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Regulation of the Replication of the Murine Immunoglobulin Heavy Chain Gene Locus: Evaluation of the Role of the 3′ Regulatory Region

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DNA replication in mammalian cells is a precisely controlled physical and temporal process, likely involving *cis*-acting elements that control the region(s) from which replication initiates. In B cells, previous studies showed replication timing to be early throughout the immunoglobulin heavy chain (Igh) locus. The implication from replication timing studies in the B-cell line MPC11 was that early replication of the Igh locus was regulated by sequences downstream of the Cα gene. A potential candidate for these replication control sequences was the 3′ regulatory region of the Igh locus. Our results demonstrate, however, that the Igh locus maintains early replication in a B-cell line in which the 3′ regulatory region has been deleted from one allele, thus indicating that replication timing of the locus is independent of this region. In non-B cells (murine erythroleukemia cells [MEL]), previous studies of segments within the mouse Igh locus demonstrated that DNA replication likely initiated downstream of the Igh gene cluster. Here we use recently cloned DNA to demonstrate that segments located sequentially downstream of the Igh 3′ regulatory region continue to replicate progressively earlier in S phase in MEL. Furthermore, analysis by two-dimensional gel electrophoresis indicates that replication forks proceed exclusively in the 3′-to-5′ direction through the region 3′ of the Igh locus. Extrapolation from these data predicts that initiation of DNA replication occurs in MEL at one or more sites within a 90-kb interval located between 40 and 130 kb downstream of the 3′ regulatory region.

The faithful duplication of the mammalian genome by DNA replication occurs in a physically and temporally ordered fashion during each round of S phase. In contrast to *Escherichia coli*, in which bidirectional DNA replication of a circular chromosome usually initiates at a single defined origin of replication (10, 26), the significantly larger genome in higher organisms requires DNA synthesis to initiate at multiple origins. Questions regarding where replication initiates and what controls replication initiation are fundamental in understanding the ordered program of DNA replication.

DNA replication in eukaryotes does not initiate from all origins simultaneously; rather, replication origins fire in a programmed manner at fixed intervals during S phase. A relationship between transcriptional activity and early timing of replication has been observed, suggestive of potential coregulation of the two processes. Housekeeping genes, accordingly, replicate relatively early in S phase in most cell types (34). Tissue-specific genes also generally replicate early in S phase in cell types in which they are expressed; however, they often replicate late when they are not transcribed. The β-globin locus, for example, is early replicating in murine erythroleukemia cells (MEL) and late replicating in other cell types, such as lymphocytes (13, 18). Significantly, the same origin, located between the δ- and β-globin genes, is utilized in both erythroleukemia cells and lymphocytes (references 1 and 20 and references therein).

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For the mouse immunoglobulin heavy chain (Igh) locus, the temporal order of Igh gene replication has been assessed by measuring the relative concentrations of *Eco*RI restriction fragment segments in 5-bromouracil-labeled DNA (BU-DNA) during various intervals of S phase (4). The murine Igh locus is comprised of variable region genes, including V (variable), D (diversity), and J (joining) segments, and constant region genes. (The Igh genes are arranged on chromosome 12 as follows: telomere-V-D-J-C_H-centromere. The terms "downstream" and "3′" are used in this article with reference to the transcriptional orientation of the Igh genes in B cells.) In non-B cells, such as MEL, the 3′-most constant region gene, namely, Cα, replicates earliest, with successive upstream genes replicating at progressively later intervals. Replication timing results are consistent with a single replication fork progressing at a rate of about 2 kb/min and initiating from an origin of replication located downstream of Cα (4). The specific location of this putative origin has not yet been determined.

In B cells (except for MPC11 [see below]), in which Ig genes have undergone a series of DNA recombination events (VDJ joining and class switching), all of the Igh gene segments examined replicate early in S phase (4). Early replication of the Igh locus in B cells can be explained by either the utilization of multiple origins or the rapid movement of a replication fork from a single origin. There is some evidence for the existence of multiple origins in B cells. Our studies using neutral-alkaline two-dimensional (2D) gel electrophoresis are consistent with replication forks from two or more origins within the Igh locus proceeding in both directions through the Cγ2b gene (13a). Furthermore, a putative origin of replication was postulated to be associated with the intronic enhancer, Eμ (2).

Analysis of the MPC11 mouse plasmacytoma line indicated that the early timing of replication of Igh genes in B cells

necessitated the presence in *cis* of sequences downstream of the Igh locus. In MPC11, as in other plasmacytomas, in addition to the expressed Igh locus on chromosome 12, there is a characteristic chromosomal translocation involving the Igh locus and the *myc* locus from chromosome 15. Igh sequences in the different chromosomal contexts exhibited dramatic differences in replication timing (5). The productively rearranged Cγ2b gene (chromosome 12) replicated early in S phase. In contrast, the Cγ2b gene, which is dissociated from the downstream portion of the Igh gene cluster as a result of its juxtaposition with c-*myc*, replicated late in S phase. The reciprocal translocation product, which contains *myc* gene segments associated with downstream Igh sequences, replicated earlier than *myc* sequences on the unrearranged chromosome 15 (5). These studies implicated the presence of regulatory regions located downstream of the Igh gene cluster which control early replication of associated sequences in B cells.

