayed by liquid scintillation spectrometry.

Preparation and Isolation of BrdUrd–DNA. The procedure used for isolating DNA was as described (10) but with the following changes. Cells were harvested and lysed in the presence of 0.15 M NaCl/0.015 M EDTA/50 mM Tris·HCl, pH 9.0/0.1% sodium dodecyl sulfate and 36 μg of proteinase K per ml for 60 min at 60°C–65°C. Purification was accomplished by extracting with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Pancreatic ribonuclease at 100 μg/ml (Worthington) and ribonuclease T1 at 100 units/ml (Worthington) were used to hydrolyze RNA. Finally, DNA was extracted once more with phenol/chloroform/isoamyl alcohol before cleaving it to completion with *Eco*RI. Preparative ultracentrifugation was used to separate the BrdUrd-substituted DNA from the unsubstituted DNA and the purity of BrdUrd–DNA was demonstrated by isopycnic centrifugation. The details of these methods are described elsewhere (9–11, 13).

Gel Electrophoresis and DNA Hybridizations. Equal amounts (4 μg) of *Eco*RI-cleaved BrdUrd–DNA from each class (I–IV) of S phase cells was electrophoresed in 0.8% agarose for 16–18 hr at 1.0 V/cm and transferred to DBM-paper by a modified method (15). After prehybridization for 4–6 hr, the DBM-paper transfers were hybridized with 5–10 $\times 10^6$ cpm of nick-translated histone probes in the presence of 10% dextran sulfate/50% formamide/(0.18 M NaCl/0.01 M Na phosphate/1 mM EDTA)/(0.02% bovine serum albumin/0.02% polyvinyl pyrrolidone/0.02% Ficoll) (M_r, ≈400,000) for 17–20 hr at 37°C. The transfer was then washed in 0.36 M NaCl/0.02 M Na phosphate/2 mM EDTA/0.1% $NaDodSO_4$ at 37°C and then in 0.018 M NaCl/0.001 M Na phosphate/0.1 mM EDTA/0.1% $NaDodSO_4$ at 52°C. The background counts were monitored using a Geiger counter, and, if necessary, the transfers were washed at a higher stringency at 65°C in low salt concentration. Finally, the transfers were exposed to Kodak XAR-5 film and a Dupont Lightning Plus intensifying screen for 2–4 days at −70°C. The relative intensities of the signals were calculated by microdensitometric analyses to determine the replication of histone genes in specific intervals of S phase.

Characteristics of DNA Probes. The isolation and characterization of λ Charon 4A recombinants containing human histone genes have been described (1, 16, 17). *Eco*RI/*Eco*RI or *Eco*RI/*Hin*dIII restriction fragments of the recombinant DNAs have been subcloned into the plasmid pBR322 (see Fig. 3), transformed into *Escherichia coli* strain HB101, and characterized by hybrid selection *in vivo* translation and partial sequencing. These plasmid DNAs were isolated by the sarkosyl lysis procedure and purified further by cesium chloride buoyant density gradient centrifugation as described (18). The cDNA probe pBFA28 (19) used to identify the *Eco*RI segments containing human complement protein factor B was kindly provided by D. Woods and H. Colten. The genomic probe used to identify the *Eco*RI segments containing human immunoglobulin heavy chain variable region V_H(I) genes was kindly provided by S. Korsmeyer and J. Ravetch.

RESULTS

Subfractionation of S Phase Cells. Populations of cells containing BrdUrd–DNA replicated during four different intervals (2.25 hr) of S were obtained for K562 cells by using centrifugal elutriation. Twenty K562 cell fractions in different stages of the S phase were pooled to produce four classes of cells that had synthesized BrdUrd–DNA during four different intervals of the S phase. A representative FMF profile for each size class is shown in Fig. 1.

