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Evidence that a Single Replication Fork Proceeds from Early to Late Replicating Domains in the IgH Locus in a Non–B Cell Line

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Summary

In non–B cell lines, like the murine erythroleukemia cell line (MEL), the most distal IgH constant region gene, *C*α, replicates early in S; other heavy chain constant region genes, joining and diversity segments, and the most proximal Vh gene replicate successively later in S in a 3′ to 5′ direction proportional to their distance from *C*α. In MEL, replication forks detected in the IgH locus also proceed in the same 3′ to 5′ direction for ~400 kb, beginning downstream of the IgH 3′ regulatory region and continuing to the D region, as well as within the *Vh81X* gene. Downstream of the initiation region is an early replicating domain, and upstream of *Vh81X* is a late replicating domain. Hence, the gradual transition between early and late replicated domains can be acheived by a single replication fork.

Introduction

The time during S phase in which different replicons are replicated is regulated. In general, loci that are expressed in a tissue-specific manner replicate during the first third of S in cells in which they are expressed but often replicate later in S in cells in which they are not expressed (see e.g., Goldman et al., 1984; Hatton et al., 1988; Simon and Cedar, 1996). For example, the β-globin gene cluster replicates early in the human K562 cell line in which it is expressed, but late in S in HeLa cells in which it is not expressed (Dhar et al., 1988): regardless of expression, the same initiation site is used (Aladjem et al., 1995). A similar pattern of timing of DNA replication has been observed for genes for α-fetoprotein and albumin, the T cell receptor β chain constant regions and the cystic fibrosis transductance regulator (Goldman et al., 1984; Hatton et al., 1988; Simon and Cedar, 1996). Each replicates early in S in cells in which they are expressed and late in cell lines in which they are silent. There are certain interesting exceptions to this observation. For example, the mouse α-globin cluster and genes coding for complement proteins C4 and factor B are early replicating in cell lines where they are not expressed (Hatton et al., 1988). It has been suggested that these genes replicate early in S phase because they may be located near an early replicating region (Goldman et al., 1984; Hatton et al., 1988; Simon and Cedar, 1996).

On a larger scale, mammalian chromosomes are organized in early and late replicating regions that show overall congruency with R and G bands, respectively (reviewed in Craig and Bickmore, 1993). Many of these regions have been observed by autoradiography to be replicated by clusters of replicons, which appear to be relatively synchronously activated during specific intervals early or late in the S phase (reviewed in Hand, 1978). Little is known, however, about how the transition from early to late timing of DNA replication occurs. There are no examples in mammalian cell lines of a late replicating replicon located within an early replicating region nor an early replicating replicon within a late replicating cluster. In addition, sharp transitions (with one possible exception; Tenzen et al., 1997) between early and late replicating regions have not been observed (Strehl et al., 1997; Bilyeu and Chinault, 1998). One mechanism to account for the progressive changes in replication timing between clusters of replicons activated in different intervals of S phase would be the sequential activation of replicons in the transition region. Another mechanism, for which we present evidence here, is a single replication fork progressing from the last in a cluster of early activated replicons to the first in a cluster of late activated replicons.

A unique pattern of replication timing is observed (see Figure 1) for the immunoglobulin heavy chain gene (IgH) locus (and to some extent the Ig kappa light chain locus; Hatton and Schildkraut, 1990). In non–B cells, like the murine erythroleukemia cell line (MEL), replication timing of specific segments of the IgH locus is linearly proportional to their map locations: sequences 3′ of the locus replicate very early during S phase in non–B cells (Michaelson et al., 1997), and replication of successive IgH segments, that is, Ch genes and Jh and Dh segments, becomes progressively later in S phase (Brown et al., 1987). All of the variable region segments we have examined—occupying at least 700 kb at the 5′ side of the IgH locus—replicate during the second half of S (Calza et al., 1984). Replication timing is different in cells of the B lineage, in which DNA rearrangements and expression of antibody genes exclusively occur. In both mature B cells and pre–B cells, sequences immediately 3′ of the IgH locus, the entire IgH coding locus, and the expressed Vh genes all replicate early in S phase (Brown et al., 1987; Michaelson et al., 1997; O. V. E., unpublished data). In pre–B cells, early replication also extends to the entire Vh gene locus (Hatton et al., 1988).

To begin to understand the molecular mechanism that regulates the timing of DNA replication in the immunoglobulin heavy chain constant region gene (IgH-C) locus, we examined the direction of replication fork movement through this locus in MEL (non–B) cells by neutral/alka-

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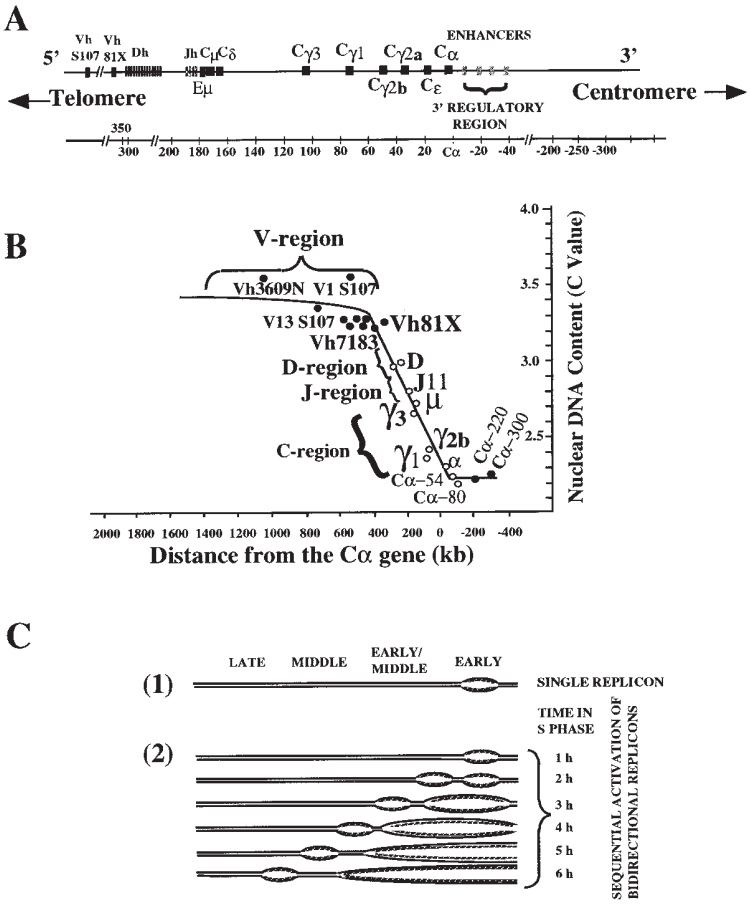

