Rate of Replication of the Murine Immunoglobulin Heavy-Chain Locus: Evidence that the Region Is Part of a Single Replicon

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We measured the temporal order of replication of *EcoRI* segments from the murine immunoglobulin heavy-chain constant region (IgC_H) gene cluster, including the joining (J) and diversity (D) loci and encompassing approximately 300 kilobases. The relative concentrations of *EcoRI* segments in bromouracil-labeled DNA that replicated during selected intervals of the S phase in Friend virus-transformed murine erythroleukemia (MEL) cells were measured. From these results, we calculated the nuclear DNA content (C value; the haploid DNA content of a cell in the G1 phase of the cell cycle) at the time each segment replicated during the S phase. We observed that IgC_H genes replicate in the following order: α , ϵ , γ 2a, γ 2b, γ 1, γ 3, δ , and μ , followed by the J and D segments. The C value at which each segment replicates increased as a linear function of its distance from C α . The average rate of DNA replication in the IgC_H gene cluster was determined from these data to be 1.7 to 1.9 kilobases/min, similar to the rate measured for mammalian replicons by autoradiography and electron microscopy (for a review, see H. J. Edenberg and J. A. Huberman, *Annu. Rev. Genet.* 9:245-284, 1975, and R. G. Martin, *Adv. Cancer Res.* 34:1-55, 1981). Similar results were obtained with other murine non-B cell lines, including a fibroblast cell line (L60T) and a hepatoma cell line (Hepa 1.6). In contrast, we observed that IgC_H segments in a B-cell plasmacytoma (MPC11) and two Abelson murine leukemia virus-transformed pre-B cell lines (22D6 and 300-19P) replicated as early as (300-19P) or earlier than (MPC11 and 22D6) C α in MEL cells. Unlike MEL cells, however, all of the IgC_H segments in a given B cell line replicated at very similar times during the S phase, so that a temporal directionality in the replication of the IgC_H gene cluster was not apparent from these data. These results provide evidence that in murine non-B cells the IgC_H, J, and D loci are part of a single replicon.

Because of the complexity of the mammalian genome, advances in our knowledge of the mechanisms involved in the initiation and organization of DNA replication have proceeded slowly. Electron microscopic and autoradiographic results have indicated that chromosomal DNA is organized into numerous replication units (replicons) that range in size from 50 to 330 kilobases (kb) and appear to initiate in synchronous clusters. Replication usually proceeds bidirectionally from origins spaced 45 to 180 kb apart. The rate of replication fork movement has been estimated from many studies to be within the range of 1.5 to 3 kb/min (for a review, see references 8 and 20). However, the above observations provide little information concerning the replication of specific loci. It is not clear whether the techniques used select for replicons of a certain size class or to what extent they select for single-copy or repeated sequences.

Considerable uncertainty also exists about the nature of mammalian origins of replication. DNA segments have been isolated from human and mouse genomes which can function in a manner similar to that of presumptive yeast origins (autonomously replicating sequences) in supporting the extrachromosomal replication of plasmids in yeast cells (21, 28). However, it has yet to be established that these autonomously replicating sequences function as replication origins in the context of the mammalian chromosome. In synchronized African green monkey cells, Kaufmann et al. (14) isolated and cloned nascent DNA synthesized at the very onset of the S phase to obtain origin-enriched sequences. Many of the clones carrying origin-enriched sequences contained middle or highly repetitive DNAs (42), and one clone

contained mitochondrial origin sequences. Two independently isolated clones contained the same middle repetitive sequences. Thus, as expected of replication origins, these clones represent specific sets of sequences rather than a random sampling of genomic DNA.

Studies by Heintz et al. (12) on methotrexate-resistant Chinese hamster ovary cells suggested that a 135-kb amplified region (amplicon) containing the dihydrofolate reductase gene may in fact be a replicon. Furthermore, the initiation of DNA synthesis in each amplicon was shown to be confined to a small subset of restriction segments during the first 40 min of the S phase. These studies suggested that the early replicating fragments contain or flank origins of replication.

While specific origins appear to exist in the genome, the available evidence suggests that only a fraction of them are active in a single cell type (36). For example, more origins are active in early *Drosophila melanogaster* embryonic cells than at later stages in development (3). The arrest of cells in the S phase with fluorodeoxyuridine results in the initiation of DNA synthesis from additional sites after release from the block, suggesting the existence of potential origins that are not normally active (37). These authors (37) found that such potential origins are spaced about 12 kb apart. In a particular cell type, only a fraction of these origins may be used.