In HeLa cells, DNA replicated during different intervals of S was obtained by labeling with BrdUrd after release from a double-thymidine block. The subsequent progression of cells through the S phase was observed by following the uptake of [^{14}C]thymidine into DNA (Fig. 2). Immediately after release, an aliquot of the culture was grown in the presence of BrdUrd for 2 hr. At consecutive 2-hr intervals, aliquots from the synchronized culture were removed and grown in the presence of BrdUrd during four different intervals of S, and DNA was prepared. After digestion with *Eco*RI, the BrdUrd–DNA was separated from the unlabeled DNA as described (10).

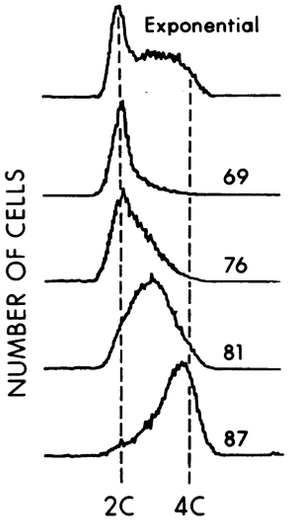

FIG. 1. FMF analysis of selected fractions after centrifugal elutriation of K562 cells. The FMF profiles are histograms giving the number of cells (ordinate) with the DNA content (measured by fluorescence) indicated on the abscissa. C is the haploid DNA content of K562 cells in G_1 phase. The numbers to the right of each profile (fraction number) are proportional to the flow rate of the Eagle's medium used for elutriation of that fraction. The exponentially growing K562 cells whose FMF profile is shown at the top were fractionated into 27 different populations of increasing size. Each fraction contained a population of cells in which BrdUrd–DNA was synthesized during a time interval whose mean was later in S than the mean of the corresponding time interval in each preceding fraction of smaller sizes. Successive fractions were pooled to produce four classes of cells in which BrdUrd–DNA was synthesized during a different selected interval of S phase. A representative profile from each of the four cell classes is shown above.

Detection of Segments Containing Histone Genes. All of the probes used are described in Fig. 3. We used five DNA segments from four cloned clusters of human core histone genes that have been previously identified and characterized. These included one *H2A* pseudogene, an *H2B* pseudogene, two different *H3* genes, and two different *H4* genes. In addition, an H1 DNA segment derived from a genomic DNA sequence containing one each of the core and *H1* histone genes was used as a probe. The restriction maps (Fig. 3) show the sizes of the *Eco*RI segments detected by each of these probes.

Time of Replication of Histone Gene Segments. For each cell line, the BrdUrd–DNA synthesized during each of the four intervals of S was fractionated electrophoretically on agarose gels and transferred to DBM-paper. For each DBM-paper transfer, a segment of cloned genomic human histone DNA was nick-translated and used as a radioactive probe. The concentration of segment replicated during each S phase interval was determined by hybridization and microdensitometry of autoradiograms. Representative autoradiograms are shown in Fig. 4, and the relative amount of each *Eco*RI segment replicated during each S phase interval was quantitated and is presented in Fig. 5.

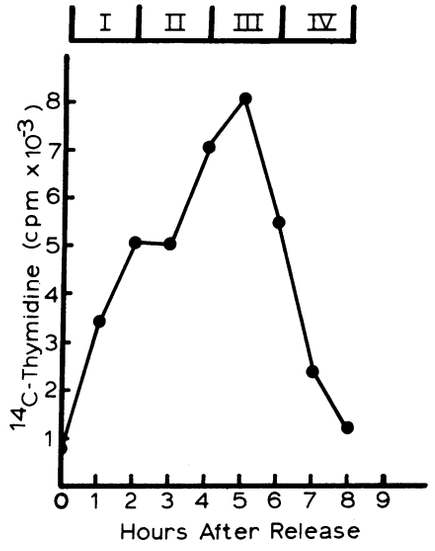

FIG. 2. DNA synthesis in HeLa S3 cells after release from two cycles of 2 mM thymidine treatment. Cells were synchronized by treatment with two cycles of 2 mM thymidine as described (14). Synchrony was monitored by pulse-labeling cells for 30 min with [^{14}C]thymidine every hour after release from the second thymidine block and assaying 10% trichloroacetic acid-precipitable radioactivity.

