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The temporal order of replication of murine immunoglobulin heavy chain constant region sequences corresponds to their linear order in the genome

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ABSTRACT

The time of replication during the S phase in a murine erythroleukemia (MEL) cell line was determined for immunoglobulin heavy chain constant region Cα,Cγ2b and Cμ sequences whose boundaries are defined by EcoR1 restriction endonuclease sites (EcoR1 segments). Logarithmically growing cultures of MEL cells with an S phase of about 7.5 hours were pulse labelled with 20 μg/ml of 5-bromodeoxyuridine (BUdR). The cells were then fractionated by centrifugal elutriation into 10-12 distinct populations containing cells in different stages of the cell cycle. Flow microfluorimetric (FMF) analysis of DNA content, measurements of cell volume and autoradiography after ^{3}H-thymidine pulse labelling were used to determine position in the cell cycle. Fractions were pooled to represent four selected intervals of S in which BU-DNA was synthesized for 2.5 hrs or less. Newly replicated DNA which had incorporated BUdR into one strand was isolated, cleaved with EcoR1, and separated on neutral Cs_2SO_4 gradients. Equal amounts of BU-DNA replicated during these four intervals of S were electrophoresed in 0.8% agarose gels, transferred to diazotized aminobenzyloxymethyl paper and hybridized with ^{32}P probes containing the Cα, Cγ2b and Cμ genes and flanking sequences. The relative amounts of segments replicated were assessed by quantitation of the appropriate bands on the autoradiograms by microdensitometry. The results indicate that the 2.8 kb Cα, 6.6 kb Cγ2b and 12 kb Cμ EcoR1 segments in these MEL cells replicated during defined intervals of the first half of the S phase. The order of replication of these EcoR1 segments as the cells proceeded through S was Cα, Cγ2b, Cμ, corresponding to the linear order of the genes determined by restriction endonuclease mapping.

INTRODUCTION

Evidence has been presented for temporally ordered replication of the mammalian genome although base sequence specific origins of replication have not been identified in mammalian cells, . Studies in Physarum polycephalum (1) and HeLa cells (2) have indicated that most of the DNA replicated in one temporal interval of the S phase is replicated in the same temporal interval in the following S phase. The interval of replication for satellite sequences is the same in genomic DNA from different mouse strains, and ribosomal DNA sequences are also replicated during defined intervals of S phase (for review and pertinent references, see 3). Several recent studies discussed below have

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focused on the replication of nonreiterated portions of the genome.

We have devised a method to obtain DNA restriction segments replicated during selected intervals of the S phase (4,5) by pulse labelling exponentially growing cells with 5-bromodeoxyuridine (BUdR) and obtaining synchronized cell fractions from different phases of the cell cycle by centrifugal elutriation. This scheme avoids the potential artifacts of drug treatment and provides large numbers of cells for our studies. We have used the elutriation method to show that α-globin sequences are located in a restriction segment of DNA which replicates early during S in Friend virus transformed murine erythroleukemia (MEL) cells (5). We have shown that both the β-major and β-minor globin genes which are separated by 15 kb in murine cells (6), are also found on different early replicating regions of DNA (Braunstein and Schildkraut, unpublished results). Additional studies have shown that sequences which hybridize to globin probes are also replicated primarily in early S (7). Finally, studies on integrated SV40 DNA indicate that the programmed time for replication of this single-copy DNA sequence depends on its location in the genome (8).

We have begun to study the temporal order of replication of the immunoglobulin heavy chain region for several reasons. Since the geographic relationship of these sequences spanning a distance in the genome of almost 200 kb is well established (e.g. see 9), it is possible, for the first time, to determine the temporal replication during S of clustered, functionally related genes whose relative locations in the genome are precisely known. The organization of these constant region genes in the Balb/c mouse is 5'-J_H-6.5kb-Cμ-4.5 kb-Cδ-55 kb-Cγ3-34 kb-Cγ1-21 kb-Cγ2b-15 kb Cγ2a-14 kb-Cε-12 kb-Cα 3' (eg. see 9). In addition, the DNA sequence organization of this part of the genome for replication may be of relevance to understanding the mechanism of heavy chain class switching from one immunoglobulin type to another. This class switch is associated with deletion of sequences from the genome during lymphocyte differentiation (10-14).

We report here the measurement of the temporal replication of closely mapping EcoR1 segments from the immunoglobulin heavy chain constant region. These segments contain or are adjacent to the Cα, Cγ2b and Cμ genes and the J_H region. Our results demonstrate that there is a defined temporal order of replication for these segments in the MEL cell line. These segments replicate during the first half of the S phase in the temporal order Cα, Cγ2b, Cμ which corresponds to the linear order of the genes determined by restriction endonuclease mapping.