An attempt to identify these replication regulatory sequences in B cells as well as to localize the origin of Igh replication in MEL has required cloning of the sequences downstream of the Cα gene. This has been unexpectedly difficult due, in part, to the extensive palindromic nature of this region which has only recently been elucidated (6, 31). We and others have described a complex 3′ regulatory region spanning 40 kb downstream of Cα. Four B-cell-specific enhancers, each of which is associated with DNase I hypersensitivity, have been identified in the 3′ regulatory region (8, 21, 24, 25, 27, 29, 31), as shown in Fig. 1. The 3′ enhancers are thought to play roles in regulating isotype class switching (7), in maintaining high levels of Igh expression in plasma cells (22), and in potentially contributing to the regulation of such processes as VDJ recombination and somatic mutation. Stable transfection studies led to the inference that three of the four enhancers, namely, hs1,2, hs3B, and hs4, had the properties of a locus control region (LCR) (24). In its various roles, the 3′ regulatory region likely has effects on the chromatin structure of the entire locus, having an impact not only on transcription and rearrangement events but potentially also on DNA replication. Additionally, given the previously observed association between DNase I hypersensitivity sites and origins of replication (3, 9, 19, 32), this constellation of DNase I hypersensitivity sites was a candidate for a region containing an origin of replication.

Significantly, a role for the regulatory region located upstream of the human β-globin locus (LCR) in the control of DNA replication initiation of the human β-globin gene cluster has been observed. This was accomplished through examination of a somatic cell hybrid (T-MEL) in which the Hispanic thalassemia chromosome, which contains a deletion of a 35-kb region encompassing the ~10-kb human β-globin LCR, was introduced into a mouse erythroid cell (MEL) background. The β-globin locus in the Hispanic chromosome in T-MEL is not only DNase I resistant and transcriptionally inactive but also late replicating. In contrast, the β-globin locus in a normal human β-globin chromosome placed in the MEL cell context (N-MEL), like its normal mouse β-globin counterpart, is DNase I sensitive, transcriptionally active, and early replicating (12, 15). These results suggested a role for the LCR in determining replication timing. Furthermore, the region encompassing the β-globin LCR appears to play a role in origin usage since replication of the human β-globin locus in T-MEL cells, in which the LCR and adjacent sequences are deleted, no longer initiates from the origin within the locus but rather from an origin downstream of the locus (1).

The studies described here assess the involvement of the 3′ Igh regulatory region (putative LCR) on DNA replication timing and control at the mouse Igh locus in B cells by examining a myeloma variant in which the entire 3′ regulatory region has been deleted on one allele. We find that the Igh locus on this allele continues to replicate early despite the absence of the 3′ regulatory region, suggesting that, in contrast to the β-globin locus, *cis*-acting elements, other than the 3′ regulatory region, affect early replication timing. Furthermore, we have assessed the involvement of the 3′ regulatory region as an origin of replication in MEL by analyzing the replication timing of and direction of replication forks in sequences downstream of the regulatory region. Our studies indicate that the 3′ regulatory region is part of the Igh replicon in MEL and predict that the region in which replication originates lies between 40 and 130 kb downstream of the 3′ regulatory region.

MATERIALS AND METHODS

Cell culture. LP1.2 cells (17) were grown in Iscove's modified Dulbecco's medium (Biowhittaker, Walkersville, Md.), supplemented with 15% heat-inactivated fetal bovine serum (Life Technologies, Bethesda, Md.), and 100-U/ml penicillin, and 100-μg/ml streptomycin (PEN-STREP) at 37°C with 5% CO_2. S107 cells were grown in Dulbecco's modified Eagle medium (JRH Biosciences, Lenexa, Kans.), supplemented with 10% donor horse serum (JRH Biosciences), PEN-STREP, 10% NCTC (Life Technologies), and nonessential amino acids at 37°C with 10% CO_2. M12.4.1 cells were grown in RPMI medium (Biowhittaker), containing 10% heat-inactivated fetal bovine serum, PEN-STREP, and 50 μM β-mercaptoethanol at 37°C with 5% CO_2. MEL were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, PEN-STREP, and 2 mM glutamine. All cells were grown in suspension cultures.

5-Bromodeoxyuridine incorporation and centrifugal elutriation. LP1.2 cells (2×10^9) were grown in the presence of 5-bromodeoxyuridine (Sigma, St. Louis, Mo.) at a concentration of 20 μg/ml for 2.25 h. The cells were then fractionated by centrifugal elutriation as described elsewhere (4) in Iscove's modified Dulbecco's medium containing 1% serum at 1,350 rpm in an elutriator rotor for a J6B centrifuge at room temperature and collected in 20 fractions. The S-phase population of cells, as determined by fluorescence-activated cell sorter analysis on samples from each fraction, was divided into four pools, which were termed Early, Early-Middle, Late-Middle, and Late S. Each pool contained ~2×10^8 to 5×10^8 cells.

BU-DNA isolation and genomic Southern analysis. Genomic DNA from LP1.2 was prepared from the various S-phase fractions and subsequently digested with the restriction enzyme *Eco*RI. BU-DNA was separated from non-BU-DNA by means of Cs_2SO_4 density gradient centrifugation as described elsewhere (4). The concentration of BU-DNA was determined by A_{260} and confirmed by a fluorescence assay. Digested DNA (8 μg) from each S-phase pool as well as from random LP1.2 DNA was separated by electrophoresis on a Tris-acetate-EDTA agarose gel and then transferred to a Hybond-N^+ membrane (Amersham, Arlington Heights, Ill.). Hybridization was performed as previously described (31). The relative concentration of *Eco*RI fragments was quantitated by densitometric analysis using ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.).