The transcriptional activity of DNA also appears to influence the activation of origins. For example, some genes replicate early in the S phase in cell types in which they are expressed and at later times in cell types in which they are not expressed (6, 9, 10; Hatton et al., manuscript in preparation). It has also been suggested that the initiation of replication from an upstream origin is required for transcriptional activation of a gene (31, 34). A recent study (13)

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suggested, however, that replication from an origin located upstream of a gene may be a necessary but not sufficient condition for its expression. The mechanisms by which specific origins are activated are unclear and may relate to such factors as the physical state of chromatin.

In an effort to characterize a replicon and to identify potential replication origins, we measured the temporal order of replication of the murine immunoglobulin heavy-chain constant region (IgC_H) genes in a number of cell lines. This region has been completely mapped (16, 32, 39), and since the IgC_H, joining (J), and diversity (D) segments encompass approximately 300 kb (see Fig. 1), it is large enough to include several average-sized replicons (8, 20). In an earlier study of three IgC_H genes in Friend virus-transformed murine erythroleukemia (MEL) cells fractionated by centrifugal elutriation, we observed that the temporal order of replication of C α , γ 2b, and μ corresponded to their linear order on the chromosome (4). C α sequences replicated earliest in the S phase, followed in order by γ 2b and μ . We expanded that study to examine the replication times of additional *Eco*RI segments in the IgC_H gene cluster. These segments include sequences in the vicinity of the IgC_H genes α , ϵ , γ 2a, γ 2b, γ 1, γ 3, δ , and μ , as well as the J and D loci. The resolution of the study with MEL cells was also increased by dividing the S phase into eight intervals rather than four as in the previous study and decreasing the length of incubation in the presence of bromodeoxyuridine (BUdR) from 2.5 to 1.5 h. We also examined the temporal order of replication of the IgC_H segments in several other murine non-B cell and B cell lines. The IgC_H, J, and D segments replicated progressively later as a linear function of their distance from C α in MEL cells. Similar results were obtained in fibroblast (L60T) and hepatoma (Hepa 1.6) cell lines. In contrast, in plasmacytoma (MPC11) and pre-B (22D6 and 300-19P) cell lines, all IgC_H segments examined replicated within a narrow interval of the S phase, at least as early as C α in MEL cells.

MATERIALS AND METHODS

Cell cultures. The MEL DS19, plasmacytoma (MPC11), and L60T cell lines were maintained as described previously (6). The Abelson murine leukemia virus-transformed (1) pre-B cell lines, 22D6 and 300-19P (kindly provided by F. Alt), and a T-cell lymphoma, RL δ 11 (kindly provided by A. Silverstone [33]), were grown in suspension cultures (Falcon flasks) in RPMI medium in the presence of 10% heat-inactivated fetal calf serum and 5×10^{-5} M β -mercaptoethanol. BALB/c 3T3 cells and a hepatoma cell line, Hepa 1.6, were grown in monolayers in Dulbecco modified Eagle medium containing 10% fetal calf serum.

Growth in the presence of BUdR and centrifugal elutriation. Growth in the presence of BUdR, centrifugal elutriation, and flow microfluorimetric (FMF) analysis of propidium iodide-stained MEL and MPC11 cells were as described previously (4–6, 9). BUdR labeling and centrifugal elutriation conditions for Hepa 1.6, 22D6, 300-19P, and RL δ 11 were as described elsewhere (Hatton et al., in preparation; S. Stuart, F. W. Alt, and C. L. Schildkraut, manuscript in preparation). With each cell line, the S-phase population was divided into four pooled fractions having modal cellular DNA contents in the approximate range of 2.0 to 2.3 C (class I), 2.3 to 3.0 C (class II), 3.3 to 3.6 C (class III), and 3.6 to 4.0 C (class IV), where C is the haploid DNA content of a cell in the G1 phase (6). In another experiment with MEL cells, bromouracil-labeled DNA (BU-DNA) was obtained from narrower intervals of

the S phase by incubating the cells with BUdR for a shorter time (1.5 instead of 2.5 h) and dividing the S-phase population into eight size classes instead of four. In these experiments, a Beckman JE10X rotor with a larger capacity than the one used previously (4) was utilized. With the larger rotor, approximately 4×10^9 cells were elutriated.

Isolation of BU-DNA. The preparation of high-molecular-weight DNA, digestion with *Eco*RI, and the separation of BU-DNA from non-BU-DNA by their buoyant density differences in Cs₂SO₄ gradients were as described previously (4, 6). The concentration of BU-DNA was determined by measuring both the A₂₆₀ and fluorescence (15). The *Eco*RI segments of BU-DNA were separated by agarose gel electrophoresis and transferred to diazobenzoyloxymethyl paper (38). The filter-bound DNA was then hybridized to the nick-translated ³²P-labeled probes for various mouse immunoglobulin genes.