Figure 1. The Immunoglobulin Heavy Chain Region Links Early and Late Replicated Zones of the IgH Locus

(A) Map of the IgH locus in non–B cells (MEL). The germline configuration of the IgH locus on murine chromosome 12q32 is shown (Shimizu et al., 1982). The entire locus is approximately 3 Mb in size and contains more than 100 variable region genes (Vh) organized in fifteen families, four joining segments (Jh), twelve diversity segments (Dh), and eight constant region genes (Ch), represented by filled squares. The 3′RR and the array of Ch, Dh, and Jh gene segments occupies ~400 kb and is referred to as the IgH-C locus. Only two variable gene families, VhS107 and Vh7183, are shown above. *Vh81X* is the most 3′ member of the most proximal variable region gene family (Vh7183), all members of which are located in an interval of 100–150 kb. The Eμ enhancer is located between the Jh segments and *C*μ gene (Gillies et al., 1983; Friedman and Brewer, 1995). Downstream of the *C*α gene, four enhancers, represented by shaded squares, comprise the 3′RR (Saleque et al., 1997). In non–B cells, such as MEL, the locus is unrearranged and transcriptionally inactive. The 3′RR is in an inactive chromatin configuration and does not display any DNase I hypersensitivity (reviewed in Birshtein et al., 1997). The scale in kb is shown below the map. The position of the *C*α gene is indicated at zero. The left to right, 5′ → 3′, orientation of the IgH locus is with respect to transcription of IgH genes in B cells.

(B) Time in S when replication of the murine IgH locus occurs in MEL (non–B) cells. The y axis indicates the nuclear DNA content (C values) at which IgH segments replicate as a function of their distance in kb (x axis) from the *C*α gene. C is the haploid DNA content of the cells at the time in S phase when a particular DNA segment replicates. For example, at the G1/S boundary the C value is 2.0, and after DNA replication is completed, the C value is 4.0. The *V1* gene, a member of the VhS107 family, is located 200–250 kb 5′ of *Vh81X*. The other three members of this family are spread over a region of at least 200 kb further upstream. The *Vh3609N* gene is about 700 kb upstream from *Vh81X*. Some of the data (open circles) presented here have been published previously (Brown et al., 1987; Michaelson et al., 1997). Replication timing was carried out as previously described (Brown et al., 1987). Probe positions (determined to within at least ± 50 kb) and timing data for segments located at *C*α−300 and *C*α−220 as well as positions for segments upstream of *Vh81X* are newly obtained.

(C) Models for the replication of the IgH-C locus in non–B cell lines. Each small bubble represents a single bidirectional replicon. Larger bubbles represent regions where forks from two or more replicons have converged. (1) Only one bidirectional replicon is activated near the beginning of S phase, and a single replication fork progresses in the direction of the Vh region gene families at a rate of about 1.5 kb/min. Constant region genes replicate during the first half of the S phase, D region genes in the middle S phase, and V region genes replicate during the second half of S. (2) Several bidirectional replicons are activated sequentially in the S phase in the IgH locus. Positions in this figure are approximately as shown on the map in (A).

line (N/A) two dimensional (2D) gel electrophoresis (Nawotka and Huberman, 1988). Together with the results of neutral/neutral (N/N) 2D gel electrophoresis (Brewer and Fangman, 1987; Friedman and Brewer, 1995), the data presented here are consistent with a model of a single replication fork that replicates IgH-C and may progress as far as 400 kb. These studies have enabled us to identify a putative initiation region(s) from which replication forks originate. In MEL cells, the IgH-C locus represents a gradual transition between early and late replicated zones. It remains to be determined whether a single replicon represents a general method for producing the transition between early and late replicating temporal domains of mammalian chromosomes.

Results

The IgH-C Locus Represents a Gradual Transition between Early and Late Replicating Regions

Recent studies have generated a complete contig of the IgH locus in overlapping yeast and bacterial artificial chromosomes (YACs, BACs) (C. C. and R. R., unpublished data), providing the basis for a complete map of the locus (Figure 1A), determination of the distances between the genes, and additional probes, all of which were essential for the further studies of DNA replication in this locus presented here. Utilizing these newly identified probes for sequences both 3′ of and throughout the IgH locus, we were able to assess the relationship

between distances and timing, as summarized in Figure 1B. The Cα gene replicates near the beginning of S, and replication along the locus becomes progressively later in proportion to the distance from Cα. Importantly, downstream of Cα (starting at about Cα−80, that is, 80 kb downstream of Cα), the temporal pattern of DNA replication is not proportional with respect to distance. Both Cα−220 and Cα−300 replicate very early in S: Cα−220 at C = 2.28 and Cα−300 at C = 2.3. Hence, a region of at least 250 kb, as assessed by four sequences downstream of Cα (Cα−54, −80, −220, and −300) replicates very early in S phase.

In the variable region, we find that *Vh81X*, which is the most 3′ member of the Vh7183 family and is located 20–60 kb 5′ of the most 5′Dh gene, replicates at 3.3 C, with the same linear relationship of distance from Cα to time (Figure 1A). However, this relationship is no longer evident for other variable region genes, including the other six members of the Vh7183 family, the four members of the VhS107 family, and the most distal gene analyzed, *Vh3609N* gene (which is located ~700 kb 5′ of *Vh81X*); all replicate in approximately the same interval late in S phase, from 3.3 C to 3.6 C.

