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Decreased replication origin activity in temporal transition regions

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In the mammalian genome, early- and late-replicating domains are often separated by temporal transition regions (TTRs) with novel properties and unknown functions. We identified a TTR in the mouse immunoglobulin heavy chain (Igh) locus, which contains replication origins that are silent in embryonic stem cells but activated during B cell development. To investigate which factors contribute to origin activation during B cell development, we systematically modified the genetic and epigenetic status of the endogenous Igh TTR and used a single-molecule approach to analyze DNA replication. Introduction of a transcription unit into the Igh TTR, activation of gene transcription, and enhancement of local histone modifications characteristic of active chromatin did not lead to origin activation. Moreover, very few replication initiation events were observed when two ectopic replication origin sequences were inserted into the TTR. These findings indicate that the Igh TTR represents a repressive compartment that inhibits replication initiation, thus maintaining the boundaries between early and late replication domains.

Introduction

In mammalian cells, groups of neighboring origins of DNA replication usually fire at similar times in S phase, generating large early or late replication domains spanning megabases. Recent genome-wide studies determined that many early and late replication domains are separated by a several hundred–kilobase region, which we termed a temporal transition region (TTR), where the DNA sequences are replicated progressively later in S phase as they become closer to late-replicating domains (Hiratani et al., 2008; Desprat et al., 2009). These TTRs are widely distributed in the mammalian genome and likely have novel functions in replication regulation and genome organization.

We previously identified a TTR at the mouse immunoglobulin heavy chain (Igh) locus (Fig. 1 A). In non–B cells, Igh replication is accomplished via a triphasic program: the sequence downstream of the Igh locus is replicated from early origins, whereas the heavy chain variable (Vh) region is replicated from late origins in S phase (unpublished data); the sequences in between (corresponding to the location of the Igh–diversity [Dh], joining [Jh], and constant [Ch] segment [DJC] cluster) are replicated by a single replication fork that initiates in early S and terminates in late S phase, forming a TTR (Fig. 1 B, top left; Ermakova et al., 1999). An Igh TTR is also present in late stages of B cell development (mature B and plasma cells; Fig. 1 B, middle left). However, during early B cell development, the TTR disappears, and the entire Igh locus replicates early in S phase in pro– and pre–B cells (Fig. 1 B, bottom left; Zhou et al., 2002b). The shift from triphasic to solely early replication is achieved by the activation of many origins located at apparently random positions within the Igh–DJC cluster (Fig. 1 B, bottom right, double arrows; Norio et al., 2005), which are silent in non–B cells (Fig. 1 B, top right). Thus, the mouse Igh locus...
A

The mouse IgH locus (chromosome 12 ~3.3 Mb)

Gene loci downstream of the IgH

Centromere

Telomere

5'

Vh (2.5 Mb)

Dh Jh Ch

Eμ 3'RR

Crip Mta2 Jagged2

3' RR

Gene loci downstream of the IgH

PacI

190 kb

Swa I

~430 kb

B

Non-B cells

Time in S phase

Early

Late

IgH locus (kb)

Non-IgH downstream

Triphasic replication program

Vh 5'06N

S107

Dh Jh Ch

Eμ

3' RR

Silent origins

Plasma cells

Time in S phase

Early

Late

IgH locus (kb)

Non-IgH downstream

Triphasic replication program

Vh 5'06N

S107

Dh Jh Ch

Eμ

3' RR

Activated origins

Pro-B cells

Time in S phase

Early

Late

IgH locus (kb)

Early replication program

Vh 5'06N

S107

Dh Jh Ch

Eμ

3' RR

C

Swa I segment (180 kb)

γ3 γ1 γ2B ε α γ2A

D

Pme I segment (~430 kb)

Crip1 Mta1 Jagged2

Crip2

Location of the 10.7 kb downstream initiation sequence
provides an excellent system to study the regulation of origin activation in the TTR. In this study, we used the Igh TTR as a model to investigate whether TTRs are chromosomal regions with origin-suppressing activity and whether silent origins within the TTR can be reactivated.