Calculation of relative concentrations of *Eco*RI segments in BU-DNA. Hybridization, washing of filters, and autoradiography were as described previously (4). The relative concentrations of *Eco*RI segments were determined from the normalized band intensities on X-ray film measured from areas of densitometric scans by using a Numonics Electronic Graphics calculator or a Cambridge Instruments Quantimet 920 (4, 9). All exposures were within the linear response range of the X-ray film. The error due to the unevenly distributed background on the X-ray film was estimated to be less than 10%.

DNA probes. The map of the IgC_H gene cluster in MEL cells shown in Fig. 1 (derived from references 16, 32, and 39) illustrates the probes used in this study and the *Eco*RI segments to which they hybridize. Other *Eco*RI segments in this region are not shown. p3' α (cloned in pSP65; provided by J. Stavnezer) consists of a 1.3-kb *Bam*HI-*Bgl*II segment located between 2.8 and 4.2 kb downstream from the C α membrane exon in I/St strain mice. This probe hybridizes to a 20-kb segment in MEL cells and a 10-kb segment in the other cell types we examined. To our knowledge, the segments hybridizing to p3' α have not been previously mapped. M67-19 (17) and p α (J558)¹³ (19) were provided by K. Marcu. pySa-3 (provided by L. Eckhart) is a 4.5-kb *Eco*RI segment subcloned into pBR325 from the CHM1g- γ 2a-9 phage clone originally isolated by Shimizu et al. (32). γ 2b(11)⁷ (30) contains cDNA (about 1 kb) cloned into the *Eco*RI site by AT tailing and was provided by K. Marcu. pBR1.4 (provided by K. Marcu) contains a genomic insert from a 6.6-kb *Eco*RI segment adjacent (5') to the *Eco*RI segment containing the γ 2b gene (17). MEP10 was isolated from a partially *Eco*RI-digested BALB/c mouse embryo DNA library and hybridizes to a 6.8-kb *Eco*RI segment containing the γ 1 gene and an adjacent (5') 16-kb segment (27). pS γ 3 (17) and p μ (3741)⁹ (30) containing cDNA (about 1 kb) inserted into the *Eco*RI site of pMB9 by AT tailing were kindly provided by K. Marcu. J_H800 (provided by F. Alt) contains an insert of 800 base pairs located 3' of J₄. pJ₁₁ (19) contains a genomic insert from the J_H region including J₃ and J₄. The D-region probes (cloned into pBR322) were kindly provided by F. Alt; DSP2.3 contains a rearrangement of DSP and J, and p6-1 (see Table 3) contains a rearrangement of DFL and J (1).

RESULTS

Isolation of newly replicated BU-DNA from different intervals of the S phase in elutriated MEL cells. To obtain DNA that replicated during selected intervals of the S phase, we incubated exponentially growing MEL cells in the presence

FIG. 1. Map positions of *EcoRI* segments from IgC_H, J, and D loci in MEL cells whose replication times were examined. The probes used to identify each of the *EcoRI* segments are indicated above the map. When these probes hybridized to more than one segment in the vicinity of a gene, we indicated these segments by number (in brackets below the map). The map distances are based on those determined for BALB/c mice (16, 32, 39). The sizes of the *EcoRI* segments in MEL cells were the same as those reported for BALB/c mice with one exception. A 4.5-kb C_α segment which hybridized to M67-19 and pa(J558)¹³ in several mouse strains was not present in MEL cells. In this cell line, these probes hybridized to 2.8- and 1.7-kb bands, respectively (4). Not included in the published maps of the BALB/c IgC_H gene cluster is a 20-kb segment in MEL cells which hybridized to p3'α, a 1.3-kb *Bam*HI-*Bgl*II segment cloned in pSP65 and located 2.8 to 4.2 kb 3' of the C_α membrane exon in I/St strain mice (G. Stavnezer, manuscript in preparation). In other murine cell lines examined in this study, p3'α hybridized to a 10- to 14-kb segment. In addition, the position of the 10-kb (D₁₀) segment (F. Alt, personal communication) and the relative positions of 4.0- and 3.8-kb D segments (D_{4.0} and D_{3.8}) were not known at the time a map of the D region was published.