Together, these data indicate that in MEL, the timing of DNA replication is regulated differently for the IgH locus and its flanking sequences. A region of at least 300 kb downstream of Cα is early replicating, while the Vh genes upstream of the constant region are late replicating. Between these early and late replicating regions, sequences extending over a 400 kb region from Cα−80 through the Ch, Jh, and Dh gene segments to the first Vh gene, *Vh81X*, replicate progressively later in S phase.

Models for the Replication of IgH-C Constant Region Genes

Replication timing data for IgH sequences in MEL cells could best be explained by two different models (shown in Figure 1C). The first (Figure 1C [1]) involves activation of a single replicon that is located in the earliest replicating region downstream of Cα. One replication fork would then proceed through the eight constant region genes, J and D regions, and finally the most 3′ heavy chain variable gene (*Vh81X*). IgH sequences in this interval would comprise a single replicon. The second model (Figure 1C [2]) involves the sequential activation of more than one bidirectional replicon in the IgH-C locus during the S phase. These models can be distinguished by analysis of the direction of replication fork progression as assessed by N/A 2D gel electrophoresis (Nawotka and Huberman, 1988). If replication forks originate from a single downstream origin, forks will progress in only one direction through the IgH-C locus (Figure 1C [1]). In contrast, replication forks from multiple bidirectional replicons activated sequentially would progress outward in both directions.

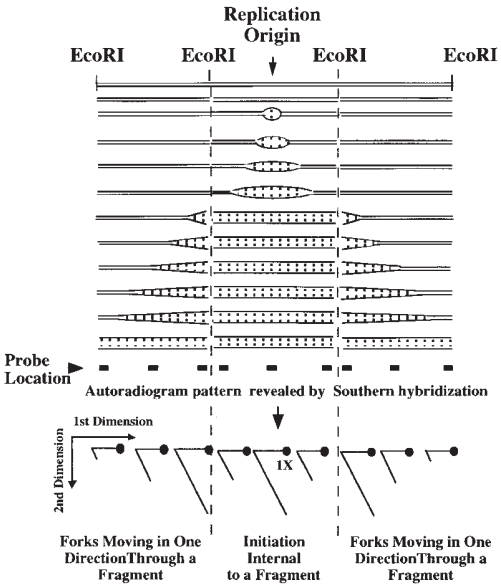

Figure 2. Neutral/Alkaline (N/A) 2D Gel Electrophoresis Detects the Direction of Replication Fork Movement and Site of Initiation of DNA Replication

In the upper half of the figure are schematic representations of the different replicative intermediates that are generated in three adjacent restriction fragments. The origin of bidirectional replication is located in the restriction fragment in the center, and forks proceed outward from the origin in both directions. Unbroken lines represent parental strands, and dotted lines represent nascent strands. Below the replicating strands, black rectangles indicate the locations of the probes used for the N/A 2D gel electrophoresis. The lower portion of the figure shows the Southern transfer hybridization patterns expected from each probe. In the first dimension, DNA is separated primarily according to mass. The second dimension is performed at alkaline pH so that nascent strands separate from parental strands and migrate according to their size, as shown at the bottom of the figure (Nawotka and Huberman, 1988). The 1X spot is indicated and represents the linear molecules of the same size as the restriction fragment. The direction of replication fork movement is revealed by hybridization to probes located at different positions relative to the ends of the restriction fragments. If forks move from the right to the left end of the restriction fragment (shown on the left part of the figure), the probe from the right end of the restriction fragment detects nascent strands of all sizes. The probe from the left end will detect only long nascent strands. The probe from the middle of the restriction fragment detects only nascent strands that are not less than half the size of the restriction fragment. If the forks move through the restriction fragment from left to right (shown on the right part of the figure), the probe from the left end detects nascent strands of all sizes and the probe from the right end detects only long nascent strands. The restriction fragment in the middle contains an origin of bidirectional replication, so that forks move from the center to both left and right of the restriction fragment. In this instance, probes from either end of the restriction fragment detect only nascent strands, which are not smaller than half of the size of the restriction fragment. The probe in the middle of the segment detects nascent strands of all sizes that originate from the initiation site.

Direction of Replication Fork Movement through the IgH-C Locus, Revealed by N/A 2D Gel Electrophoresis

Due to the complexity of the mammalian genome and the extremely low abundance of molecules actively replicating single copy loci, a series of steps to enrich for replication intermediates was crucial prior to electrophoresis (see Experimental Procedures). First, MEL cells from an exponentially growing culture were fractionated by size by centrifugal elutriation (Brown et al., 1987), and cells in early, early middle, and middle late fractions

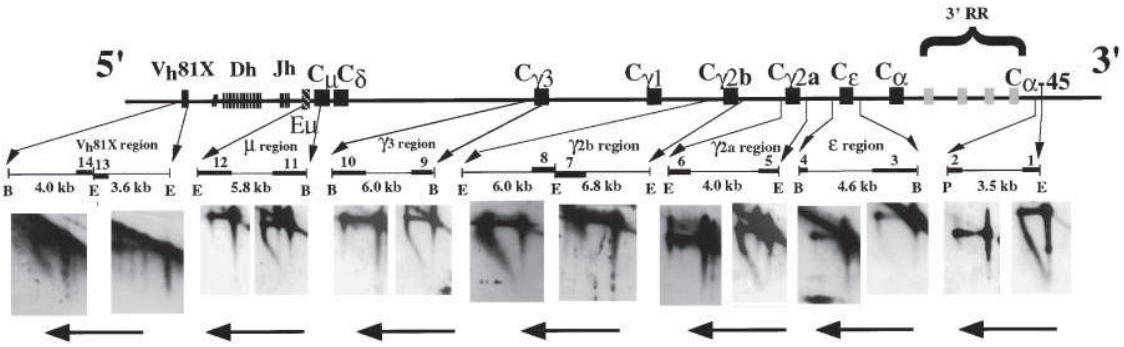

Figure 3. Replication Forks Originate Downstream of the 3′ RR and Progress in the Direction of the Vh Region in MEL Cells