2D gel electrophoresis. Matrix-associated DNA was prepared as described elsewhere (11). Additional steps to enrich for replication intermediates were as follows. Mouse cells contain significant amounts of high-molecular-weight (HMW) DNA that purifies along with the matrix-associated DNA and contains predominantly mouse satellite DNA. The satellite DNA is not digested by the restriction enzymes routinely used in the preparation of matrix-associated DNA. In order to eliminate the satellite DNA from the matrix-associated DNA fraction, sucrose gradient centrifugation in 0.8 M Na-EDTA-Tris (pH 8.0) was performed. The nuclear matrix fraction was layered on top of a 10 to 40% continuous sucrose gradient and centrifuged for 20 h at 83,000 × *g*, and 1-ml fractions were collected by pumping the gradient out from the bottom. The fractions were analyzed for size by agarose gel electrophoresis. In general, the first four to five fractions contained only HMW DNA (>23 kb), while fractions 5 to 15 contained DNA from 2 to 20 kb. Fractions not containing HMW DNA were pooled, and benzoylnaphthyl-DEAE–cellulose chromatography was performed (16, 23). DNA from the caffeine fraction was further examined by 2D gel electrophoresis.

Neutral-alkaline 2D gel electrophoresis was performed as described elsewhere (23, 28).

Immunofluorescence. Cells (LP1.2, S107, and M12.4.1) were washed twice in 1% bovine serum albumin in phosphate-buffered saline (PBS). Approximately 10^6 cells were cytocentrifuged onto each slide. The slides were air dried and then fixed in 5% acetic acid and 95% ethyl alcohol for 20 min at −20°C. The slides were rinsed twice in PBS and then incubated with varying dilutions of fluorescein isothiocyanate-labeled goat anti-mouse IgA antibody (Southern Biotechnology, Inc., Birmingham, Ala.) for 20 min at room temperature in a dark humid environment. Slides were then washed three to four times in PBS and mounted with Fluoromount G (Southern Biotechnology, Inc.). Slides were viewed by fluorescent microscopy on a Zeiss Axiophot light microscope. Quantitative anal-

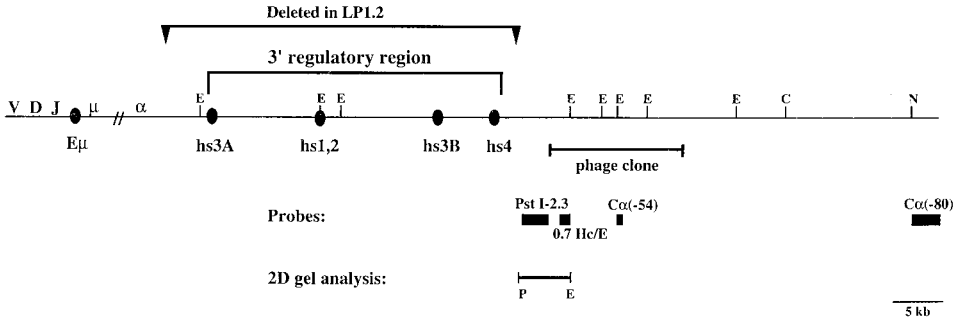

FIG. 1. Map of region 3′ of mouse Igh locus. Filled ovals represent enhancer elements. Enhancers in the 3′ regulatory region include hs3A (25), hs1,2 (8, 21, 29), hs3B (24), and hs4 (24, 27). The ~35-kb deletion in the mutant myeloma cell line LP1.2 includes all elements in the 3′ regulatory region (17, 27), as indicated by the arrows. The phage clone from which Cα(−54) was derived (see Materials and Methods) is shown. The location of probes used in these studies, as described in Materials and Methods, is shown. The fragment tested in 2D gel electrophoresis (Fig. 4) is indicated. E, *Eco*RI; C, *Cla*I; N, *Nco*I; P, *Pvu*II; Hc, *Hin*cIII. Not all sites are shown.

ysis was performed with a Bio-Rad MRC 600 laser scanning confocal microscope. NIH Image 1.55 software was used on a Macintosh Quadra 950 for quantitative analysis.

DNA probes. The following probes from the region 3′ of mouse Igh Cα were used in these studies. The probe *Pst*I-2.3 (17, 27) is a 2.3-kb *Pst*I fragment, located ~3 kb downstream of the Igh 3′ regulatory region, that was isolated from an LP1.2-derived clone. A 0.7-kb *Hin*cII-*Eco*RI fragment (0.7 Hc/E) (GenBank accession no. AF019135) that lies downstream of *Pst*I-2.3 was used to screen a partially *Sau*3A-digested 129-OLA library cloned in the *Bam*HI site of Charon 35 (originally received from Oliver Smithies and given to us by R. Kucherlapati and W. Edelmann, Albert Einstein College of Medicine). Within a 13-kb positively hybridizing phage insert was a 0.4-kb *Eco*RI/*Bam*HI fragment, Cα(−54) (GenBank accession no. AF005484), which is located ~12 kb downstream of the 3′ regulatory region and ~54 kb downstream of Cα in the BALB/c mouse. The probe Cα(−80) (GenBank accession no. AF017981 and AF017982) is a 2.3-kb fragment that was derived from the Sp6 end of a BAC clone (BAC145B12; see below) and resides ~80 kb 3′ of Cα in the BALB/c mouse. Figure 1 shows the location of these probes.

The β-globin probe is a 1-kb *Bam*HI/*Nco*I fragment spanning bp 52816 to 53852 (33) of mouse β-globin minor.