of BUdR (20 μg/ml). Cell populations in various stages of the S phase were then separated on the basis of cell size, which is proportional to DNA content, by centrifugal elutriation as described previously (4, 9). Fractions of elutriated cells with similar DNA contents (determined by FMF analysis) were pooled such that the S phase was divided into four or eight size classes in different experiments. The length of the BUdR pulse was 2.5 h for the four-interval experiment and 1.5 h for the eight-interval experiment. Cells from each size class contained BU-DNA which had replicated during the 1.5- or 2.5-h BUdR pulse prior to elutriation and hence during different intervals of the S phase. DNA from these cells was digested with *EcoRI*, and BU-DNA was separated from non-BU-DNA by preparative Cs₂SO₄ density gradient centrifugation (4). Equal amounts (4 μg) of *EcoRI*-digested BU-DNA from different intervals of the S phase was fractionated by agarose gel electrophoresis and transferred to diazobenzyloxymethyl paper (38). The filter-bound DNA was sequentially hybridized to ³²P-labeled cloned segments corresponding to different IgC_H segments (Fig. 1). Autora-

diagrams obtained from exposure of X-ray film to diazobenzyloxymethyl paper revealed bands of differing intensities in the lanes containing DNA that replicated during selected intervals of the S phase. The *EcoRI* segments flanking the γ2b gene are shown in Fig. 2. The intensities of these bands were proportional to the relative concentrations of the *EcoRI* segments that replicated in the presence of BUdR. The concentration of a particular segment was greatest in BU-DNA from the interval of the S phase during which it replicated. With these methods, we have been able to use the DNA samples described here to detect genes that replicate during the early as well as the late S phase (6; Hatton et al., in preparation). In the present study, this approach was used to determine the temporal sequence of replication of different IgC_H segments.

Determination of the nuclear DNA contents (C values) at which various IgC_H segments replicated in MEL cells. As a cell progresses through the S phase, the nuclear DNA content (C value) increases from 2 to 4 C. Thus, the C value

FIG. 2. Relative concentrations of sequences flanking the γ2b gene (γ2b-1; Fig. 1) in BU-DNA from eight intervals of the S phase. Exponentially growing MEL cells were pulsed with BUdR (20 μg/ml) for 1.5 h. The cells were then fractionated according to size by centrifugal elutriation. Samples were pooled into eight size classes representing cells from different intervals of the S phase. *EcoRI* digests of BU-DNA (4 μg) from each size class (lanes 1 to 8) were fractionated on a 0.8% agarose gel and transferred to diazobenzyloxymethyl paper. A ³²P-labeled probe (pBRI.4) detected the 6.8-kb *EcoRI* segment flanking γ2b (3').

FIG. 3. Linear increase in DNA content and cell size as MEL cells progress through the S phase. MEL cells in the G₁ phase were collected by centrifugal elutriation and incubated at 37°C. At 30-min intervals, samples were removed for analysis of cellular DNA content (C value) by FMF analysis or of cell size (determined with a Coulter Electronics Channelyzer). Similar results were obtained in an independent elutriation experiment.

FIG. 4. Relative abundance of cells that had a given DNA content during the 1.5-h BUdR pulse in each of eight elutriated cell populations. The FMF profiles are histograms showing numbers of cells (ordinate), with the DNA content (measured by fluorescence) indicated on the abscissa. The numbers to the right of each profile indicate the flow rate (milliliters per minute) of the medium used for elutriation of those fractions. The fractions were pooled to produce eight classes of cells. BU-DNA synthesized during a different interval of the S phase was isolated from each of these cell classes. The shaded areas indicate the proportions of cells in each elutriated population that had a given nuclear DNA content (2.2 C) at some time during the BUdR pulse.

is a convenient measure of how far a cell has advanced through the S phase. We verified that the DNA contents in MEL cells increased throughout the S phase at a constant rate by the following experiment. A population of MEL cells in the G1 phase was collected by centrifugal elutriation and maintained at 37°C in medium containing 10% fetal calf serum. At 30-min intervals, samples were removed; their DNA contents were determined by FMF analysis, and their cell sizes were determined with a Coulter Electronics Channelyzer. The average cell size increased linearly throughout the G1 and S phase (Fig. 3). The average DNA content remained at 2 C for a 2-h interval, corresponding to the

length of the G1 phase in MEL cells (E. Brown, unpublished results). After 2 h, the cells entered the S phase synchronously. Upon entry into the S phase, the average DNA content per cell increased linearly to a value of 4 C over a period of 8.5 h. These results show that the C value in MEL cells increased at a constant rate throughout the S phase. Thus, all S-phase cells will have increased their DNA contents by 0.35 C during a 1.5-h BUdR pulse (17.6% of an 8.5-h S phase during which the DNA content increases by 2 C). This constant rate of increase in the DNA content allowed us to determine the specific C values at which IgC_H segments replicated by using the strategy described below.

First, the relative concentrations of *Eco*RI segments in the BU-DNA from different pooled elutriated populations were determined from their band intensities on autoradiograms. The relative concentration of a segment that replicated at a given C value in the BU-DNA of a particular elutriated population was proportional to the relative percentage of cells in that population which had a nuclear DNA content equal to this C value at some time during the BUdR pulse.