MATERIALS AND METHODS

Cell growth and separation according to cell size: MEL cells (DS19 kindly provided by Dr. P. Marks) derived from clone 745A of Dr. C. Friend, were grown at 37°C in spinner flasks using Dulbecco's modified Eagle's medium (DME) containing 7.5% fetal bovine serum (FBS) (Gibco) to a cell density of about 6 x 10^5 cells/ml. Under these conditions the duration of S phase is about 7.5 hrs and the doubling time is about 12 hours. The cells were grown in the presence of BUdR at a concentration of 20 µg/ml for either 1.75 or 2.5 hrs. All work with BU-DNA was carried out under light from gold fluorescent bulbs (GE F40GO). Cells were harvested by centrifugation at 450xg for 15 min. In one of these experiments, cells grown in BUdR for 2.5 hr were subsequently grown in medium containing 10 µM thymidine for 0.5 hr. The harvested cells were resuspended in DME with 1% FBS at a concentration of at least 4 x 10^7 cells/ml. A total of 1 x 10^9 cells were then injected into the sample mixing chamber of an elutriator rotor maintained at a speed of 2,000 rpm in a J21 centrifuge (Beckman Instruments). Cell fractions were elutriated by increasing flow rates of 15-35 ml/min DME containing 1% FBS using a Masterflex pump (Cole Parmer). During elutriation, the DME and the centrifuge chamber were maintained at 22°C. The cell size of each fraction was monitored using a Coulter Model ZBI particle counter and Model C-1000 particle size analyzer. The DNA content per cell was measured by flow microfluorimetry using a Becton-Dickinson flourescence activated cell sorter (FACS II) on samples of 1 x 10^6 cells suspended in 0.1% sodium citrate containing 50 µg/ml propidium iodide (15,16). The fractions eluting at different pump speeds were then pooled, according to DNA content. Fractions with cells having a modal per cell DNA content between 2.0-2.3C, 2.3-3.0C, 3.3-3.6C, and 3.6-3.8C, were pooled yielding four cell classes. The cells of each class had previously synthesized BU-DNA during different but slightly overlapping selected intervals of the S phase.

DNA isolation and cesium gradient centrifugation: DNA was isolated by a modification of the procedure of Walker and McLaren (17). Cells were suspended in 0.3M sodium trichloroacetate and lysed in 0.2% Na dodecyl sulfate (SDS) at room temperature or alternatively, the cells were resuspended in 0.05 M Tris, 0.15 M NaCl plus 0.015 M EDTA at a final pH of 9.5 and incubated in the presence of 1% SDS for 2 min at 65°C. The lysate was extracted by shaking with phenol saturated with 0.2 M Tris, pH 8.0, and then twice with chloroform-isoamyl alcohol (24:1). The DNA was spooled using 2 volumes of ethanol, redissolved and incubated for 1 hour at 37°C in the presence of pancreatic

RNAse A (50 μg/ml) and RNase T1 (20 units/ml) and then for 1 hour in the presence of pronase (100 μg/ml). The phenol and chloroform-isoamyl alcohol extractions and ethanol precipitation were repeated, and the DNA was redissolved and dialyzed against 10 mM Tris, 1 mM EDTA, pH 8.0. The DNA from each fraction was incubated for two hours at 37°C with about 2 units of EcoR1 (Miles) restriction endonuclease per μg of DNA. Then about two additional units of EcoR1 were added and the incubation continued for two hours. The resulting digest was heated at 65°C for 10 minutes and analyzed for completeness of digestion by electrophoresis in a 0.8% agarose gel with TAE (0.4 M Tris; 0.004 M Na acetate; 0.002 M EDTA, pH 8.2) as the running buffer. Electrophoresis was carried out at about 1.0 volt/cm in the presence of 1.0 μg/ml ethidium bromide using a gel whose dimensions were 14 x 15.5 x 0.7 cm and photographed on a U.V. transilluminator. The EcoRI cleaved DNA samples prepared from the four size classes of cells were then analyzed for the percentage of BU-DNA present by analytical centrifugation in Cs_2SO_4 and separated into BU-substituted and unsubstituted DNA by preparative ultracentrifugation for 40 hrs at 35,000 rpm in a Ti 45 rotor in 0.01 M Tris, 0.001 M EDTA, Cs_2SO_4 (n_D = 1.3710) pH 8.2. These separated DNAs were collected, dialyzed 3 times for 24 hrs against 0.01 M Tris, 0.001 M EDTA, pH 8.2 and concentrated by ethanol precipitation. The BU-substituted and unsubstituted DNA samples were each analyzed for the efficiency of separation by analytical density gradient centrifugation in a Beckman Model E ultracentrifuge.