Restriction fragments (size is indicated below each fragment) examined by N/A 2D gel electrophoresis are shown below the map. The position of the probes at 3′ or 5′ ends of the restriction fragments are indicated by the solid rectangles, and the probes are summarized in Table 1. Autoradiograms are shown under each restriction fragment. DNA was obtained from cells in the early and early middle stages of S phase, except for the analysis of the 3.5 kb PvuII/EcoRI fragment (DNA prepared from exponentially growing cells) and for the *Vh81X* segment (DNA prepared from the middle portion of S phase). In other experiments (data not shown), the 3.5 kb PvuII/EcoRI fragment was used to analyze DNA obtained from cells in the early and early middle stages of S; and γ*3* region and μ region segments have been examined using DNA prepared from exponentially growing cells. In each case, we observed the same results. Each 3′ end probe detects nascent strands of all sizes, while 5′ end probes detect only long nascent strands. This indicates that replication forks originate downstream of the 3′ RR and continue to progress in the 3′ to 5′ direction. For several segments (ε region, γ*2a* region, and γ*2b* region), after hybridization using the 5′ end probe, the transfer was stripped and a 3′ end probe was used. Note that probes 7 and 8, as well as 13 and 14, are located in close proximity to each other flanking an EcoRI site. In these two regions (γ*2b* and *Vh81X*), the 3′ and 5′ end probes detect two neighboring segments rather than the same segment. Thus, for these two pairs, the pattern is reversed, and the small nascent strands are observed in the autoradiogram on the left. Arrows under autoradiograms indicate the direction of replication fork movement. E, EcoRI; B, BamHI; P, PvuII.

of S phase were collected separately. DNA associated with the nuclear matrix was then isolated and applied to a sucrose gradient. DNA devoid of high molecular weight fractions was then separated by benzoyl naphthyl DEAE– (BND-) cellulose chromatography. Figure 2 schematically represents how N/A 2D gel electrophoresis reveals replication fork movement from a bidirectional origin of DNA replication.

Figure 3 shows the direction of replication fork movement through IgH sequences in MEL, as examined by N/A 2D gel electrophoresis. For example, the most 3′ segment of this region, a 3.5 kb PvuII/EcoRI fragment in the 3′RR (extreme right in Figure 3), has been examined as follows. After N/A 2D gel electrophoresis, DNA from the gel was transferred to a membrane and hybridized to probes 1 and 2 for either end of the 3.5 kb PvuII/EcoRI segment (*C*α−45) on two different blots, or sequentially with intermediate stripping. Linear molecules of the size of the restriction segment (1X spot, see Figure 2) are detected at the upper right part of the autoradiogram (shown below the segment). The horizontal line emanating from the 1X spot represents the parental strands. The vertical line that originates from the 1X spot reflects nicked molecules and is generally indicative of the quality of the DNA preparation. Nascent strands are found along a diagonal line emanating from parental strand molecules. The probe from the 3′ end of the fragment detects nascent strands of all sizes. As seen on the adjacent autoradiogram, the probe from the 5′ end of the segment detects only large nascent strands. These results show that replication forks move in the 3′ to 5′ direction through the 3.5 kb PvuII/EcoRI segment, implying that they originate from a region located further 3′, that is, downstream of this segment. These data are consistent with the timing data shown in Figure 1B.

As shown in Figure 3, we have used twelve probes (Table 1) to examine seven segments of IgH-C sequences. A probe for the 3′ end of each segment detected nascent strands ranging from ~300 bp to the full length of the linear segment while a probe for the 5′ end detected only nascent strands of large sizes. The exclusive identification of large replicative intermediates at the 5′ end of the segment is not due to the preferential loss of small products during DNA preparation because these small products can be detected at the 3′ end of the same segment on the same DNA transfer (Figure 3). These results show that replication forks progress from the 3′ end to the 5′ end of each of the seven segments.

To further extend these studies, we have also performed N/A 2D gel electrophoresis on two segments containing the *Vh81X* gene and have found that replication forks also progress in the 3′ to 5′ direction (Figure 3). Since the *Vh81X* gene replicated later in S phase, we examined replication intermediates from exponentially growing cells as well as from cells in the middle part of S phase. Together with studies on timing (Figure 1B), which indicate that *Vh81X* replicates with the same linear relationship as do the Ch genes, this result suggests that a single bidirectional replicon replicates Ch, Jh, Dh, and the *Vh81X* gene, although we cannot exclude activation of an additional replicon that is located in the Dh region. These results are most consistent with a single bidirectional replicon with an initiation region that is located downstream of *C*α−45.

Several important controls have been carried out to validate the 2D gel techniques used here. The majority of the N/A gels (and many of the N/N 2D gels [see below]) were performed two or more times using independent matrix preparations with the same result. In addition, we showed that, as expected in N/A 2D gel electrophoresis, a probe from the middle of a segment detected