BAC cloning. A 129/Sv bacterial artificial chrosome (BAC) library (Bruce Birren, Whitehead Institute) was screened initially in pools by PCR using primers derived from Cα exon 3 and subsequently by single-plate spot hybridization with Cα exon 3 PCR product (6a). One of the Cα-containing BAC clones, BAC145B12, was shown also to contain Cα(−54) sequences by PCR. To clone the ends of the BAC, plasmid rescue was performed whereby the BAC was digested with *Cla*I and subsequently religated, leaving a 16-kb insert and releasing all internal *Cla*I fragments. The SP6 terminal 2.3-kb *Nco*I/*Not*I (*Not*I is a vector site) fragment [which we termed Cα(−80)] was subcloned into pUC18. Field inversion gel electrophoresis on a partial *Cla*I-complete *Not*I digest was performed with a Bio-Rad FIGE electrophoresis system. To determine the distance between Cα(−54) and Cα(−80), Extend Long Template PCR (Boehringer Mannheim, Indianapolis, Ind.) was performed with primers from the 3′ end of Cα(−54) and the downstream portion of the BAC with BAC145B12 as a template.

RESULTS

Isolation of sequences downstream of the Igh 3′ regulatory region. As noted in the introduction, analysis of replication timing in the mouse plasmacytoma cell line MPC11 implicated the region downstream of the Igh locus as responsible for early replication (5). Furthermore, the same general region is implicated as an origin of Igh DNA replication in MEL, as inferred from the work of Brown et al. (4). The ~40-kb region immediately downstream of Cα that spans the 3′ Igh regulatory region, as mentioned in the introduction, has only recently been fully described (8, 21, 24, 25, 27, 29, 31). In order to clone sequences downstream of the regulatory region, we screened a 129-OLA phage library (Materials and Methods) with probe 0.7 Hc/E from the extreme 3′ end of the 23-kb *Eco*RI fragment (Fig. 1). A 13-kb phage clone containing 0.7 Hc/E-hybridizing sequences which extended approximately 20 kb 3′ of the Igh 3′ regulatory region was isolated. However, of the 11 kb of sequences beyond the 0.7 Hc/E, all were repetitive in nature (Alu, L1, and VL30), except for a single unique 0.4-kb *Eco*RI/*Bam*HI fragment. The 0.4-kb fragment is located ~54 kb downstream of Cα, and thus the probe was named Cα(−54).

Additional downstream sequences were sought by identification of a Cα(−54)-hybridizing BAC clone (BAC145B12) from 129/Sv mouse DNA (see Materials and Methods). In order to determine the extent of DNA sequences contained within this clone, sequences from either end were isolated and analyzed. One end contained repetitive sequences from the L1 family, precluding precise localization; from the other end, a unique 2.3-kb fragment was identified. The first indication that this unique fragment was located downstream of the Igh gene cluster came from the observation that a panel of Igh-containing yeast artificial chromosome (YACs) was negative for hybridization to this fragment (data not shown). The determination that the 2.3-kb fragment was, in fact, linked to Cα(−54) was demonstrated by cohybridization of Cα(−54) and the 2.3-kb fragments to a single 80-kb fragment generated by partial restriction enzyme digestion of the BAC, after separation by field inversion gel electrophoresis (data not shown). Additionally, PCR analysis enabled us to link VL30 sequences at the 3′ end of the phage clone with the downstream portion of the BAC clone and to determine how far apart these sequences were located (data not shown). Together, these experiments allowed us to conclude that the BAC extends 22 kb beyond the Cα(−54)-hybridizing phage clone described above. We have designated the 2.3-kb fragment as Cα(−80), denoting its distance from Cα (Fig. 1).

Igh 3′ regulatory region does not control DNA replication timing in B cells. The implication from the altered timing of replication of the Cγ2b gene as a result of the MPC11 translocation was that sequences in the 3′ region of the Igh locus might control replication timing in B cells (5). To test whether the Igh 3′ regulatory region might be responsible for directing early replication of the Igh locus in B-lymphoid lines, we took advantage of the mutant myeloma cell line LP1.2 (low producing). The LP1.2 line has a reduction in Cα Ig expression compared to its parent, W3129, accompanied by a ~34-kb deletion in the expressed allele 3′ of Cα, which encompasses all four enhancers identified in the 3′ regulatory region (17, 27). These data implied that 3′ regulatory sequences were required for high levels of Igh expression in plasmacytoma cells. The residual low levels of expression presumably reflected retention of the intronic enhancer, Eμ.

We first established that deletion of the Igh 3′ regulatory region in LP1.2 resulted in uniformly decreased levels of Ig, as contrasted with a variegated phenotype, as previously observed