Gel electrophoresis and determination of concentration of constant region gene EcoR1 segments in BU-DNA: DNA concentrations were determined from optical density at 260nm and by the fluorescence method of Kissane and Robbins (18). Results from both methods were in agreement. Equal amounts (5 μg) of EcoR1 segments of BU-DNA replicated during selected intervals of S were then electrophoresed for about 16 hrs and transferred to diazobenzyloxymethyl (DBM) paper by the method of Wahl, Stern and Stark (19). One modification was that the gel was treated with 0.15 M instead of 0.25 M HCl. Another modification (Wall and Stark, personal communication) was that the transfers were carried out for 3-5 hrs or overnight. The paper was washed for 15 minutes each twice with 0.4 M NaOH and twice with 2X SSPE (1 x SSPE is 0.18 M NaCl, 0.01 M Na_2HPO_4, 1mM EDTA, pH 7.0). The DBM paper was then prehybridized for at least 2 hrs and hybridized in the presence of 10% (wt/vol) dextran sulfate as described (19) with about 0.5 x 10^6 cpm/lane of ^{32}P-dCTP nick translated constant region probes for 16 hrs at 37°C with some agitation. The transfers

were washed, autoradiographed at -70°C for 1-7 days using Kodak XR5 film and a Dupont Lightning Plus intensifying screen. The relative intensities of the bands were determined by averaging the peak areas of several microdensitometer tracings of the autoradiogram of each lane. Each band on the autoradiogram was traced along its full width and the results were averaged to eliminate small variations due to inhomogeneity in the image. The genomic and cDNA clones used in these studies were generously provided by Dr. K. Marcu. Plasmids pμ(3741)[9] (20) and pγ2b(11)[7] (21) contained cDNA (about 1 kb) inserted into the EcoR1 site of pMB9 by AT tailing. The Cα probe (M67-19, provided by L. Harris and K. Marcu) contained 0.6 kb of the 3' end of the 4.5 kb Cα EcoR1 genomic segment described by Marcu et al. (20). pJ11 (20) contains a genomic insert from the J_H region. pBR1.4 (22) contains a genomic insert from a 6.6 kb EcoR1 segment adjacent (5') to the EcoR1 segment containing the Cγ2b gene.

RESULTS

Characterization of elutriated cell populations in different stages of S

The results presented below were obtained from three independent synchrony experiments each of which was analyzed for the temporal replication of all three immunoglobulin constant region genes. Exponential MEL cells were grown for either one third or one fourth of the S phase (in different elutriation experiments) in the presence of BUdR (20 μg/ml). The cells, concentrated by centrifugation and resuspension, were injected into the sample mixing chamber of the elutriator rotor. The rate of flow of the elutriation medium was increased in successive steps to obtain 16 fractions containing cells of increasing size. For each fraction of cells, we assessed the percent of cells at each stage in the cell cycle by flow microfluorometric (FMF) analysis and by autoradiography after brief incorporation of ^{3}H-thymidine. These results, as well as measurements of the percent mitotic cells and kinetics of progression of the elutriated cells through the cell cycle showed that for the fractions containing predominately S phase cells, the modal DNA content increased linearly with cell volume (Furst and Schildkraut, manuscript in preparation).

Fractions of cells were pooled to produce four cell classes (see Fig. 1) in different stages of S, so that BU-DNA isolated from them had previously been synthesized during four different intervals of S. The first fractions (not shown) obtained at the lowest pump speeds contained cells in G1. These fractions were discarded. For the first interval (class I), fractions 75-78

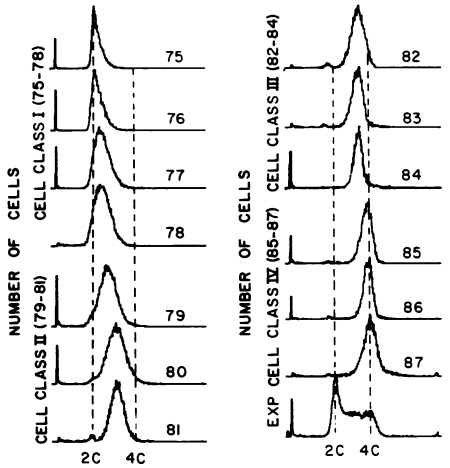

Figure 1. FMF analysis showing cellular DNA content of fractions of cells obtained by centrifugal elutriation. The FMF profiles are histograms giving the number of cells (ordinate) with the DNA content (measured by flourescence) indicated on the abscissa. C is the haploid DNA content of MEL cells in G1. An FMF profile (designated EXP) was obtained from the exponential culture before elutriation. 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. Similar profiles were obtained from the fractions of the cells in the three independent elutriation experiments from which BU-DNA was prepared for these studies. The fractions were pooled, as indicated above, to produce four classes of cells. BU-DNA synthesized during a different interval of the S phase was isolated from each of these cell classes.