Concomitant with the activation of origins in the TTR, gene transcription and histone modifications at the Igh locus change dramatically during early B cell development (Chowdhury and Sen, 2001). We asked whether introduction of these changes can lead to the activation of silent origins in the TTR. To answer this question, we established a versatile in vivo system in mouse embryonic stem (ES) cells to modify the endogenous Igh TTR in a systematic and specific manner. Using this system, we show that a high level of transcription or elevated histone H3 acetylation (H3ac) and H3 lysine 4 trimethylation (H3K4me3) within a few kilobases in the TTR are not sufficient to activate latent origins. Moreover, very few initiation sites were observed when two normally active origins were inserted in the TTR. These results indicate that the Igh TTR represents a repressive compartment that prevents replication initiation. Compared with local changes, global changes in higher order chromatin structure and nuclear compartmentalization appear to play an important role for the activation of origins in the TTR. Thus, this study provides important insights toward the elucidation of the properties of TTRs.

Results

Developmentally regulated origins are silent in the >400-kb TTR in wild-type (WT) mouse ES cells

We used mouse ES cells to study the developmental regulation of origin activation at the endogenous Igh locus. ES cells were chosen because of their amenability to genetic manipulation by homologous recombination. Our previous results showed that origins in the 3′ part of the TTR (Igh–DJC cluster) are silent in several non–B cells but activated in pro–B cells (Fig. 1 B, right; Norio et al., 2005). In this study, we show that origins are not detected throughout the entire TTR in ES cells during early development. This allowed us to ask whether the TTR is repressive to replication initiation and whether silent origins can be activated by transcriptional activation and histone modifications.

We used single-molecule analysis of replicated DNA (SMARD), a definitive technique for directly examining DNA replication, to address these questions (Norio and Schildkraut, 2001). SMARD relies upon sequential labeling of replicating DNA in exponentially growing cells with two halogenated nucleoside analogues (IdU followed by CldU; Fig. S1, A and B). The replicated DNA molecules that incorporated these analogues are detected by immunostaining and visualized by fluorescence microscopy (red fluorescence for IdU and green for CldU). Because IdU is incorporated first and CldU second, the transition from red to green in replicated DNA molecules indicates the direction of the replication fork. A red region flanked by green patches indicates a replication initiation site. An asymmetric pattern of FISH signals was used to identify the DNA molecules of interest and their orientation. Distinct patterns of replication can be observed if many DNA molecules are aligned according to the positions of FISH signals on their physical map (Fig. S1 C). Because all of the labeling is performed under physiological conditions, the results directly reflect the replication process in vivo.

To investigate whether the developmentally regulated origins in the 3′ part of the Igh TTR are also silent at a very early stage of development, SMARD was performed on the ~180-kb Swal DNA segment of the Igh locus from WT mouse ES cells (the positions of the segments analyzed are indicated below the map in Fig. 1 A). Similar to other non–B cells, no replication initiation was observed in 408 red and green (RG) Swal molecules analyzed (Fig. 1 C and Fig. S1 D). In addition, we had previously found that there was no initiation site in the 190-kb PacI segment located 5′ to the Swal segment (Norio et al., 2005). In both the Swal and PacI segments, replication forks proceeded exclusively from 3′ to 5′ across the Igh TTR, suggesting that there are origins downstream of the Igh locus. To test this hypothesis, an ~430-kb Pmel segment containing the 3′ end of the Igh locus and ~300 kb of downstream sequence was analyzed by SMARD. As predicted, multiple initiation sites (Fig. 1 D,
astersisks) were observed in this segment, forming an initiation zone of several hundred kilobases. An active initiation region is located ∼70–90 kb downstream of Ca. This corresponds to the region where a replication origin was previously shown to be present by 2D gel analysis in non–B cells (Zhou et al., 2002a). Approximately one in five molecules exhibited Initiation in this region. A 10.7-kb sequence within this region was cloned for additional detailed experiments (Fig. 1 D, red arrow). Collectively, the aforementioned results demonstrated that the developmentally regulated origins in the Igh TTR are silent in WT mouse ES cells.

In vivo system for targeted modification of the genetic and epigenetic status at the Igh locus

Because the origins in the Igh TTR are silent in WT ES cells but activated during B cell development, we asked whether TTRs are chromosomal regions that suppress replication Initiation and whether changes in local factors can reverse their suppressive activity. To answer this question, we developed a versatile in vivo system to alter both the genetic and epigenetic status of the Igh locus systematically in mouse ES cells. This cassette consists of hygromycin phosphotransferase (HY) thymidine kinase (TK) flanked by two inverted variant loxP sites (L1 and 1L, also known as Lox511; Feng et al., 1999). HYTK is a fusion gene of HY and Herpes Simplex Virus TK driven by a CMV promoter. Hygromycin was used to select for the clones with successful homologous recombination (positive selection). The maps are not drawn to scale. (B) Next, the HYTK cassette is exchanged with another cassette containing a sequence of interest by RMCE. In this example, the incoming cassette contains multiple Gal4 UAS sites (double arrows) and an EGFP reporter gene (green box) also flanked by two inverted L1 sites. Cells without exchange are selected against with gancyclovir. Using this system, different DNA sequences can be Inserted at the target sites to modify the genetic status of the endogenous Igh locus systematically in mouse ES cells. (C) With the inserted sequence shown, different regulatory proteins fused with the Gal4 DBD can be tethered to the Gal4 UAS site [VP16 in this case]. The bound proteins then act to regulate gene expression (EGFP reporter gene in this case) and modify the epigenetic status of the target site.