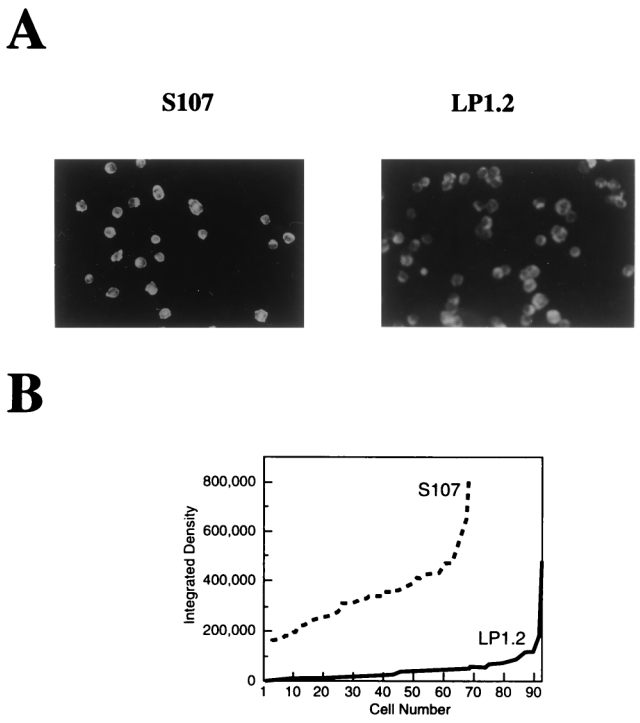

FIG. 2. Relatively uniform expression of Cα in LP1.2. (A) A sample field from slides of (high-IgA-expressing) S107 and (low-IgA-expressing) LP1.2 cells stained with αIgA antibody (see Materials and Methods) as viewed by light microscopy is shown. Cytoplasmic staining is relatively uniform in LP1.2 cells compared to control S107 cells. (B) Quantitative analysis of fluorescence intensity in LP1.2 compared to S107 cells as analyzed by confocal microscopy. Quantitation of integrated densities of LP1.2 cells (91 cells) and S107 cells (66 cells) was accomplished with NIH Image 5.5 software. Integrated density refers to the sum of the pixel values for a given cell. Cells are numbered from lowest- to highest-expressing cell for each of the two cell lines examined, and cell number refers to individual cells based on this assignment.

for other loci in which segments of LCRs were deleted (14, 30). It was possible that LP1.2 might contain multiple cell populations with different transcriptional states, each potentially having distinct replication timing characteristics. An immunofluorescence assay was employed to ascertain whether Ig gene expression was uniform in LP1.2. LP1.2 slides were prepared by cytocentrifugation and then treated with a fluorescein isothiocyanate-αIgA antibody (Fig. 2). High-IgA-expressing S107 cells and IgG2b-expressing (non-IgA-expressing) M12.4.1 cells were used as positive and negative controls, respectively. Results were analyzed by fluorescence microscopy as well as by subsequent quantitative analysis (see Materials and Methods). The LP1.2 cells exhibited relatively uniform levels of IgA expression with the exception of ~1 to 2% of the cells, as was observed for the S107 cells, which exhibited a higher overall level of expression as expected. Given the overall uniform phenotypic expression, the LP1.2 cells were suitable for our studies.

To analyze the timing of replication of the Igh locus in LP1.2, BU-DNA from LP1.2 replicated during successive intervals of S phase was obtained, as described in Materials and Methods. The *Eco*RI-digested Early, Early-Middle, Late-Middle, and Late S-phase DNA samples were subjected to genomic Southern blot analysis. As expected from previous studies (17, 27), the probe *Pst*I-2.3 detected a ~30-kb unrearranged fragment, in addition to the 6.2-kb fragment of interest on the expressed allele encompassing the 3′ LCR deletion (Fig. 1 and 3A). Assessment by hybridization of the relative concentrations of *Eco*RI segments in the BU-DNA from different intervals of S phase revealed that the highest concentrations of both segments were in Early S DNA. No significant differences were observed for the segment derived from the allele containing the deletion compared to the intact fragment. As a control, we assessed the timing of replication of sequences from the β-globin locus. As previously shown for murine B-cell lines, a probe from this region hybridized most strongly to DNA from Late S phase (Fig. 3A; Table 1). Together, these results indicated that the region 3′ of the Igh cluster maintained early timing of DNA replication even in the absence of the 3′ regulatory region.

To confirm these results, additional probes from the region beyond the deletion in LP1.2 were hybridized to the LP1.2 blot (Table 1). When probed with Cα(−54), which hybridizes to a 2.8-kb *Eco*RI fragment located ~8 kb downstream of *Pst*I-2.3, or with Cα(−80), which hybridizes to a 6.8-kb *Eco*RI fragment in MEL, the highest concentrations of newly replicated DNA were in the Early S DNA sample. Control blots from myeloma cell lines (MPC11 and S107) hybridized with Cα(−54) or Cα(−80) showed similar early replication timing (Fig. 3B; Table 1). Thus, the absence of the 3′ regulatory region did not result in a detectable change in replication timing.

Extended region 3′ of Cα is part of Igh replicon in MEL. The above results indicated that the 3′ regulatory region has no obvious role in regulating early replication in the B-cell lines. However, it is possible that the 3′ regulatory region might, in some instances, function as a region in which replication initiates. We could not easily use B-cell lines to address this possibility because early replication timing throughout the Igh locus in these cells (4) indicates the utilization of multiple adjacent origins or rapid movement of replication forks from a single origin. In MEL, our data assessing the temporal order of replication of the Igh locus pointed to a single origin downstream of Cα (4) (Fig. 4). The maximum distance at which the origin could be located with respect to the 3′-most gene had been estimated to be ~120 kb downstream of the Cα gene (4). If, however, replication from the origin does not initiate precisely at the start of S phase, or if pausing of replication forks occurs, then the origin would actually lie significantly closer than the maximum estimated distance, potentially within the 3′ regulatory region.

We addressed the possibility that DNA replication of the Igh locus could originate within the 3′ regulatory region by using recently cloned DNA downstream of the Cα gene to examine replication timing in MEL. BU-DNA that replicated during successive intervals of S phase was isolated, fractionated by gel electrophoresis, and subjected to genomic Southern blot analysis using the Cα(−54) and Cα(−80) probes (Fig. 3C). Quantitative analysis using densitometry was employed to assess the relative concentrations of DNA fragments that replicated during the four different intervals of S phase (Table 1). It was evident that while both the Cα(−54)-hybridizing 2.8-kb *Eco*RI fragment and the Cα(−80)-hybridizing 6.8-kb *Eco*RI fragment, located ~25 kb further downstream, replicated early in S phase, they both replicated relatively earlier compared to Cα (Table 1). Significantly, the times at which these two segments replicated follow the linear function previously observed for segments throughout the C_H gene region (4), as shown in Fig. 4. These results suggest that initiation commences downstream of Cα(−80), i.e., >40 kb downstream of the 3′ regulatory region. In fact, no evidence for replication initiating within this 3′ regulatory region was detected by neutral-neutral 2D gel electrophoresis using a segment within the 3′ regulatory region (13a).