were pooled. FMF analysis indicated that the modal DNA content of these cells was 2.1 C. Since these cells had previously been grown in the presence of BUdR for about 2 hrs. (1.75 or 2.5 hrs. in different synchrony experiments) and had spent part of the labeling interval in G1, the BU-DNA obtained from these cells should have been synthesized during an interval representing less than the first 2 hrs.of S. For the second interval (class II), fractions 79-81 were pooled to give cells having an average DNA content of 2.9 C. These cells had replicated DNA somewhat before and during the middle of the S phase. For the third interval (class III), fractions 82-84 were pooled to give cells having an average DNA content of 3.4 C. These cells had synthesized DNA in the presence of BUdR during the middle and third quarter of S. Finally, the fourth interval (class IV, fractions 85-87) contained cells in late S, G2 and mitosis. The average DNA content was 3.8 C and these cells synthesized BU-DNA during the last 2 hrs. of S.

Isolation of BU-DNA replicated during selected intervals of the S phase

For each of the three synchrony experiments presented here, we determined the percent of BU-DNA in each DNA preparation from the four classes of cells representing the different S phase intervals. The smallest (fractions 75-78) S phase cells collected contained DNA replicated for less than the first 2.5 hrs. of S. Although these cells were present for 2.5 hrs. in medium containing BUdR, during most of this time they were in G1 and were not synthesizing DNA. Thus, we expected the DNA isolated from these fractions containing predominately early S phase cells to contain less BU-DNA than the DNA isolated from fractions containing cells in the middle of S. The proportions (18% for class I, 49% for class II, and 39% for class III, and 40% for class IV) of BU-DNA observed agreed with the values predicted from the times spent in G1 and G2 (Figure 2, A and B). The DNA from these cells containing both BU-DNA and unsubstituted DNA was cleaved exhaustively with EcoR1, and BU-DNA was separated from the unsubstituted DNA in preparative Cs_2SO_4 gradients. We checked the efficiency of separation of the BU-DNA by analytical density gradient centrifugation in Cs_2SO_4 (see Figure 2, C-D), which showed the presence of less than 5% unsubstituted DNA in the BU-DNA for all three elutriation experiments.

Relative concentrations of heavy chain constant region EcoR1 segments in BU-DNA replicated during selected intervals of S.

Equal amounts of the EcoR1 cleaved BU-DNA that had replicated during each of the four different S-phase intervals and EcoR1 cleaved BU-DNA from unfractionated MEL cells were electrophoresed in adjacent lanes. After transfer to diazotized paper, the covalently bound DNA was hybridized in the presence of dextran sulfate with the appropriate radioactive probes. Autoradiography (Fig. 3) revealed the same size EcoR1 segments in BU-DNA as in unsubstituted DNA indicating that substitution in one strand does not alter the specificity of these EcoR1 restriction sites. In all instances, background hybridization was similar in each lane of the gel. There was no hybridization to the region corresponding to the well in each lane.

The pμ(3741)[9] probe hybridized to the 12 kb EcoR1 segment containing the Cμ gene. The γ2b(11)[7] probe hybridized to the 6.6 kb EcoR1 segment containing the Cγ2b gene and less strongly to the 23 kb EcoR1 segment containing the γ2a gene. With one exception, the sizes of the EcoR1 segment were the same as those reported previously for DNA isolated from Balb/c mice (e.g. see 9). The 4.5 kb Cα segment present in EcoR1 digested DNA from several mouse strains (20) was not observed. Instead, two sub-segments (1.7 and 2.8 kb) were

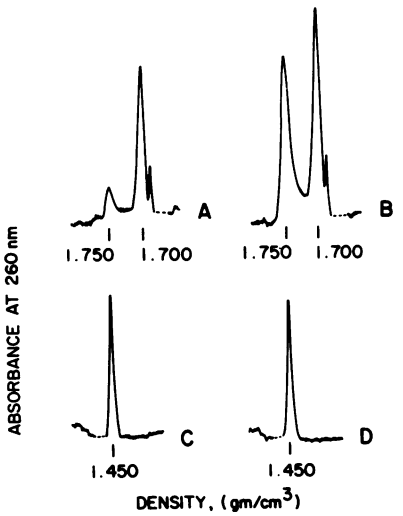

Figure 2. Isolation of BU-DNA, replicated during selected intervals of S, on density gradients of cesium salts. High molecular weight DNA was prepared from the pooled cell populations described in Figure 1 and examined by analytical equilibrium density-gradient centrifugation. Density standards were omitted from the tracings for clarity. (A,B) Centrifugation in CsCl of high molecular weight DNA from cells of class I and II, respectively. The density observed for the BU-DNA band corresponds to unifilar substitution of 96% of the thymine residues. The peak of unsubstituted DNA at 1.690 gm/cm^3 represents mouse satellite DNA. Satellite BU-DNA forms bands at 1.726 gm/cm^3 and at 1.761 gm/cm^3. The former is not resolved from main band BU-DNA. (C,D) Analytical centrifugation in Cs_2SO_4 of aliquots of the pooled BU-DNA fractions obtained after cleavage by EcoRI and preparative Cs_2SO_4 density-gradient centrifugation of DNA samples shown in A and B. Satellite DNA is not resolved from the main band in these gradients. Similar profiles were obtained from BU-DNA isolated from cells of class III and IV.