Table 1. Probes from the IgH Locus that Have Been Used

Probe Number	Probe Preparation	References
1	0.7 kb HincII/EcoRI fragment, located near the 3′-most EcoRI site in 3′RR	(Michaelson et al., 1997)
2	PstI-2.3 is a 2.3 kb PstI/PstI fragment; contains an internal PvuII site that cuts the probe into 1.6 kb and 0.7 kb segments. The 1.6 kb part of the probe is located at the 5′ EcoRI/PvuII segment.	(Gregor and Morrison, 1986; Michaelson et al., 1995)
3	2.0 kb XbaI/BamHI probe has been excised from a 4.6 kb BamHI/BamHI segment (p4.6Cϵ) containing ϵ constant region gene coding sequence; located at 3′ end of the BamHI/BamHI fragment.	(Calvo et al., 1991)
4	0.3 kb BamHI/KpnI probe, that is located at the 5′ end of the 4.6 kb BamHI/BamHI (p4.6Cϵ) segment containing ϵ constant region gene coding sequence.	(Calvo et al., 1991)
5	The 0.8 kb XbaI/EcoRI probe, located at the 3′ end of the 4.2 kb EcoRI segment (pSγ2a-3), encompassing the switch region for the *Cγ2a* gene.	(Eckhardt and Birshtein, 1985)
6	The 1.0 kb EcoRI/SacI probe located at the 5′ end of the 4.2 kb EcoRI segment (pSγ2a-3), encompassing the switch region for the *Cγ2a* gene.	(Michaelson et al., 1995)
7	The EcoRI/BglII probe is located upstream of the *γ2b* gene coding sequence. The 1.9 kb probe is derived from the 6.8 kb pγ2bR1.4 subclone and is located at the 5′ end of the EcoRI segment.	(Roeder et al., 1981; Lang et al., 1982)
8	pBR1.4 probe is located at the 3′ end of the 6.0 kb EcoRI segment that encompasses the *γ2b* switch region.	(Roeder et al., 1981; Lang et al., 1982)
9	The 1.9 kb HindIII/BamHI probe at the 3′ end of the 6.0 kb BamHI/BamHI fragment (pγ3-6/0), which contains 2.0 kb *Cγ3* gene.	(Lang et al., 1982)
10	The 2.0 kb BamHI/KpnI probe, which encompasses the *Cγ3* protein coding sequence, is located at the 5′ end of the 6.0 kb BamHI/BamHI segment (pγ3-6/0).	(Lang et al., 1982)
11	The probe from the 3′ end of the 4.5 kb EcoRI/BamHI segment (J-14) is a 2.0 kb HindIII/BamHI fragment, which contains exon CH1 and part of exon CH2 of the *Cμ* gene. In MEL cells, an EcoRI/BamHI segment (J-14) is 5.8 kb.	(Lang et al., 1982)
12	The 5′ end probe is a 900 bp EcoRI/SacI fragment located at the 5′ end of the 4.5 kb EcoRI/BamHI segment (J-14); the SacI site is located 3.3 kb from exon CH1 of the *Cμ* gene. In MEL cells, this EcoRI/BamHI segment (J-14) is 5.8 kb.	(Lang et al., 1982)
13	0.8 kb AseI fragment containing the 3′ half of the *Vh81X* gene from pVh81X	(Yancopoulos et al., 1984)
14	0.9 kb DraI fragment from pVh81X	(Yancopoulos et al., 1984)
15	The probe is 2.3 kb located at *Cα*-80.	(Michaelson et al., 1997)
16	The hs 4 enhancer probe	(Michaelson et al., 1995)
17	The hs3 enhancer probe is 1.2 kb XbaI/Sau3A.	(Saleque et al., 1997)
18	J-11 probe is 2.0 kb BamHI/EcoRI fragment	(Lang et al., 1982)
19	1.6 kb probe prepared from BAC395D22 is located at the 3′ end of the HindIII segment.	(Sidow et al., 1997; present study)
20	A 1.8 kb NotI fragment containing the 3′ end of the Serrate gene	(Lan et al., 1997)
21	The 500 bp probe from the 3′ end (Sp6 end) of BAC 397G20	(present study)
22	1.0 kb probe is from the 3′ end of BAC 225H9.	(present study)
23	A 1 kb EcoRI/BamHI fragment of the *Vh3609N* gene	(Tutter et al., 1991

nascent strands of intermediate sizes and not the smallest sizes (data not shown). Furthermore, there is no technical limitation that preferentially results in the detection of forks proceeding exclusively in the 3′ to 5′ direction. Based on studies of the murine β-globin locus in which an initiation region has been mapped by three different techniques (M. Aladjem et al., personal communication; L. H. N., O. V. E., and C. L. S, unpublished data), we expected replication forks to proceed predominantly in a 5′ to 3′ direction (opposite to IgH) when probing the 3′ side of an initiation region. When the same DNA transfers used to study the IgH region were probed for the β-globin locus, we detected these predicted forks (L. H. N., O. V. E., and C. L. S., unpublished data).

While we have been able to obtain probes spanning the locus, unique probes are limited in number due to the unusual repetitive content of this locus. In addition to a relatively high content of Line1 elements (Herring et al., 1998), the IgH locus is comprised of families of related and cross-hybridizing elements, that is, the Ch, Jh, Dh, and Vh gene families. Therefore, we have not been able to examine overlapping segments throughout the IgH locus, and we cannot exclude the possibility that there are additional smaller replicons in the regions not yet examined. In addition, in a few instances, we have used the same probe to detect the 3′ end of one segment as well as the 5′ end of an overlapping segment. In a few instances, we have also used a probe at the 3′ end of one segment and the 5′ end of an adjacent segment. Based on timing data, putative smaller replicons would have to be activated sequentially in S phase. Moreover, if there are additional bidirectional replicons in the locus, it seems likely that replicative intermediates other than those containing a single replication fork (i.e., molecules containing replication bubbles or converging replication forks) would be detected in the studies using N/N 2D gel electrophoresis, as described below.

Shape of Replicative Intermediates in the IgH Locus Revealed by N/N 2D Gel Electrophoresis

We examined ten segments between the *Cα* gene and the J region, and we have only detected replication intermediates that migrate as molecules containing a single replication fork (Figure 4). Importantly, we did not detect initiation and termination events, an observation

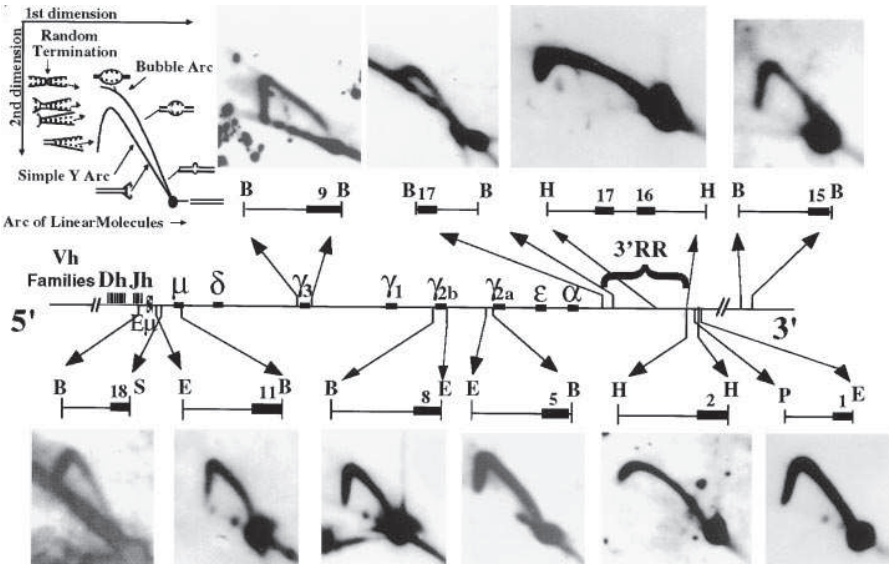

Figure 4. Initiation and Termination Events Have Not Been Detected in MEL by N/N 2D Gel Electrophoresis