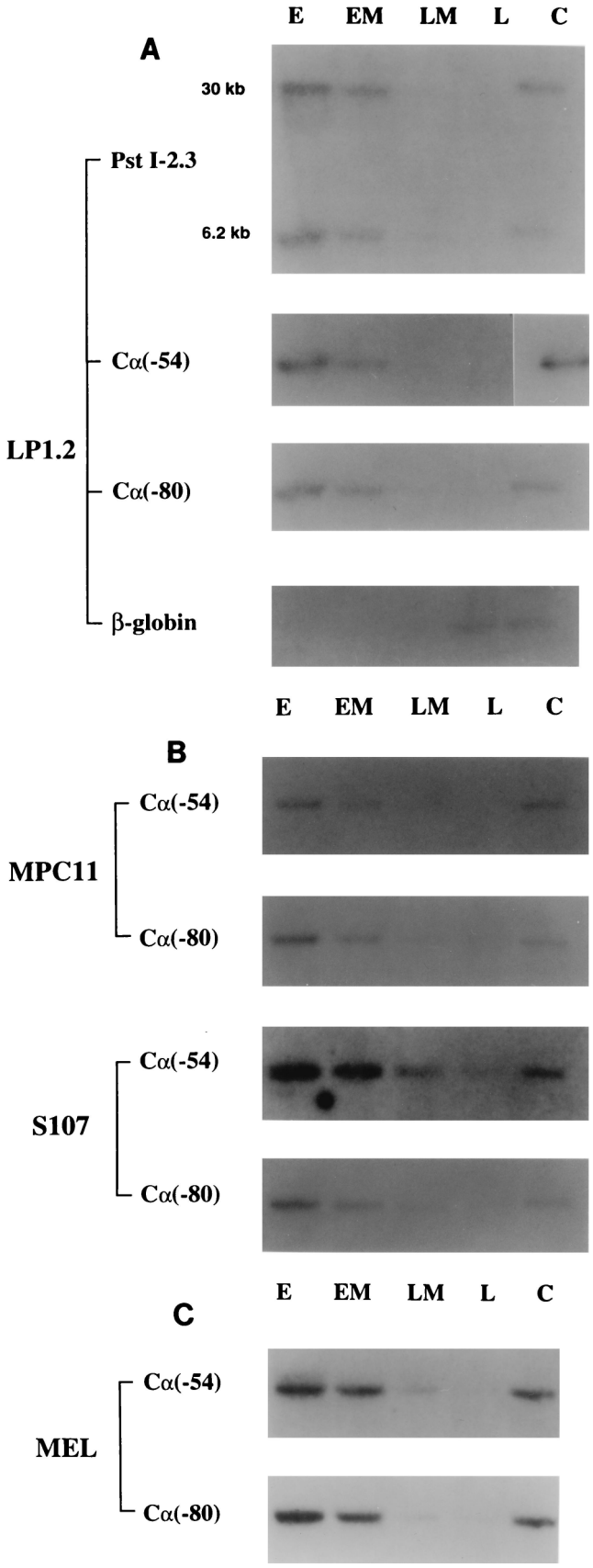

FIG. 3. Early replication of fragments downstream of Cα. Genomic Southern blot analysis of BU-DNA replicated during selected intervals of S: Early (E), Early-Middle (EM), Late-Middle (LM), and Late (L). Equal amounts of DNA were loaded in each lane. C refers to control DNA, indicating that total genomic DNA was loaded in these lanes. DNA from the following sources was used: mutant myeloma cell line (LP1.2) (A), B-cell lines (MPC11 and S107) (B), and non-B-cell line (MEL) (C). For MEL, five separate transfers were hybridized, and a representative blot is shown. Blots used for panels B and C were prepared as described elsewhere (4, 5). Probes used are as indicated. For description and location of probes, see Materials and Methods and Fig. 1.

Further support that replication initiates downstream of the 3′ regulatory region came from analysis of the direction of replication forks in this region. Neutral-alkaline 2D gel electrophoresis (Fig. 5) demonstrated that in MEL replication proceeded in the 3′-to-5′ direction. These data indicated that in MEL replication of the Igh cluster did not originate within sequences corresponding to the 3′ regulatory region.

DISCUSSION

Early replication of sequences 3′ of the Igh locus in B cells occurs independent of the 3′ regulatory region. In B cells, where the Igh locus is rearranged and expressed, the entire locus replicates early in S phase. Previous studies in the MPC11 cell line demonstrated that a translocation event, resulting in the dissociation of the Igh locus from its 3′ sequences, resulted in a shift from early to late replication of the Cγ2b gene, while the reciprocal c-*myc* gene, associated with 3′ Igh sequences, replicated correspondingly earlier (5). The implication from these studies was that *cis*-acting downstream Igh sequences are likely to influence the early replication timing of the adjacent genes.

We have tested whether the 3′ regulatory region is required for early replication timing of Igh sequences in B cells. We utilized the LP1.2 cell line, which contains a 34-kb deletion, including the four enhancers of the 3′ regulatory region plus ~4 to 5 kb upstream and ~2 to 3 kb downstream of the terminal enhancers (27). Deletion of this segment has been associated with a reduction in transcription of the locus in these cells (17). Our results show that the timing of replication of sequences that we have tested downstream of Cα in LP1.2 is not distinguishable from that of the same region in other B-cell lines. Hence, the 3′ regulatory region is not required for early replication of this portion of the Igh locus in B cells.