detected, using appropriate probes, in DNA from MEL cells cleaved with EcoRI (see Fig. 4). The M67-19A probe used in our studies hybridized to the 2.8 kb band. Thus, we determined the temporal replication of sequences about 2 kb from the Cα gene and sequences surrounding the Cγ2b and Cμ genes. The pJ11 probe was used to detect the 6.2 kb EcoRI segment containing the J_H region. The pBR1.4 probe was used to identify the 6.6 kb EcoRI segment adjacent (5') to the EcoRI segment containing the Cγ2b gene.

The relative concentration of each EcoRI segment containing immunoglobulin genes was determined from the areas of the corresponding bands on microdensitometer tracings of the autoradiograms. In reconstruction experiments, we have demonstrated (5) that these areas are directly

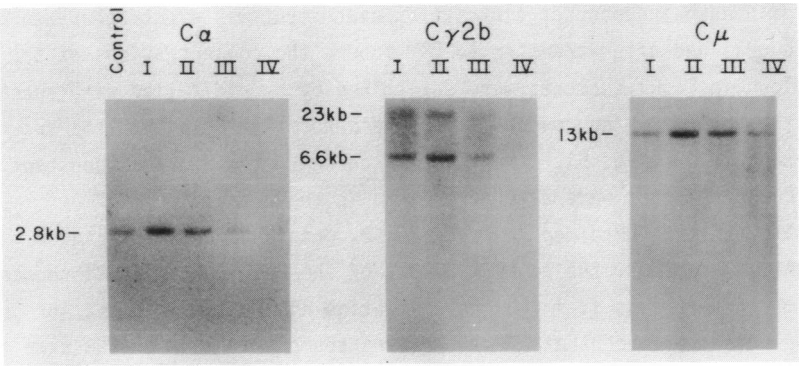

Figure 3. Autoradiograph of EcoR1 digest of BU-DNA after fractionation on an 0.8% agarose gel after transfer to DBM paper and hybridization to ^{32}P-labeled immunoglobulin heavy chain constant region probes. A HindIII digest of λ DNA was run in each gel as a molecular weight standard. The pattern of EcoR1 cleaved BU-DNA visualized by ethidium bromide staining of the gels before transfer was identical to unsubstituted DNA run as a control in a neighboring lane. In each gel, a sample of BU-DNA and unsubstituted DNA from unfractionated cells were also run as a control. One of these controls is shown above. Each lane contained 5 μg of mouse DNA and the hybridization mixture contained about 4 x 10^6 cpm/lane. Similar autoradiographs were obtained for DBM transfers prepared with BU-DNA from the other elutriation experiments in this study.

proportional to the concentration of the EcoR1 segments present in the BU-DNA.

Three independent experiments were carried out each involving independently maintained cultures of MEL cells that were grown in the presence of BUdR for either 1.75 or 2.5 hrs before different classes of cells were obtained by elutriation. As previously described (5) there was no significant difference in the results whether or not a thymidine chase was used.

Each set of four BU-DNA samples from the different classes of cells

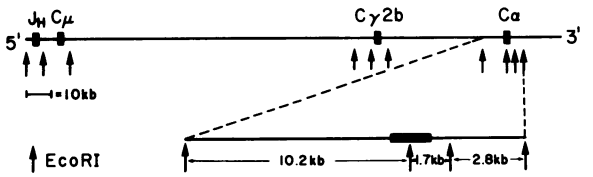

Figure 4. The top line shows the organization (see e.g. 9) of the three constant region genes for which the temporal replication of surrounding EcoR1 segments have been determined here. The lower line shows the position of an additional EcoR1 site in DNA from MEL cells that has not been observed in DNA from other mouse strains (20).

obtained in each independent elutriation experiment was electrophoresed on separate gels and after transfer to DBM paper, the concentrations of the immunoglobulin EcoR1 segments were determined by hybridization with specific radioactive probes. The concentrations were normalized so that the sum for each gene was 100 (Fig. 5). The relative concentrations of the constant region EcoR1 segments were also determined in the unsubstituted (nonreplicated) DNA obtained from the Cs_2SO_4 gradients shown in Fig. 2. In this DNA, as expected, the concentrations of the Cα and Cγ2b EcoR1 segments were highest for class IV in which replication did not occur (data not shown).

The relative concentration of the gene segment in each BU-DNA sample indicates the percent of cells in each class in which the segment replicated. From this we have calculated the nuclear DNA content (as a multiple of C, the haploid DNA content of MEL cells in G1) at the time that the cells replicate the gene segment. This is described below first for the Cα gene and then as a general method for use with the other constant region genes.