The six different *Eco*RI segments containing histone genes replicated predominantly during the first two intervals of the S phase in both the K562 and HeLa cell lines. In most instances, the histone genes were present in highest concentration during the second S phase interval (BrdUrd–DNA from cells of class II). If a particular histone gene segment replicated at the same time in each cell in the culture, the DNA content of the K562 nucleus at this time would be about 2.3 C. This indicates that in K562 cells these histone genes do not replicate immediately upon entrance of the cells into the S phase. Similar results were obtained in HeLa cells, although the replication of the histone genes appeared to be somewhat earlier.

To demonstrate that complete transfer of all four DNA samples occurred and that the DNA was available for hybridization with radioactive probes to single copy DNA, the replication times of several other genes were measured. The DBM-paper transfers used for these control studies contained the same BrdUrd–DNA samples replicated during the four S phase intervals. Both very early and very late replicating DNA segments were observed. The human factor B complement gene replicated predominantly during the first interval of S phase and a human heavy chain variable region gene replicated during the latest interval of S phase (Table 1). These results demonstrate our ability to detect genes replicating during the first and last intervals of S phase. Each DBM-paper transfer was hybridized with several different probes both individually and combined. In addition, similar

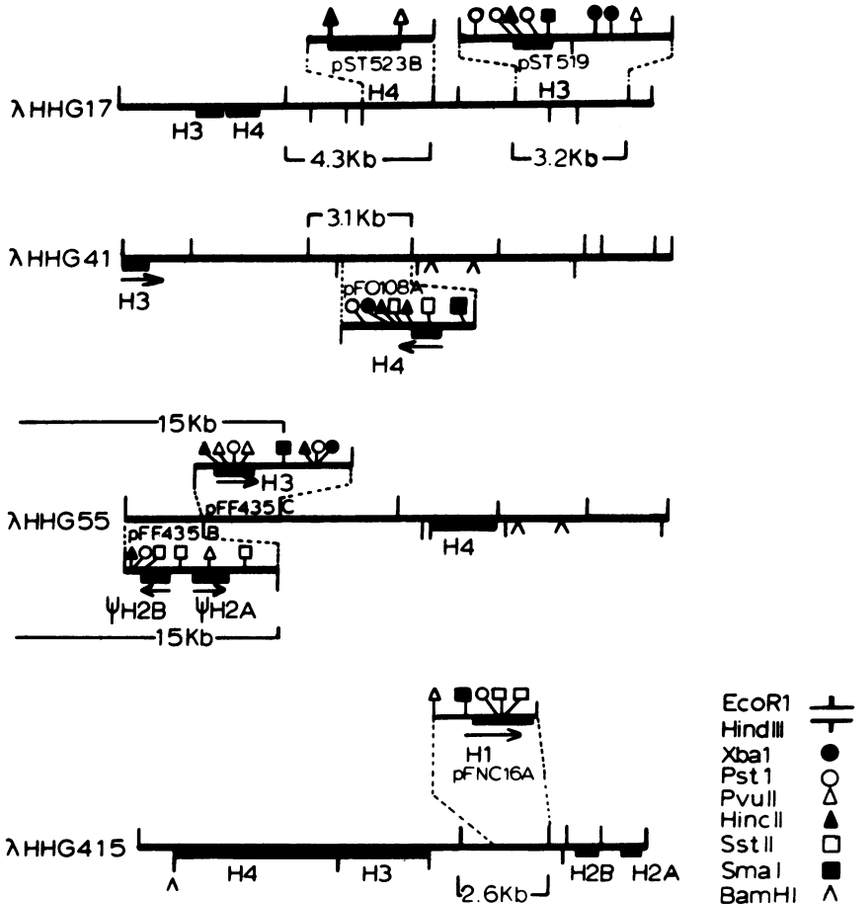

FIG. 3. Organization of histone coding sequences in four human genomic DNA segments containing histone genes. The organization of human genomic DNA segments from which hybridization probes for analysis of human histone gene replication were derived are shown. Illustrated in expanded form are the fine structure maps of the subcloned sequences used as hybridization probes. Direction of transcription for the histone genes is indicated by solid arrows, pseudogenes are designated, and sizes of *Eco*RI-generated genomic DNA segments that hybridize to the various histone gene probes are indicated by brackets. Note that *Eco*RI-digested genomic DNA segment hybridizing to pFF435B and pFF435C extends beyond the artificial *Eco*RI site at the left end of the λHHG55 insert.