To determine the percentage of cells in each elutriated population which replicated at a given C value during the BUdR pulse, we used the FMF profiles. In the FMF profiles shown in Fig. 4, the shaded area (which encompasses 0.35 C) illustrates a population of cells in each interval which replicated at a given C value during the BUdR pulse. We determined the percentages of total cells in each interval which were included in the shaded area [(shaded area/total area in profile) × 100]. The total number of cells in each pooled interval was then normalized, and the relative percentage of cells which replicated at a given C value (i.e., in the shaded area) during the BUdR pulse was determined. Using this analysis (with the aid of a computer), we prepared a table (not shown) of the normalized percentage of cells in each cell population (representing each selected interval of the S phase) which replicated when their nuclear DNA contents were at a particular C value. This was done for 100 selected C values.

To determine the C value at which a segment replicated, we matched its relative concentration in BU-DNA from each pooled interval with the normalized percentage of cells which replicated at 100 selected C values. For example, in Table 1 we list the relative concentrations of three IgC_H segments in BU-DNA from each of eight intervals and the C values for which the normalized percentage of cells in each interval agreed most closely with these data. From these data, we determined that γ 2a, γ 1, and J₁₋₄ replicated at 2.43, 2.57, and 2.71 C, respectively. Using this analysis, we determined the C values at which the IgC_H, J, and D segments detected by the probes in Fig. 1 replicated.

To determine whether the FMF profiles are a true reflection

TABLE 1. Determination of the C values at which IgC_H segments replicated during the S phase^a

<i>Eco</i> RI segment	Probe	Relative concn, as a normalized %, of segment in BU-DNA determined from hybridization or calculated from FMF profiles (shown in parentheses) for the following selected intervals of the S phase							
		1	2	3	4	5	6	7	8
5.0-kb flanking γ 2a (5')	pS γ 2a-3	17.1 (16.8)	22.0 (23.6)	22.8 (24.6)	19.3 (18.9)	10.0 (9.9)	6.2 (4.1)	1.9 (1.6)	0.6 (0.5)
16-kb flanking γ 1 (5')	MEP10	9.9 (10.0)	17.4 (19.8)	26.0 (24.1)	20.5 (22.4)	10.7 (14.3)	11.5 (6.0)	3.6 (2.6)	0.3 (0.6)
6.6-kb J ₁₋₄	pJ ₁₁	5.9 (5.6)	11.8 (15.5)	22.2 (21.5)	17.0 (24.3)	14.7 (19.2)	6.5 (8.6)	8.3 (4.6)	2.7 (0.8)

^a Eight classes of cells (see Materials and Methods) that had synthesized BU-DNA during eight different intervals of the S phase were chosen. The relative concentrations (expressed as a percentage of the total concentration in all eight intervals) of the *Eco*RI segments indicates their temporal order of replication. The number of cells in each class was normalized to 100, and a subpopulation containing a given C value during the BUdR pulse was expressed as a percentage of all such cells. To determine the C value at which an *Eco*RI segment replicated, we compared its distribution in the BU-DNA from each size class (measured by hybridization) with the relative distribution of cells (measured by FMF analysis) which had a given C value in each size class. We chose as the C value at which an *Eco*RI segment replicated a nuclear DNA content at which the sum of the differences between the two sets of data (determined by a computer) was the smallest. The C values at which the three segments replicated were determined to be 2.43C (γ 2a), 2.57C (γ 1), and 2.71C (J₁₋₄).

FIG. 5. Increase in nuclear DNA contents (C values) at which IgC_H segments replicated as a linear function of their distance from C_α in MEL cells. The 3' end of the 20-kb *Eco*RI segment which hybridized to p3'α (Fig. 1) in MEL cells was designated as 0 kb. The abscissa shows the distance (in kilobases) of the midpoint of each *Eco*RI segment from this reference point. D_{4.0} and D_{5.4} were treated as one combined segment in this analysis. The C value at which each segment replicated was determined from its relative concentration in BU-DNA from each elutriated cell class. (A) Temporal replication determined by analysis of BU-DNA from four intervals of the S phase. (B) Temporal replication determined by analysis of BU-DNA from eight intervals of the S phase.

tion of the distribution of DNA content per cell rather than artifacts from instrumentation, we also examined the distribution of cell sizes in each elutriated population. It has been observed that a linear relationship exists between cell sizes (determined with a Coulter Electronics Channelyzer) and DNA contents (determined by FMF analysis) of elutriated populations (A. Furst and C. Schildkraut, unpublished data). It was therefore possible to determine C values from cell sizes. We found the standard deviations of the distributions of C values in elutriated populations determined by cell sizes and DNA contents to be in close agreement (data not shown). Therefore, the FMF profiles represent the actual distribution of C values in elutriated populations.