Temporal order of replication of EcoR1 segments containing constant region genes

The EcoR1 segment adjacent (3') to the Cα gene is present in the BU-DNA isolated from each of the four classes of cells shown in Fig. 1. The different concentrations of the gene present in these four BU-DNA samples indicates that the Cα segment was replicated in different proportions of the cells from each of the four cell classes. We assumed that the particular nuclear DNA content at which the Cα gene segment replicates should determine the proportion of the cells in a particular cell class replicating the Cα segment. We then calculated, the approximate nuclear DNA content that would be most consistent with the relative concentrations of EcoR1 segments (Fig. 5) and the distribution of cellular DNA content in each cell class (Fig. 1).

Before elutriation, each cell had synthesized DNA in the presence of BUdR so that the portion of the genome synthesized was about 0.67C (except for those cells that were in G1, G2, or M during the BUdR labeling period). We have observed (Furst and Schildkraut, unpublished results) that under our conditions, the nuclear DNA content increases approximately linearly with time as S proceeds. We used a computer generated average FMF profile for each of the four cell classes to calculate the average DNA content per cell (NC) as a multiple, N, of the haploid DNA content, C, of a cell in G1. These cells had synthesized BU-DNA from the time their DNA content was (N-0.67)C until the time their DNA content was NC. Based on this calculation, a comparison was

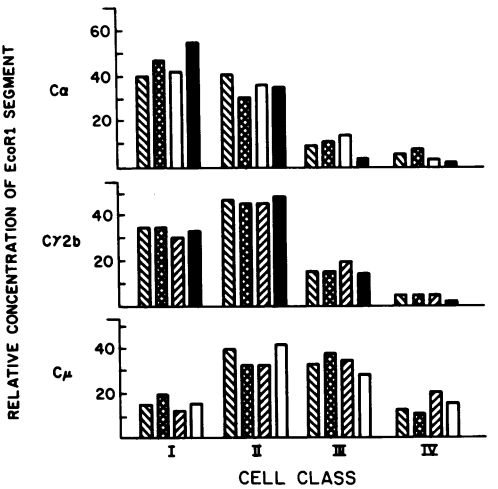

Figure 5. Relative concentrations of heavy chain constant region gene EcoR1 segments in BU-DNA replicated during selected intervals of S. Each DBM transfer was separately hybridized with each of the constant reigon gene probes to produce autoradiographs such as those shown in figure 3. The probe was then removed from the DBM transfers by treatment with NaOH and hybridized with a different probe. In each gel, at least two DNA samples isolated from exponentially growing cells were present as a control (not shown). The relative concentration of the EcoR1 segment containing the constant region gene in BU-DNA has been shown to be proportional to the intensity of the band produced in the autoradiograph (5). Results are shown for three separate elutriation experiments carried out with independently grown MEL cultures. The relative concentrations have been normalized so that their sum is 100. In one experiment, (FMF profiles shown in Fig. 1 and density gradient centrifugation shown in Fig. 2) separate aliquots of the BU-DNA samples were electrophoresed on three separate gels and transferred to DBM paper. The hatched bars represent the average results after hybridization to each of these DBM transfers. In a second experiment, cells were grown for 1.75 hr in the presence of BUdR (closed bars) while in a third experiment, 2.5 hr of growth in the presence of BUdR was followed by growth for 30 min in the presence of 10 μm thymidine (open bars).

BU-DNA was synthesized by most of the cells in classes I and IV for less than 2.5 hrs since most of these cells spent a portion of this time in G1 or G2. Thus, the BU-DNA synthesized in the cells of classes I and IV represented a smaller portion of the genome than the BU-DNA synthesized in the cells of the classes II and III. The portion of the genome represented in the BU-DNA was included in the algorithm of the computer program used for the analysis of this data. The calculated C values vary by ±0.1C depending on the value chosen for the interval of replication for the BU-DNA isolated from class I, however, this does not affect the temporal order of replication. The unique value of C at which each of the constant region segments replicates that is most consistent with the observed concentration of the segments was calculated as described in the text. The average data for one elutriation experiment shown above (hatched) was used in this calculation. For Cα, the value was 2.3C, for Cγ2b, 2.4C and for Cμ, 2.65C with a standard deviation of 10%. Similar calculations, assuming that more than one value of cellular DNA content determines the time of replication of each gene segment, require that most of these values are within ± 0.2 C of the unique C values.

made of the proportion of cells (in each cell class) in which BU-DNA had been replicated when their DNA content had reached a particular value of C. We determined the value of C at which the EcoR1 Cα segment replicated that would be most consistent with the concentrations of this segment observed in each of the cell classes (Fig. 5). This value of C was chosen for the gene replication time.