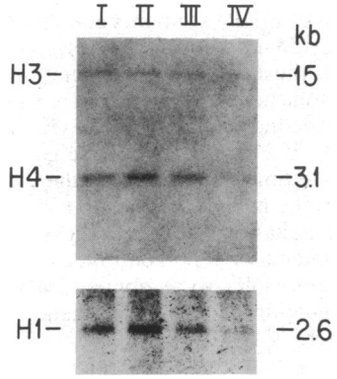

FIG. 4. Autoradiograms of *Eco*RI segments containing histone genes of K562 cells after hybridization with ^{32}P-labeled probes. *Eco*RI-cleaved BrdUrd–DNA replicated during the four different intervals of S phase was prepared from K562 cells and separated according to size by agarose gel electrophoresis. After transfer to DBM-paper, the BrdUrd–DNA was hybridized with ^{32}P-labeled nick-translated probes. The relative concentrations of *Eco*RI segments replicated during each interval of S phase were obtained from densitometric analysis of the autoradiograms shown above. These results are quantitated in Fig. 5. (*Upper*) Mixture of two nick-translated probes (pFF435C and pFO108A) was used to detect the *Eco*RI segments containing the *H3* and *H4* histone genes. (*Lower*) pFNC16A probe was used to detect the *Eco*RI segment containing the *H1* histone gene. kb, Kilobases.

results were obtained when the same probe was used on duplicate DBM-paper transfers (Fig. 4).

We conclude that histone genes that produce different RNA transcripts replicate at similar times during the S phase in human cells.

DISCUSSION

We have shown that in two cell lines human histone genes replicate predominantly during the first half of the S phase, although they are not the earliest genes to replicate. Two entirely different techniques were used to obtain DNA replicated at different times during S phase. For both the HeLa and K562 cell lines, other genes were observed to replicate earlier during S phase than the histone genes. The temporal order of replication of members of other multigene families has been examined (9–11). Globin genes have been shown to replicate early during S phase; however, one α-globin pseudogene is late replicating (11). This pseudogene has a different chromosomal location from the other members of the α-globin gene family.