Low level transcription of HYTK was not sufficient to activate origins in the Igh TTR

We selected the 180-kb SwaI segment, which is located at the 3’ of the TTR and contains silent origins (Fig. 1 B, right; Norio et al., 2005) to determine whether silent origins can be activated using the aforementioned strategy. Because the SwaI segment is replicated by a single replication fork in ES cells, even rare Initiation events within the segment as a result of targeted modification will be detected by SMARD. The segment between Cc and Cα was chosen for gene targeting (Fig. 3 A) because it contains a relatively long, unique sequence and, importantly, has activated origins nearby in pro–B cells (Fig. 1 B, bottom right, blue asterisks). We targeted the exchangeable cassette (HYTK) to the early part of the TTR between Cc and Cα in one allele of the Igh locus (insertion site is indicated by a red arrow in Fig. 1 B, top left), and the success of homologous recombination was verified by Southern blotting (Fig. 3, A and B).
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Targeting Gal4-VP16 to the Igh locus induced significant transcriptional activation of the reporter gene but did not activate origins in the TTR

To determine whether a high level of transcription could activate origins, the HYTK cassette was exchanged by RMCE with another transcription unit containing 14 copies of Gal4 UAS followed by an EGFP reporter gene (Gal4EGFP) driven by the CMV promoter. The success of RMCE was confirmed by PCR (Fig. 4 A) and Southern blotting (Fig. S2, A and B). An EGFP probe was used to confirm that the cassette was inserted as a single copy into the Igh locus (ES46) was less than one copy per cell, whereas the mean number of β2 microglobulin (β2m) transcripts was ~10 copies per cell, as measured by real-time quantitative RT-PCR.

Next, we examined whether insertion of the transcription unit HYTK could activate the latent origins flanking the insertion site in ES cells. SMARD did not reveal any replication initiation site in the SwaI segment from the ES cells with the HYTK inserted between Ce and Ca (Fig. 3 D). As in the WT ES cells, all replication forks progressed exclusively in the 3’ to 5’ direction. This demonstrated that insertion of a transcription unit was not sufficient to activate origins nearby. However, the Igh TTR in mouse ES cells does not exhibit H3K4me3, which is a mark of open chromatin structure (Mikkelsen et al., 2007). Thus, it was possible that the chromatin structure in the TTR was not very open, repressing transcription of HYTK to a level that may be sufficient to confer hygromycin resistance but not high enough to activate replication origins. Indeed, real-time quantitative RT-PCR showed that HYTK transcription was relatively low, as the mean number of HYTK transcripts was less than one copy per cell, which is significantly lower than a constitutively expressed gene, β2 microglobulin (~10 copies/cell; Fig. 3 C). Therefore, it was possible that the failure of the HYTK transcription unit to activate neighboring origins in the TTR was caused by its low level of transcription.

Targeting Gal4-VP16 to the Igh locus induced significant transcriptional activation of the reporter gene but did not activate origins in the TTR

We used Gal4 DDB fusion proteins directed to the Gal4 UAS sites adjacent to the EGFP reporter gene to increase the level of EGFP transcription. Expression vectors encoding Gal4 DDB fused with three different proteins with the potential to

Figure 3. Low level expression of the HYTK transcription unit did not activate origins. (A) The map of the targeted allele of the Igh locus after insertion of the L1HYTK1L cassette. The gene-targeting vector contains an 8.8-kb KpnI segment (green) of the Igh locus, including the Ce (orange box) and Ca exon (blue box) of the Ca gene. HYTK was inserted into the SfoI site of the 8.8-kb KpnI fragment. Genomic DNA was digested with BglII (fragment sizes are indicated above and below) and screened with a probe (red bar) 5’ to the KpnI fragment. (B) Southern blotting after BglII digestion, demonstrating L1HYTK1L cassette insertion between Ce and Ca. The probe recognizes an 11-kb BglII fragment in the WT allele and a 10.2-kb fragment in the allele with a HYTK cassette inserted. A WT ES cell (ESC; left) and an ES colony in which homologous recombination occurred (right) are shown. (C) The transcription level of the HYTK was minimal when inserted between Ce and Ca. The mean number of HYTK mRNA molecules in ES cells with the HYTK cassette (ES46) was less than one copy per cell, whereas the mean number of β2 microglobulin (β2m) transcripts was ~10 copies per cell, as measured by real-time quantitative RT-PCR.