The concentration of the Cα gene EcoR1 segment was highest in BU-DNA from the cells of classes I and II (Fig. 5). The results are consistent with the Cα gene replicating early during S. We calculated that if this segment replicated only at a unique cellular DNA content, then the cellular DNA content at that time was 2.3 ± 0.2C. In agreement with the results shown in Fig. 5, 40-50% of the cells in the first two cell classes incorporated BU into the newly replicated Cα gene. Since cell class I is more homogeneous, the concentration of the Cα gene should be higher in this DNA. In addition, the concentration of Cα sequences in the BU-DNA of class III cells places an upper limit on the cellular DNA content at the time this gene replicated. Most of the cells (84%) of class III replicated BU-DNA at the time their DNA content was greater than 2.4 C. The concentration of the Cα sequence in BU-DNA from class III cells was four fold lower than in class I cells. Therefore, the cellular DNA content at the time this gene replicated was less than 2.4 C.

The nuclear DNA content at the time that the Cγ2b and Cμ EcoR1 segments replicated was calculated by the same procedure described above for the Cα gene. As noted in the legend to Fig. 5, this calculation provides a range of values for the nuclear DNA content that is consistent with the time each of these EcoR1 segments replicated. Further studies will allow us to determine whether each gene segment can replicate at more than one time within this range or whether each gene replicates when a unique nuclear DNA content is reached. The temporal order of replication that is most consistent with the data presented here for this MEL cell line is Cα, Cγ2b, Cμ.

The temporal replication of two additional EcoR1 segments was measured in duplicate synchrony experiments. For the J_H sequences, the relative concentrations of the EcoR1 segments present in BU-DNA replicated in the four different cell classes was 15 in class I, 40 in class II, 30 in class III and 15 in class IV. These values were similar to those obtained (Fig. 5) for the Cμ EcoR1 segment which is adjacent (3') to the J_H segment in DNA from the Balb/c mouse (see Fig. 4). The relative concentrations of the EcoR1 segment adjacent (5') to the γ2b EcoR1 segment were also measured. The relative concentrations were 33 in class I, 38 in class II, 19 in class III and 10 in

class IV. These values were similar to those obtained for the γ2b segment (Fig. 4).

DISCUSSION

We have measured the temporal replication of the immunoglobulin heavy chain constant region segments Cα (2.8 kb), Cγ2b (6.6 kb) and an adjacent (5') segment (6.6 kb), Cμ (12 kb), and J_H (6.2kb) defined by EcoRl restriction sites. In the MEL cell line, these sequences replicate in the temporal order Cα, Cγ2b, Cμ, during the first half of the S phase. By measuring the relative concentrations of these segments in BU-DNA samples replicated during different intervals of S, we have shown that each of these genes is located on (or for the Cα gene, directly adjacent to) a genomic segment that replicates in a different, restricted time interval in S.

It is difficult to assess from these experiments to what extent these constant region segments replicate at slightly different times in different cells or on different chromosomes. The temporal order of gene replication determined for the closely mapping genes in these studies requires the measurement of small quantitative differences (dependent on several variables) in concentration of EcoRl segments. One variable that should be particularly noted is the possible difference in EcoRl segment concentration due to size specific losses during DNA preparation. To assure minimum random shearing of DNA during isolation, we extracted by gently shaking, and high molecular weight was confirmed by low voltage electrophoresis of each preparation in 0.3% agarose gels as previously described (23). The length of the DNA prepared by the method used here was at least 75 kb.

Another technical problem, which limits our ability to discriminate between a model of single initiation sites or one primary and several secondary replication origins relates to the resolution of our elutriation method. However, our calculations of the expected distribution of temporally replicated segments based on the distribution of the cells with different per cell DNA content in each interval of S (see Figure 1) gives us results consistent with the hypothesis of a single initiation point for replication. In future experiments, we plan to define more exactly the width of the interval during which these genes are replicated by using a BU-labeling period that is much shorter than 2.5 hrs and by pooling fewer fractions to produce a greater number of cell classes. In one experiment performed to increase the resolution of the method, (solid bars in Fig. 5), the cells received a shorter (1.75 hr) BU-pulse. The concentrations of the Cα and Cγ2b genes were lower in

the cells of the classes III and IV, as would be expected for genes replicated during the first half of S. Our results indicate that less than 25% of the Cα and Cγ2b EcoR1 segments in these cells replicate during the second half of S. We are currently measuring the levels of immunoglobulin transcripts in MEL cells in order to determine the relationship between gene expression and time of replication for these genes.