Average rate of replication of the IgC_H gene cluster in MEL cells. We determined the C values at which each IgC_H segment replicated in experiments in which the S phase was divided into four or eight intervals. In each experiment (Fig. 5A and B), we observed that the C values at which various IgC_H, J, and D segments replicated varied as a linear function of their distances from the C_α domain (Fig. 1). From these data, we determined that the average cellular DNA content increased 0.68 C in the time required for replication of the entire 300-kb IgC_H gene cluster. Since the DNA content of MEL cells increases from 2 to 4 C in 8.5 h, the average rate of increase in the C value is 4×10^{-3} C/min. Thus, replication of the IgC_H gene cluster took place in 160 min at an average rate of approximately 1.8 kb/min. This

FIG. 6. Increase in nuclear DNA contents (C values) at which IgC_H segments replicated as a linear function of their distance from C_α in other non-B cell lines. (A) L60T. (B) Hepa 1.6. The *Eco*RI segment (α-4) which hybridized to p3'α was 10 kb in these cell lines. The 3' end of this segment was designated as 0 kb in these cell lines. The 1.7- and 2.8-kb C_α segments (α-2 and α-3) found in MEL cells (Fig. 1) are replaced by a 4.5-kb segment in these cell lines.

value agrees well with many other estimates for the rate of fork movement in mammalian cells (8, 20).

Determination of the temporal order of replication of IgC_H segments in other murine cell lines. Similar experiments (four intervals) were carried out with fibroblast (L60T) and hepatoma (Hepa 1.6) cell lines. In each cell line, we observed that IgC_H segments replicated in a distinct temporal order, starting with C_α and proceeding in a 5' direction to the D region at rates of 1.8 kb/min (Fig. 6A and B). Similar results (data not shown) were obtained with the BALB/c 3T3 and RL δ 11 cell lines. However, in a B-cell plasmacytoma (MPC11), we observed little or no change in the relative concentrations of IgC_H segments in BU-DNA from four intervals of the S phase as a function of distance from C_α (Table 2). In these cells, all segments replicated earlier in the S phase than C_α did in MEL cells. We also observed that the C values at which the IgC_H segments replicated did not increase as a function of distance from C_α in two pre-B cell lines (22D6 and 300-19P) (Table 3).

DISCUSSION

A wide body of evidence has indicated that DNA replicons are initiated in a temporally defined order during the S phase

TABLE 2. Replication of IgC_H segments in MPC11 cells

<i>Eco</i> RI segment (kb) ^a	Probe	Relative concn of segment in BU-DNA from cell class:			
		I	II	III	IV
α-2, α-3 (4.5)	M67-19	72	17	7	4
5' Flanking γ2a (6.5)	pSγ2a	66	21	8	5
γ2b-2 (6.6)	γ2b(11) ⁷	66	21	8	4
J _H (3.5) ^b	pJ ₁₁	68	21	8	3

^a See Fig. 1. In this cell line, the *Eco*RI site between segments α-2 and α-3 is absent, and one 4.5-kb segment is observed.

^b Data represent the averages of the results from two hybridizations.

TABLE 3. Replication of IgC_H segments in pre-B cells

Cell line	<i>Eco</i> RI segment (kb) ^a	Probe	Relative concn of segment in BU-DNA from cell class:			
			I	II	III	IV
22D6	α-4 (14)	p3'α	64.9	30.6	3.7	0.9
	α-2, α-3 (4.5)	M67-19	65.9	26.6	9.5	2.1
	5' Flanking	pSγ2a-3	64.5	25.1	9.6	0.9
	γ2a (5.0)		70.5	25.9	2.9	0.9
	γ2b-2 (6.8)	γ2b(11) ⁷	60.1	33.7	5.3	0.8
	γ2b-1 (6.8)	pBR1.4	61.0	31.7	6.4	0.9
	γ3 (23)	pSγ3	70.3	28.3	1.3	0
	μ (19.0)	pμ(3741) ⁹	53.0	33.0	12.0	2.5
	J _H (3.0)	pJ ₁₁	69.3	25.5	5.0	0.1
	J _H (4.5)	pJ ₁₁	59.3	34.9	5.5	0.3
	DFL (4.4)	p6-1	56.0	37.0	6.9	0.4
300-19P	α-2, α-3 (4.5)	M67-19	53.5	40.0	6.2	0.2
	γ2b-2 (6.8)	γ2b(11) ⁷	46.3	46.7	7.0	0
	γ2b-1 (6.8)	pBR1.4	53.4	46.6	0	0
	γ3 (23)	pSγ3	47.2	35.0	11.7	5.9
	μ (19.0)	pμ(3741) ⁹	57.0	27.2	11.8	3.9
	J _H (5.0)	J _{H800}	57.2	36.0	6.7	0.2
	J _H (5.0)	pJ ₁₁	54.8	39.3	5.9	0
	DSP (4.0, 5.5)	DSP2.3	45.3	40.7	5.7	8.1

^a The sizes of the *Eco*RI segments in these pre-B cell lines are the same as those in germ line DNA (Fig. 1), except for the rearranged J segments and the α-2 and α-3 segments.