(D) No replication initiation sites were detected after insertion of HYTK between Ce and Ca. The map of the 180-kb SwaI fragment is shown at the top of the figure. The insertion site of the HYTK cassette is indicated by a green arrow. The blue bars below the map indicate the position of the FISH probes used to identify the Igh molecule and determine its orientation. All replication forks proceeded from 3’ to 5’ in the 180-kb SwaI segment after insertion of the HYTK cassette. 27 out of a total of 110 molecules analyzed are shown (arrowheads). Error bars indicate mean ± SD of several repeated experiments.
activate transcription (the acidic transcriptional activator VP16 and two transcriptional coactivators, CBP and PCAF) were transiently transfected into the ES cells with the Gal4EGFP cassette. When the expression level of the EGFP reporter was measured by FACS, strong activation was only observed with Gal4-VP16 (Fig. 4 B). No synergistic effects were detected between Gal4-VP16 and Gal4-CBP or Gal4-PCAF. To confirm that the transcriptional activation was mediated by both of the Gal4-VP16 fusion partners, equivalent amounts of vector expressing Gal4-VP16, Gal4 DBD alone, or VP16 fused with Lac repressor were transiently transfected into the ES cells with the Gal4EGFP cassette. Only Gal4-VP16 significantly activated the transcription of the EGFP gene, whereas the Gal4 DBD alone or VP16 fused with Lac repressor did not (Fig. 4 C). The relative activation of the EGFP reporter by the Gal4-VP16 was >25-fold over baseline as measured by the percentage of EGFP+ cells normalized for transfection efficiency. Gal4-CBP, Gal4-PCAF, Gal4 DBD, and Lac-VP16 all increased EGFP transcription only about fivefold. This might be nonspecific activation, as the transfected cells may grow slower than untransfected cells, thus accumulating more EGFP mRNA and protein. These results suggested that Gal4-VP16 activated the EGFP reporter gene by specifically binding to the Gal4 UAS sites rather than by the overexpression of VP16 or Gal4 DBD.

To test whether a high level of transcription is sufficient to activate origins, ES cells with the Gal4EGFP cassette were transiently transfected with the Gal4-VP16 expression vector. The EGFP+ cells expressing the highest levels of EGFP (20% of the cells) were isolated by FACS and analyzed by SMARD. However, no replication initiation was observed in the SwaI segment (Fig. 4 D). As in the WT ES cells, replication forks proceeded exclusively from 3’ to 5’ in all of the molecules analyzed.

It is possible that the lack of an effect of transient Gal4-VP16 expression on origin activation was related to silencing or loss of the Gal4-VP16 expression vector. Because EGFP protein possesses a long half-life in cells, the intracellular abundance of EGFP protein may not correlate with the level of EGFP gene transcription. Thus, after transient Gal4-VP16 transfection, the sorted EGFP+ cells, which had a high level of EGFP protein, may not have had a high level of EGFP transcription. To rule out this possibility, we expressed EGFP at a steady high level by establishing an ES cell line stably expressing Gal4-VP16 (Fig. 5 B). After being sorted by FACS, the brightest EGFP+ ES cells (Fig. 5 C and Fig. S3 A) were amplified and stably expressed Gal4-VP16 (Fig. 5 D). After sorting the SWA+ FACS-sorted ES cells by FACS, strong activation was only observed with Gal4-VP16 (green arrow). 12 out of a total of 34 molecules analyzed are shown (arrowheads). Error bars indicate mean ± SD of three experimental repeats. (D) Transient expression of Gal4-VP16 did not activate origins. All of the DNA replication forks progressed in one direction from 3’ to 5’ in the 180 kb Swal fragment of the Igh TTR from FACS-sorted EGFP+ ES cells transiently transfected with Gal4-VP16. The insertion site of the Gal4EGFP cassette is indicated by a green arrow. 12 out of a total of 34 molecules analyzed are shown (arrowheads). Error bars indicate mean ± SD of several repeated experiments.