Work from several laboratories has led to a complete map of the locations of eight murine heavy chain constant region genes and their flanking DNA sequences. The entire region in Balb/c mouse DNA has been cloned (about 200 kb) and all of the sites for several restriction endonucleases have been mapped (see 9 and reference therein). The arrangement of these constant region genes appears to be similar in C57B1/6 DNA (9). Similarities between the size of the EcoR1 segments obtained from other mouse strains and those from Balb/c have been reported (17). With the exception of the Cα segments, we have not detected any differences in the sizes of six EcoR1 constant region segments from those observed in Balb/c DNA. This suggests that the distances between the Cα, Cγ2b and Cμ genes are very similar in Balb/c and MEL DNA. The results described above suggest that the Cα and Cμ genes are also about 170 kb apart in the DNA from MEL cells (derived from the DBA/J mouse). From our calculations, the replication times during S of the Cα and Cμ sequences differ by as little as one hour or as much as two hours. Several possible models are consistent with these data. Our data are consistent with a single bidirectional fork beginning near the Cα gene and progressing in the 5' direction, towards the Cμ gene at a rate of approximately 1.8 kb per minute which is within the range observed for most mammalian cells (for review see 24). Consistent with these results we, have also found that the β-major and β-minor globin genes, which are much closer together (15 kb apart) than the constant region genes studied here, showed a very small difference in replication times (Braunstein and Schildkraut, unpublished results). Our results are not offered as a proof that sequence specific origins of replication exist in mammalian cells because the ordered replication of the immunoglobulin heavy chain sequences we have studied may be a result of nuclear architecture.

Another model consistent with our results is that these genes are located on more than one replicon. Our results indicate that if these genes were on three separate adjacent replicons completely contained within the cloned 200 kb region (see Fig. 4), they would not be simultaneously activated. In this case, they would differ from many other clustered replicons (reviewed in

24). Our data indicates that irrespective of the number of different replicons on which these specific segments are located, there is a defined temporal order of replication for these sequences.

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REFERENCES

1. Braun, R., and Willi, H. (1969) Biochim. Biophys. Acta 174, 246-252
2. Mueller, G.C. and Kajiwara, K. (1966) Biochim. Biophys. Acta 114, 108-115
3. Balazs, I., Brown, E.H. and Schildkraut, C.L. (1974). Cold Spr. Harb. Symp. Quant. Biol. 38, 239-245
4. Brown, E.H. and Schildkraut, C.L. (1979) J. Cell. Physiol. 99, 261-277
5. Furst, A., Brown, E.H., Braunstein, J.D. and Schildkraut, C.L. (1981) Proc. Natl. Acad. Sci. USA 78, 1023-1027
6. Haigwood, N.L., Jahn, C.L., Hutchinson, C.A. and Edgell, M.A. (1981) Nucleic Acids Res. 9, 1133-1150
7. Epner, E., Rifkind, R. and Marks, P. (1981) Proc. Natl. Acad. Sci. USA 78, 3058-3062
8. Marchionni, M.A. and Roufa, D.J. (1981) Cell 26, 245-258
9. Shimizu, A., Takahashi, N., Yaoita, Y. and Honjo, T. (1982) Cell 28, 499-506
10. Cory, S., Jackson, J. and Adams, J.M. (1980) Nature 285, 450-461
11. Davis, M., Calame, D.M., Early, P., Livant, D., Joho, R., Weissman, I. and Hood, L. (1980) Nature 283, 733-739.
12. Kataoka, T., Kawakami, T., Takahashi, N. and Honjo, T. (1980) Proc. Natl. Acad. Sci. USA. 77, 919-923
13. Lawton, A., Kearney, J., Cooper, M. (1980) In Biological Regulation and Devlopment, 2, Organization and Cell Function, R. Goldberg, ed. (New York:Plenum Press), pp. 437-466
14. Maki, R., Trauneker, A., Sakano, H., Roeder, W. and Tonegawa, S. (1980) Proc. Natl. Acad. Sci. USA 77, 2138-2142
15. Crissman, H.A. and Steinkamp, J.A. (1973) J. Cell Biol. 59, 766-771
16. Krishan, A. (1975) J. Cell Biol. 66, 188-193
17. Walker, P.M.B. and McLauren, A. (1965) J. Mol. Biol. 12, 394-409
18. Kissane, J.M. and Robbins, E. (1958) J. Biol. Chem. 233, 184-188
19. Wahl, G.M., Stern, M. and Stark, G.R. (1979) Proc. Natl. Acad. Sci. USA 76, 3683-3687
20. Marcu, K.B., Banerji, Penncavage, N.A., Lang, R. and Arnheim, N. (1980) Cell 22, 187-196
21. Schibler, U. Marcu, K.B. and Perry, R.P. (1978) Cell 15, 1495-1509

22. Lang, R.B., Stanton, L.W. and Marcu, K.B. (1982) Nucleic Acids Res. 10, 611-630
23. Fangman, W.L. (1978) Nucleic Acids Res. 5, 653-665
24. Edenberg, H.J. and Huberman, J.A. (1975) Ann. Rev. of Genetics 9, 245-284