The relative positions of the restriction fragments that have been examined are indicated, and the probes within restriction fragments are shown as black rectangles. A description of the probes (see numbers) is presented in Table 1. Like N/A 2D gel electrophoresis, the N/N 2D gels also separate the DNA molecules according to their size in the first dimension. In the second dimension, the gel conditions maximize the effect of the shape of DNA molecules on their migration. This technique can detect replication intermediates that correspond to initiation events, and to termination and to single replication forks proceeding through the segment (Brewer and Fangman, 1987; Friedman and Brewer, 1995). Only replication intermediates in which forks progress from one end of the segment to the other end were detected in the IgH locus. E, EcoRI; B, BamHI; H, HindIII; P, PvuII; S, SacI.

that argues against the presence of bidirectional sequentially activated replicons in the IgH-C locus. As a control for these studies, using nuclear matrix-associated MEL cell DNA and N/N 2D gel electrophoresis with the same techniques as in the present study, we were able to detect replicative intermediates containing bubbles indicative of a replication origin in the mouse rDNA locus (P. Galgano and C. L. S., unpublished data). Thus, the absence of detectable replication intermediates containing replication bubbles in the IgH locus is not due to a limitation of the technique but, rather, is characteristic of the locus itself.

Other investigators used an estimation of the abundance of nascent strands to identify a specific, relatively weak origin of DNA replication within 500 bp of the intronic enhancer, Eμ, in both B cells and in fibroblasts (Ariizumi et al., 1993). Neither of the 2D gel electrophoresis techniques used here detected this origin. N/A 2D gel electrophoresis did not identify any replication forks that would originate from an Eμ-containing DNA segment. Nor did we detect this origin by N/N 2D gel analysis, even when we examined an Eμ DNA segment (Figure 4, BamHI/SacI segment) in which the putative origin of DNA replication is located within the middle third of the examined segment, where initiation is best detected. Thus, if this Eμ-associated origin is present in MEL cells, it must be weak.

Direction of Replication Fork Progression Changes in the Early Replicating Region Located Downstream of the 3′ RR

Probes located at the 3′ end and at the 5′ end of two overlapping segments at *C*α−100 (probe 22) and *C*α−220 (probe 21) and one segment at *C*α−300 (probes 20 and 19) were used to determine replication fork direction (Figure 5). Nascent strands of all sizes were detected in all instances, indicating that replication forks progress in both directions. In contrast, replication forks progress exclusively in the 3′ to 5′ direction through *C*α−45 (Figure 3). Thus, the direction of replication fork movement changes within a region of ~55 kb, in the interval between *C*α−45 and *C*α−100, consistent with the location of an initiation region within this interval. Therefore, we have identified the region from which the replication fork originates that makes the transition between the early and late replicating boundaries of the IgH locus. Furthermore, at least one additional initiation region must be located downstream of *C*α−300, although other initiation regions may also be present in the interval between *C*α−100 and *C*α−300. Interestingly, as previously shown (Figure 1B), the replication timing of sequences downstream of *C*α−80 no longer changes with distance.

Discussion

Examination of DNA replication of the IgH gene cluster in MEL, based on previous data and data presented in this report, has shown that: (1) DNA segments between *C*α−300 and *C*α−80 replicated early during S phase; (2) segments within a 400 kb interval including Ch, Jh, Dh, and the first variable region gene, *Vh81X*, replicate progressively later in direct proportion to their distance from the putative earliest replicating sequences; and (3) other variable region gene segments detected replicate late in S. These studies indicate that the IgH replicon is

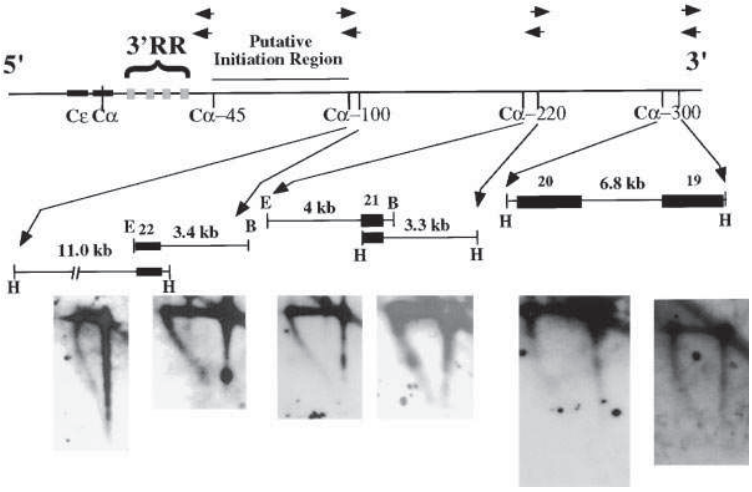

Figure 5. Direction of the Replication Fork Movement in the Region Downstream of the 3′RR

The size of examined fragments and their approximate distance from the *C*α gene, as obtained by mapping of BAC clones, are indicated. Probes utilized to assay the direction of replication fork movement are indicated by black rectangles. The autoradiograms of 2D gel electrophoresis are presented under the fragments. The direction of replication fork movement, as determined by N/A 2D gel electrophoresis, is indicated by arrows at the top of the figure. Two overlapping segments have been examined at *C*α−100. The probe (probe 22) from the 3′ end of one segment, which is also at the 5′ end of the overlapping segment, detects nascent strands of all sizes. This result demonstrates that the replication forks move in both directions through this segment. A putative initiation region has been denoted in the region where the change in direction of replication forks occurs. For the segments at *C*α−220, similar results were obtained using two different DNA transfers from independent experiments. Probes from both ends of the restriction fragment at *C*α−300 (probes 20 and 19) also detect nascent strands of all sizes, indicating that replication forks progress in both directions through this segment. E, EcoRI; B, BamHI; H, HindIII.

bounded on one side by an early replicating region and a putative initiation region located 3′ of the 3′RR and on the other side by late replicating Vh region genes.