These results can be compared to the β-globin locus replication studies. It is evident from the experiments using the human β-globin deletion mutant T-MEL that there are *cis*-acting elements that direct replication that are distinct from the origin itself. The deletion at the T-MEL locus that affects replication timing and origin usage includes the LCR and more than 20 kb of upstream sequences (12). Whether the LCR alone, however, is specifically responsible for replication timing was not ascertained.

In the case of LP1.2, the deletion more precisely excises the 3′ enhancers, and hence, it is evident that the 3′ regulatory region alone is not essential for the early timing. The possibility that the Igh 3′ region might, in some instances, play some role in replication cannot be categorically ruled out. Perhaps deletion of the Igh 3′ regulatory region in LP1.2 has an impact on replication initiation from the adjacent origin of replication, but the effect was not observable due to the simultaneous early firing of other nearby origins, which may not be under the control of the 3′ regulatory region. It is also possible that sequences in the 3′ regulatory region might be involved in the early timing of replication of the adjacent sequences in non-B cells such as MEL, although this was not tested here. Nonetheless, these findings implicate other sequences, not excluding

TABLE 1. Relative concentration of segments in BU-DNA fractions from successive intervals in S phase[a]

Cell type	Cell line	Probe[b]	Relative concn (%) of segment in BU-DNA[c] from cell class:			
			E	EM	LM	L
Non-B	MEL[d]	Cα(−54)	51.5	33.5	9.6	5.4
		Cα(−80)	57.2	32.5	6.8	3.5
B-cell myeloma	MPC11	Cα(−54)	52.2	26.2	13.8	7.8
		Cα(−80)	59.2	23.7	9.4	7.7
	S107	Cα(−54)	51.4	31.3	12.3	5.0
		Cα(−80)	58.1	28.7	9.2	4.0
B-cell mutant myeloma	LP1.2	*Pst*I-2.3 (30 kb)	57.8	29.4	7.3	5.5
		*Pst*I-2.3 (6.2 kb)	56.2	29.5	10.6	3.8
		Cα(−54)	58.5	33.0	6.1	2.4
		Cα(−80)	57.8	30.6	9.5	2.3
		β-Globin[e]	5.8	16.1	20.1	58.0

[a] S-phase fractions are: Early (E), Early Middle (EM), Late Middle (LM), and Late (L).
[b] For a description of the probes, see Materials and Methods.
[c] Values were quantified by densitometry and ImageQuant software (Molecular Dynamics).
[d] MEL data is an average of hybridization of five separate blots.
[e] β-Globin probe (see Materials and Methods) was used as a control in the LP1.2 experiments for a region that replicates late in B cells.

the origin itself, in the control of replication timing in B cells. Additional sequences essential for early replication timing might lie adjacent to the 3′ regulatory region. However, given that several kilobases immediately downstream of the 3′ regulatory region contain repetitive sequences, they seem unlikely to constitute this regulatory segment.

Clearly the regulation of DNA transcription and replication of the Igh locus is more complex than in the β-globin locus. In addition to tissue-specific regulation of gene expression, Ig genes undergo gene rearrangements and somatic hypermutation during B-cell development. These processes are regulated by multiple elements, including an intronic enhancer, Eμ, in addition to enhancers within the 3′ regulatory region. Given the additional facets associated with the Igh locus compared to the β-globin locus, it may be the case that transcription and replication are jointly regulated at the latter but not the former locus. It is intriguing to consider the possibility that additional elements, perhaps adjacent to and distinct from the LCR, control replication at the β-globin locus.

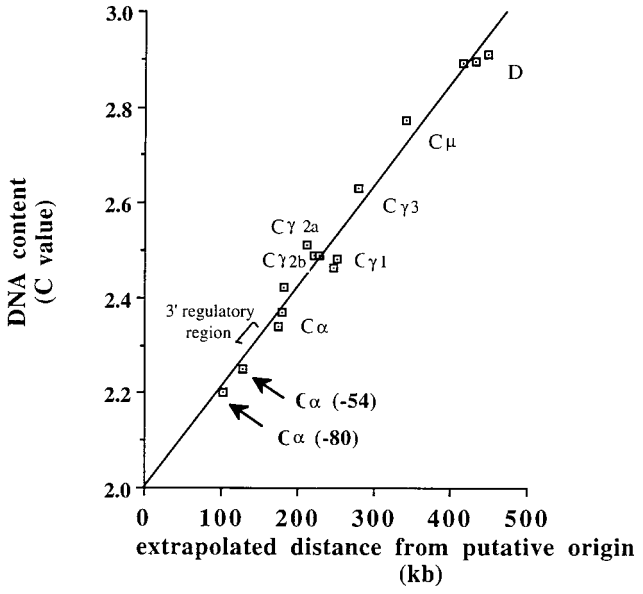

FIG. 4. Replication initiation in MEL is downstream of Igh 3′ regulatory region. C value, which designates nuclear DNA content at the time of replication (minimum value, 2.0; maximum value, 4.0), is calculated as described elsewhere (4) based on data in Table 1. The C value for Cα(−54) is 2.25, and that for Cα(−80) is 2.2. The Cricket Graph program was used to draw the extrapolated line. By extrapolation, a C value of 2.0 (indicative of replication initiation) would lie at 130 kb downstream of the 3′ regulatory region. For a description of probes Cα(−54) and Cα(−80), see Materials and Methods. The positions of additional points on the line were previously determined (4). The bracket denotes the relative position of the 3′ regulatory region.