^b Two sets of data represent the results from two independent hybridizations.

(2, 4, 6, 9, 10, 20, 36). In addition, the time at which specific genes replicate appears to be invariant from one S phase to the next, except possibly in cases in which the transcriptional state of a gene has changed. The available evidence suggests a correlation between the time some genes replicate during the S phase and their transcriptional activity (6, 9, 10; Hatton et al., in preparation). Thus, expressed genes usually replicate earlier than nonexpressed genes. The mechanisms governing this temporally programmed initiation of DNA replication are poorly understood and are the subject of much investigation.

Earlier studies with synchronized Chinese hamster cells reported that the S phase is divided into distinct early and late replicating components which are separated by a pause in DNA replication (reviewed in reference 10). In contrast, we observed that the DNA content of the MEL cell line increases linearly as the cells progress through the S phase. Thus, in MEL cells we do not find evidence for a clear delineation between the early and late halves of the S phase, as has been previously suggested for other cell lines.

We have used centrifugal elutriation to examine the temporal order of replication of *Eco*RI segments in the IgC_H gene cluster of several murine cell lines. The results indicate that in nonlymphoid cell lines, IgC_H gene segments (Fig. 1) replicate in the following order: α, ε, γ2a, γ2b, γ1, γ3, δ, and μ, followed by the J and D segments (Fig. 5 and 6). In two separate elutriation experiments with MEL cells, differing degrees of resolution were obtained by dividing the S-phase population into four (2.5-h BUdR pulse) or eight (1.5-h BUdR pulse) intervals. The results obtained in each experiment were essentially the same. The C value at which an IgC_H segment replicated varied as a linear function of its distance in the 5' direction from an *Eco*RI site located 3' of the Cα domain in MEL cells. From these data, the rate of replication of the IgC_H gene cluster was determined to be 1.7 to 1.9 kb/min. This value is within the range of earlier measurements of the rate of mammalian DNA replication

fork movement in a variety of cell types (8). These results are consistent with the segments of the IgC_H locus in MEL cells being part of a single replicon. However, the possibility that this region may contain numerous small replicons (less than 20 kb) which initiate sequentially cannot be ruled out. This latter possibility would conflict with autoradiographic studies suggesting that neighboring replicons are activated synchronously rather than sequentially (8, 20, 29). Our results are not consistent with the sequential activation of bidirectional replicons that are similar in size to the average replicon (60 kb) observed by autoradiography. We have also considered the possibility that replication of all of these IgC_H segments initiates at the same time at several different origins but that it takes longer to complete the synthesis of the replicons located further from Cα. This implies that somewhere in the vicinity of the D segments there is a segment that replicates at the same time as Cα or earlier. We have examined the replication of many segments flanking the D_{5,4} segment, and they do not replicate at the same time as Cα or earlier. We have also determined that the heavy-chain variable region segment that is closest to the D segments replicates later than the D segments (Stuart et al., in preparation).

If the IgC_H gene cluster and flanking sequences in MEL cells are part of a single replicon, the origin should be located within the earliest replicating segment(s). However, the region on the 3' side of Cα has not been extensively mapped, and probes for such a study are not presently available. Therefore, we cannot determine, at present, whether there are segments in this region that replicate earlier than Cα. However, the earliest an origin can be activated is the beginning of the S phase (at 2 C). If the linear relationship shown in Fig. 5 is extrapolated back to 2 C, we find that the origin can be no further than 120 kb upstream from the 3' α segment, assuming that replication proceeds linearly from the point of initiation. A single origin for the 300-kb gene cluster of IgC_H, J, and D localized near Cα would imply the

existence of a replicon as large as 600 kb (if replication is bidirectional and termination sites are equidistant from the origin) or 300 kb (if replication is unidirectional). Such a bidirectional replicon would be considerably larger than the mean of 50 to 330 kb observed by electron microscopy or autoradiography (for a review, see references 8 and 20). However, replicons as large as 600 kb have been reported by some investigators (41). While autoradiography suggests that 85 to 90% of replication bubbles observed are bidirectional, patterns consistent with unidirectional replication have also been observed (8). Thus, the possibility of a unidirectional replicon cannot be excluded.