We performed N/A 2D gel electrophoresis on seven segments within the 400 kb region that encompasses the IgH-C locus and two restriction segments in the vicinity of the nearest variable gene, *Vh81X*. We conclude that replication forks progress exclusively in the 3′ to 5′ direction; however, we would not detect less than about 10% of replication forks if they progressed in the opposite direction. While the lack of unique probes prevented examination of a ~100 kb region that contains the array of Dh gene segments, our studies show that the replication time of the *Vh81X* gene is consistent with the same linear relationship between replication time and distance from the *C*α gene as observed for all the Ch, Jh, and Dh segments. Additionally, N/A 2D gels indicate that replication forks progress through the segment containing the *Vh81X* gene in the same direction as observed for the other IgH segments that we have studied. These data strongly suggest that about 400 kb of the IgH gene cluster is replicated by a single replicon, which is activated at the beginning of S phase and progresses until a substantially late time (C = 3.4) in the S phase (Figure 6).

We cannot totally rule out the possibility of multiple, sequentially activated unidirectional replicons by these experiments. Except at potential initiation sites, the N/A 2D gel electrophoresis patterns would be identical for a single bidirectional replicon and for multiple sequentially activated unidirectional replicons. In addition, N/N 2D gel electrophoresis should detect initiation or termination events in particular fragments, and our experiments detected neither. It remains possible, however, that if initiation or termination occurred in different segments in different cell populations, their frequency at any single location could be so low as to preclude detection.

On the other hand, there is limited evidence for unidirectional replicons in mammalian cells. Autoradiography studies have estimated that only 5% of mammalian replicons could be unidirectional (Hand, 1975). Although unidirectional replication has been described for plasmid replication of prokaryotes, mitochondrial DNA, and some viral genomes (Kornberg and Baker, 1992), it has not been reported in yeast or for the few replicons studied on the molecular level in mammalian cells. In addition, replicons in mammalian cells that are located in proximity to each other are usually activated simultaneously, but not sequentially (Huberman and Riggs, 1966; reviewed in Hand, 1975). Therefore, we conclude that our data are most consistent with a single replication fork proceeding through the IgH-C locus in a 3′ to 5′ direction. This replicon is activated near the beginning of S phase in cells, like MEL, in which the IgH locus is not expressed. Evidence for relatively large replicons has been previously presented (e.g., Yurov et al., 1977; reviewed in Hand, 1978).

Localization of Initiation Events

Replication forks progress in both directions in sequences located at *C*α−100, *C*α−220, and *C*α−300 downstream of the IgH-C locus (Figure 5). These data suggest the presence of an initiation region or a sequence-specific bidirectional replication origin in the interval between *C*α−45 and *C*α−100 (for a schematic representation, see Figure 6). These data also provide evidence for at least one additional replicon, which originates downstream of *C*α−300. Other replicons may be located between *C*α−100 and *C*α−300. If discrete initiation sites are present, we should detect segments where replication forks progress predominantly in one direction. Indeed, at one location (*C*α−120), the majority of replication forks proceeds in one direction (data not shown). Alternatively, the identification of replication forks moving in both directions could be explained by an initiation zone. Forks progressing in both directions have previously been detected in rDNA (Little et al.,

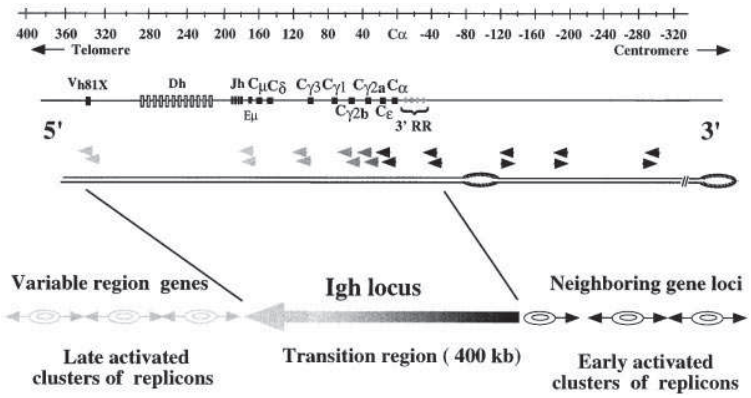

Figure 6. Summary of Replication Fork Movement in the Murine IgH Locus in MEL (Non–B) Cells

The structural organization of the locus is shown in the upper part of the figure. Segments that have been assayed by N/A 2D gel electrophoresis are shown by pairs of arrows (schematically represented in the middle part of the figure); each arrow represents the location of the probe. These data are consistent with a single replication fork progressing in only one direction through the IgH-C locus from 3′ of 3′RR to 5′(*Vh81X*) for ~400 kb. Replication forks originate downstream of the 3′RR, within an ~55 kb region (*C*α−100 to *C*α−45) where we detected a switch in the direction of replication fork progression. Further 3′, replication forks move in both directions, indicating the presence of a second replicon in the region located at least 300 kb downstream of *C*α. The bubbles represent either site-specific initiation or a delocalized initiation zone. The IgH-C region, Jh, Dh, and first variable gene (*Vh81X*) represent a transition between early and late replicated regions (shown at the bottom). A single replication fork apparently originating from the early activated replicon travels for almost the entire S phase to the late replicated region. The gradient indicates the gradual change in replication timing of the IgH-C. Early and late replication is achieved by clusters of replicons activated at similar times in S. The bottom diagram is not aligned with the others in the figure.

1993) and in the DHFR initiation zones (Dijkwel et al., 1994).

Further detailed studies are in progress using N/N 2D gels to detect initiation events and to localize initiation site(s) in the IgH gene cluster. It will then be of interest to compare the IgH-associated initiation regions with others that have been intensively studied, that is, dihydrofolate reductase (DHFR) in Chinese hamster ovary cells (for review see Dijkwel and Hamlin, 1996; Kobayashi et al., 1998), ribosomal RNA genes (Little et al., 1993; Yoon et al., 1995; Gencheva et al., 1996), and the β-globin gene locus in human cells (Kitsberg et al., 1993; Aladjem et al., 1995, 1998).