Replication of the Igh locus in MEL initiates from an interval between 40 and 130 kb downstream of the 3′ regulatory region. Studies in B-cell lines did not exclude the possibility that Igh DNA replication initiated within the 3′ regulatory region. However, use of B cells to identify an origin of Igh DNA replication would be difficult because replication of the Igh locus occurs within a limited interval early in S phase and presumably involves multiple bidirectional replicons. We, therefore, turned to the MEL cell line, in which the Igh locus replicates throughout S phase, presumably as a single replicon which initiates from downstream of the locus (4). Using recently cloned probes, we have found that replication of the Igh gene cluster initiates significantly downstream of the 3′ regulatory region, as sequences 40 kb 3′ of the terminus of the 3′ regulatory region appear to lie on the same replicon as other Igh sequences.

Results from neutral-neutral 2D gel electrophoresis are consistent with the conclusions from the replication timing studies since initiation sites were not detected in this region in MEL with selected probes (13a). Furthermore, neutral-alkaline 2D gel electrophoresis analysis indicates that replication forks proceed in the 3′-to-5′ direction through the 3′ regulatory region. Should a single region of initiation be utilized, then, according to the model, replication would initiate 3′ of the regulatory region and proceed upstream at a rate of ~2 kb/min until the end of S phase. The Igh locus would thus be replicated from a single origin or initiation region and would constitute an unusually large (perhaps 1 Mb, if bidirectional) replicon. It should be noted, however, that one cannot formally exclude

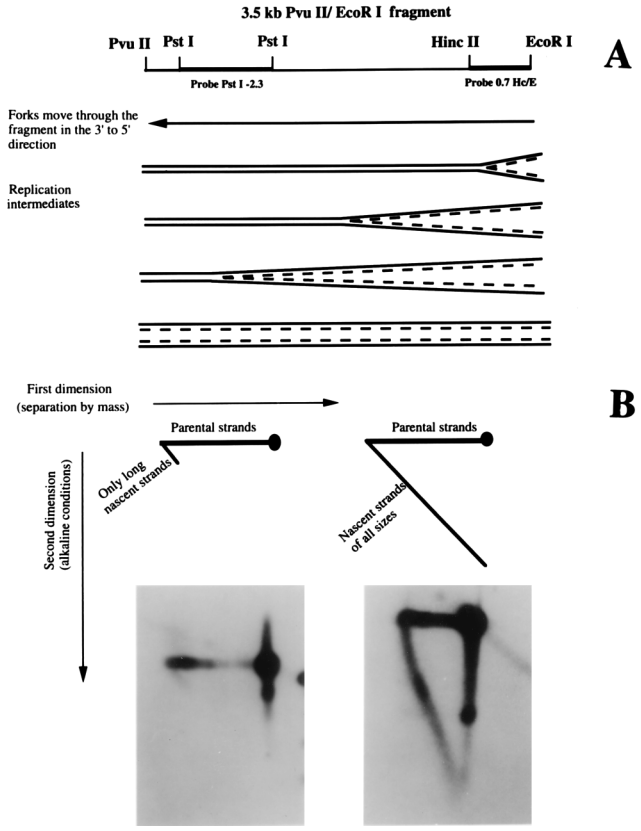

FIG. 5. Neutral-alkaline 2D gel electrophoresis of the *Pvu*II/*Eco*RI fragment located downstream of the 3′ regulatory region. For the location of the *Pvu*II/*Eco*RI fragment, see Fig. 1. To perform neutral-alkaline 2D gel electrophoresis, DNA associated with the nuclear matrix was first isolated (11) from an exponentially growing population of MEL and then fractionated by sucrose gradient centrifugation. Fractions containing DNA fragments with a molecular size of less than 23 kb were collected. DNA containing replication intermediates was purified with benzoylnaphthyl-DEAE–cellulose (16, 23), and 2D gel electrophoresis was performed. In the first dimension, DNA was separated primarily according to the mass of the fragment. The arrow in the figure shows the direction of migration of the replication intermediates in the first dimension. As a fragment is replicated, its mass doubles, so that the distance a fragment migrates is proportional to the amount of replication that has occurred. The second dimension was performed under alkaline conditions. The arrow indicates the migration of the replication intermediates in the second dimension. Under alkaline conditions, the nascent strands (dashed lines) are separated from the parental strands and migrate according to size. For forks moving through the fragment in the 3′-to-5′ direction, the probe at the 3′ end of the fragment will detect nascent strands of all sizes. The probe at the 5′ end of the fragment will detect only long nascent strands. (A) The restriction map of the fragment is shown at the top of the figure with the probes indicated by black rectangles. Below the map is a diagram showing the direction of replicative fork movement (3′ to 5′). (B) The expected neutral-alkaline 2D gel pattern that is generated by replicative intermediates if forks proceed in the 3′-to-5′ direction is shown. Hybridization results are shown below the expected patterns. When hybridization was performed with the 0.7 Hc/E probe that is located at the 3′ end of the fragment, nascent strands of all sizes were detected (right panel). However, only long nascent strands can be detected by the *Pst*I-2.3 probe (left panel), indicating that replication forks proceed from 3′ to 5′ through this fragment.

the possibility that multiple origins, which replicate exclusively in the 3′-to-5′ direction, may be sequentially fired.

Based on the extrapolation shown in Fig. 4, we predict the localization of the putative downstream origin in MEL to a 90-kb interval commencing downstream of Cα(−80), i.e., more than 40 kb and extending up to a maximum of 130 kb downstream of the 3′ regulatory region (or from 80 to 170 kb downstream of Cα). The cloning of sequences within this region is in progress. In the β-globin cluster, the same origin is utilized in both erythroleukemic and lymphoid cells. It remains to be determined whether an origin of DNA replication 3′ of the Igh locus is similarly utilized in both MEL and B-cell lines.

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