While the results of this study indicate that initiation occurs 3' of the IgC_H gene cluster, they do not imply that the replication of this region is always initiated at the same site. Instead, replication might be initiated from a number of possible sites within a specific region. In simian virus 40 DNA, multiple initiation sites for DNA synthesis have been identified within a 200-base-pair region of the genome containing the origin of replication (11). In Epstein-Barr virus, whose genome is approximately 40 times larger than that of simian virus 40, the origin region (*ori P*) encompasses 1,700 base pairs (23, 40). The *ori P* region of Epstein-Barr virus may resemble mammalian origins of replication more closely than do the origins of replication of simian virus 40 or polyomavirus, since in a lytic infection these viruses undergo exponential DNA replication during the cell cycle, leading to cell death. In contrast, plasmids containing Epstein-Barr virus *ori P* undergo a controlled replication that is more characteristic of mammalian replicons that initiate once in the cell cycle. The size and complexity of an origin of replication may increase with the size of the genome, reflecting the need to interact with a variety of controlling factors.

While we have assigned specific C values for the replication of IgC_H segments in murine cell lines, we cannot state that the replication of this region initiates at the same time in every cell or in every homolog of chromosome 12 on which the IgC_H genes are located. The C values which we have calculated may reflect an average time of replication for all of the IgC_H segments in the culture. However, the fact that we were able to discern a temporal directionality in the replication of this region suggests that replication proceeds through the IgC_H gene cluster in the same direction in the large majority of cells.

A directionality in the replication of IgC_H segments similar to that in MEL cells was also observed in other non-B cell lines, including L60T (fibroblast) and Hepa 1.6 (hepatoma). We obtained similar results (data not shown) for BALB/c 3T3 cells and RL δ 11. In contrast, we found that all IgC_H segments in a plasmacytoma (MPC11) cell line replicated earlier in the S phase than did C α in MEL cells, with no resolvable temporal order. In MPC11, there has been a DNA rearrangement that deletes the constant region genes upstream of γ 2b, so that J and C α are separated by a much shorter distance (55 kb) than in non-B cells. As shown in Fig. 5, differences in the replication times of fragments separated by less than 50 kb were detected in MEL cells. Similar results were obtained in a pre-B cell line (22D6). In another pre-B cell line (300-19P), all segments replicated as early as did C α in MEL cells. Both 22D6 and 300-19P contain an undelimited germ line IgC_H and rearrangements of D and J and produce D μ mRNA (24, 25).

It is possible that replication proceeds at a faster rate in expressed regions of the genome in which chromatin is less condensed than in more condensed, inactive regions. Thus,

in cells in which the IgC_H gene cluster is transcriptionally active, replication might proceed at too fast a rate for a temporal directionality to be clearly resolved. While no evidence exists for modulation of the rate of replication in active DNA versus inactive DNA, this possibility cannot totally be ruled out from the results of this study.

It is also possible that more origins are active in the IgC_H gene cluster in B cells than in non-B cells. As stated above, our data are consistent with the IgC_H, J, and D segments in MEL cells and other non-B cells being part of a single replicon with an origin in the vicinity of C α or further downstream. In contrast, additional origins at various sites in the IgC_H gene cluster may be activated in B cells, resulting in replication from multiple replication forks rather than one replication fork. If these origins are activated at approximately the same time in the S phase, a temporal directionality would not be observed by our methods. This explanation is in agreement with studies suggesting that the mammalian genome contains many potential origins of replication, of which only a few are utilized in different specialized cells (35, 37). There are precedents for the use of alternative origins in a particular stretch of DNA. For example, there can be several alternative origins of replication in the DNA of *Escherichia coli* bacteriophage T7 (26). The number of origins of replication has been shown to change during development in *D. melanogaster* (3). The recruitment of additional origins in the IgC_H gene cluster in B cells might indicate a need for replication initiated from a nearby origin for gene expression. Electron microscopic studies have shown that nascent transcripts are associated with early replication forks in *Physarum polycephalum* (22). In mouse mitochondrial DNA, replication and transcription initiate from the same site (7). The specific positioning of origins of replication in relation to active genes has also been suggested by others (18, 31, 34). Thus, the initiation of replication from specific origins may be an early step in the activation of a gene. A technique described in a recent study by James and Leffak (13) should prove useful in searching for closely spaced origins of replication in the expressed IgC_H gene cluster in B cells. The identification of replication origins in the IgC_H and other expressed or silent multigene families should help to elucidate the relationship between DNA replication and gene activity.

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