The IgH Locus as a Model for Understanding Transitions between Other Early and Late Replicated Chromosomal Regions

In the present study, we provide evidence that multiple replicons are activated in the early replicated region downstream of *C*α. Furthermore, our observation that Vh genes at the other end of the IgH locus that are located several hundred kb from each other replicate within a narrow interval late in S phase also suggests the presence of several replicons. Connecting these early and late replicating domains is the IgH-C locus, through which it is likely that a single replication fork progresses during most of the S phase. This replication fork appears to originate from the last in a cluster of early activated replicons and proceeds to the first in a cluster of late activated replicons (Figure 6).

In the budding yeast, *S. cerevisiae*, a single replication fork has similarly been shown to provide the transition between early and late replicating regions on chromosome III. An origin is located at ARS 305 near the middle of the chromosome (Huberman et al., 1988). Initiation occurs early in S, and replication forks then progress toward the late replicating left telomere (Reynolds et al., 1989; Dubey et al., 1991). In mammalian cells, however, our studies of the IgH cluster represent a unique analysis of the direction of replication fork progression between early and late replicating DNA sequences (Figure 6).

The IgH locus could represent a model for many other similar transition regions that are present in mammalian genomes. For example, timing of DNA replication was shown to change gradually at a R/G band boundary on chromosome 13 (between 13q14.3/q21.1) over a distance of 500 kb (Strehl et al., 1997), an observation originally interpreted to result from progressively later activation of additional origins of DNA replication. A similar gradual transition has been observed between cytogenetic band boundaries on the human X chromosome (Bilyeu and Chinault, 1998). Another transition in DNA replication timing has been identified within a 100 kb boundary between R and G bands located between classes II and III in the human major histocompatibility complex (Tenzen et al., 1997). Each of these observations could be accounted for by a single replication fork that accomplished the transition.

Evidence for a Developmentally Regulated Origin

Replication of the IgH locus is very different in cell lines of the B lineage in which IgH genes are expressed. For example, in pre–B cell lines, the entire IgH locus—from downstream of *C*α and including all of the IgH-V genes—replicates early in S, and a transition region no longer exists. Coupled with additional results of N/A 2D gel electrophoresis in which we detected replication forks moving through IgH-C genes in both directions (O. V. E., L. H. N., and C. L. S., unpublished data), we postulate that at least one additional, B cell–specific origin is activated in the IgH-C locus. In MEL cells, in which this origin(s) is silent, it is likely that the IgH locus is replicated by a single replication fork and forms a transition region between early and late replicated domains. It seems likely, therefore, that the temporal arrangement of DNA replication in mammalian cells is a dynamic developmentally regulated process, accompanied by the activation or silencing of origins of DNA replication. Further

experiments will determine the mechanisms by which silencing or activation of origins occurs.

Experimental Procedures

Cell Culture

Mouse erythroleukemia cells (MEL) were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (GIBCO BRL), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C with 5% CO_2. Cells were maintained as an exponential culture at a density between 2×10^5 and 7×10^5 cells/ml.

Centrifugal Elutriation

Centrifugal elutriation was performed as described (Brown et al., 1987). Cells were fractionated according to size at 1340 rpm in the JE 10X elutriator rotor of a J6B Beckman centrifuge at 4°C. The stage of the cells in S phase was determined by fluorescence-activated cell sorter analysis. Cells in the early and early middle S phase fractions were used to prepare matrix-associated DNA.

DNA Preparation

Nuclear matrix-associated DNA was isolated as described (Dijkwel et al., 1991). Following dialysis and precipitation, DNA from $\sim 5 \times 10^8$ cells was digested with the appropriate restriction enzyme(s) in a volume of 3 ml for 4 hr. This additional restriction digestion was necessary since the matrix fraction otherwise showed a significant amount of partial digestion products (our unpublished data). Mouse matrix-associated DNA contains a high molecular weight (HMW) fraction, which is not digested by enzymes used in its preparation. The HMW DNA (probably the satellite DNA component) binds to the BND column and interferes with the chromatography procedure.

To separate HMW DNA from the matrix-associated DNA fraction, a sucrose gradient is necessary. A 30 ml 10%–40% continuous sucrose gradient in 0.8 M NaCl, 20 mM Tris-HCl (pH 7.4), and 5 mM EDTA was prepared using a gradient maker. Matrix DNA from 1 to 5×10^8 cells in a volume of 0.5 to 5 ml was layered on top of the gradient. The tubes were centrifuged using the Beckman SW28 swinging bucket rotor at 26,000 rpm (83,000 g) at 15°C for 20–24 hr. One milliliter fractions were collected by pumping the gradient out from near the bottom of the tube (3 ml remains in the tube as bottom fraction, and 27 1 ml fractions were collected). Every second fraction was analyzed on a 0.5% agarose gel to determine which fractions contained the DNA fragments in the desired size range. The first four to five fractions contained predominantly HMW DNA and were discarded. The desired fractions, which in most experiments were fractions 5–15 (containing fragments from <1 kb up to 20–23 kb), were pooled and ethanol precipitated. The entire amount of DNA recovered from this precipitation was used for BND-cellulose column chromatography, as described (Dijkwel et al., 1991). After precipitation and resuspension, DNA eluted by the caffeine wash was quantitated by Hoechst dye binding and examined by N/N or N/A 2D gel electrophoresis.

Two-Dimensional Agarose Gel Electrophoresis

Approximately 20–24 μg DNA from the caffeine wash was subjected to N/A 2D gel electrophoresis (Little et al., 1993). N/N 2D gel electrophoresis of fragments between 2 kb and 5 kb was performed as previously described (Brewer and Fangman, 1988) using 10–14 μg of caffeine wash DNA. For restriction fragments between 5 and 19 kb, we used the conditions previously described (Little et al., 1993).

Southern Transfer and Hybridization

DNA was transferred to Hybond N Plus membranes (Amersham) as recommended by the manufacturer.

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