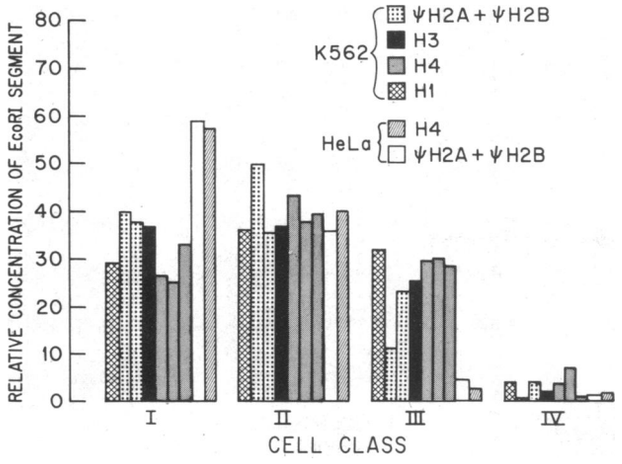

FIG. 5. Temporal replication of core and *H1* histone genes in K562 and HeLa cells. The relative concentrations of *Eco*RI segments of BrdUrd–DNA containing core and *H1* histone genes were determined by densitometric analysis of autoradiograms such as those shown in Fig. 4. The relative concentrations (normalized to 100) are compared in BrdUrd–DNA isolated from four different cell classes, each representing different but somewhat overlapping intervals of the S phase. The probes used detected *Eco*RI segments containing individual members (see Fig. 3) of subfamilies of histone genes. One *Eco*RI segment contained *H2A* and *H2B* pseudogenes (see Fig. 3). Two different probes were used to detect the H4 segments. The first set of bars shows the results using pST523B. The second and third sets are the results using pFO108A.

Table 1. Replication of complement protein factor B and heavy chain variable region genes in K562 and HeLa cells

	Size of *Eco*RI segment, kb	Relative concentration of segments in BrdUrd–DNA isolated from cell class			
		I	II	III	IV
K562 cells					
Human complement	6.0	68.0	27.1	3.9	0.9
protein factor B	1.6	75.9	17.1	6.3	0.7
	0.8	61.1	27.7	9.6	1.5
V_H(I)	3.2 + 3.4	3.8	20.2	26.9	49.1
HeLa cells					
Human complement	6.0	78.3	16.6	2.9	2.2
protein factor B	1.6	90.3	3.7	3.1	2.8
	0.8	88.7	6.0	4.1	1.2
V_H(I)	3.2 + 3.4	2	2.5	43	52.5

V_H(I), human immunoglobulin heavy chain variable region, type I; kb, kilobases.

The probes that we have used detected four different gene clusters in at least two different chromosomal locations (20). Adjacent *H2A* and *H2B* pseudogene sequences that are located on chromosome 1 replicate at a similar time to functional *H3* and *H4* histone genes in a structurally similar histone cluster located on the same chromosome. We also studied an *H1* gene that resides on a different chromosome. The *Eco*RI segment containing this gene replicated at a time similar to the core histone genes. The genomic segments we have examined contain only a small number of the more than 50 different human histone genes. We do not preclude the possibility that some of the histone genes whose temporal replication we have not yet examined will be late replicating.

Evidence has been presented to show that the maximal rate of appearance of labeled core histone mRNA occurs during the first third of the S phase in yeast cells (3) and in HeLa cells (4). The replication of human histone genes early during S phase, at the time maximal appearance of histone mRNA occurs, suggests the possibility of a functional relationship. Since the histone genes studied here are not the earliest sequences to be replicated, they are not likely to be located within a few kilobases of replication origins that are the earliest to be activated during the S phase. Thus, our results imply that replication origins of this type are not a requirement for the periodic cell cycle appearance of core and H1 histones in these human cell lines. This does not preclude the possibility that origins of replication that are activated after the initiation of the S phase play a role.

It has been shown (4) that in HeLa cells synchronized by double-thymidine block, the rate of appearance in the cytoplasm of the core histone mRNAs is maximal during the first third of the S phase. In fact, core histone mRNA appears in the cytoplasm before the histone genes replicate. A similar observation was made in yeast (3). Synthesis of histone mRNAs in HeLa cells predominantly during the initial segment of S phase is also supported by *in vitro* nuclear transcription studies (unpublished observations). The rate of appearance of the core mRNAs decreases significantly at

about the time the histone genes replicate and before the second half of the S phase. Based on the above observations, we suggest that the replication of the histone genes may play a role in the periodic expression of the core and *H1* histone genes, perhaps by mediating a change in the structural properties of the genes and/or in their flanking sequences.

The human histone genes are a multigene family, the majority being expressed in a cell cycle periodic manner. However, histone proteins have been described that are synthesized independently of DNA synthesis (21, 22). Our studies have focused on histone genes expressed during S phase. Establishing the replication interval of these human histone genes that are expressed independently of DNA synthesis may provide further insight into the functional implications of the relationship between histone gene replication and expression.

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