**Gal4-VP16 significantly increased H3ac and H3K4me3 near the Gal4 UAS site**

Active transcription is usually accompanied by elevated histone acetylation. The histone acetylation status around the insertion site between Cε and Cα in EGFP+ and EGFP negative (EGFP−) cells was investigated by chromatin immunoprecipitation (ChIP).
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The latent origins near the insertion site between C and C in the Igh TTR. To test whether TTRs are chromosomal regions that intrinsically suppress replication initiation, we generated an exchange vector with a 10.7-kb DNA sequence containing an active initiation site from the initiation zone downstream of the Igh locus (Fig. 1 D, red arrow). This 10.7-kb segment was inserted between C and C by RMCE. The insertion was confirmed by Southern blotting and PCR (Fig. S4, A–C). The WT allele (180 kb) and the allele with the 10.7-kb insert (>190 kb) ran to distinct positions on the pulsed-field gel (Fig. 6 A). They were excised separately and subjected to SMARD analysis. In most of the >190-kb molecules, replication forks progress in one direction as in the WT ES cells (Fig. S4 D). Replication initiation sites were observed only in two out of 129 RG molecules from two individual clones (Fig. 6, B and C). However, when SMARD experiments were performed on the 180-kb SwaI segment (WT allele) from the same ES cells, no origins were observed in a similar number of molecules analyzed (n = 120; Fig. 6 D). These results indicated that the 10.7-kb sequence induced minimal replication initiation, suggesting that it has some cis-regulatory elements that facilitate origin activation.

**Primers 0.1-kb 3’ to the 14x Gal4 UAS sites detected an approximately sevenfold increase in pan H3ac in the EGFP+ cells when compared with the EGFP– cells (Fig. 5 C). As a result, the distance from the Gal4 UAS sites increased, the level of H3ac diminished to background levels. The enrichment was lost beyond 1 kb to the 5’ side of the Gal4 UAS sites and beyond 2.5 kb to the 3’ side of the Gal4 UAS sites compared with EGFP– cells (Fig. 5 C).** Similar results were observed using an antibody to histone H3 lysine 9 acetylation (Fig. S3 D) or H3K4me3 (Fig. 5 D), which are two other markers for active chromatin. These results demonstrated that histone modifications induced by Gal4-VP16 were confined to a few kilobases between Ce and Co where several origins are activated in pro-B cells (Fig. 1 B, bottom right). However, no replication initiation event was observed in 158 molecules analyzed, indicating that localized histone modifications were not sufficient to activate the developmentally regulated origins in the TTR.

**The initiation efficiency of ectopic origins was low after they were inserted in the TTR**

Based on the aforementioned results, local high level of transcription or elevated histone acetylation alone cannot activate the latent origins near the insertion site between Ce and Co in the Igh TTR. To test whether TTRs are chromosomal regions that intrinsically suppress replication initiation, we generated an exchange vector with a 10.7-kb DNA sequence containing an active initiation site from the initiation zone downstream of the Igh locus (Fig. 1 D, red arrow). This 10.7-kb segment was inserted between C and C by RMCE. The insertion was confirmed by Southern blotting and PCR (Fig. S4, A–C). The WT allele (180 kb) and the allele with the 10.7-kb insert (>190 kb) ran to distinct positions on the pulsed-field gel (Fig. 6 A). They were excised separately and subjected to SMARD analysis. In most of the >190-kb molecules, replication forks progress in one direction as in the WT ES cells (Fig. S4 D). Replication initiation sites were observed only in two out of 129 RG molecules from two individual clones (Fig. 6, B and C). However, when SMARD experiments were performed on the 180-kb SwaI segment (WT allele) from the same ES cells, no origins were observed in a similar number of molecules analyzed (n = 120; Fig. 6 D). These results indicated that the 10.7-kb sequence induced minimal replication initiation, suggesting that it has some cis-regulatory elements that facilitate origin activation.
Figure 6. Initiation from the 10.7-kb origin sequence downstream of the IgH locus was minimal after insertion into the TTR. (A) Demonstration of the insertion of the 10.7-kb initiation zone sequence between Ccr and Cα by Southern blotting (SwaI digestion) after pulsed-field gel electrophoresis. A probe from the SwaI segment–specific probe detected a 190-kb SwaI fragment after the insertion of the 10.7-kb sequence as well as the 180-kb WT allele. (B) One SwaI molecule with DNA replication initiation sites was detected out of a total of 73 molecules after the 10.7-kb sequence was inserted between Ccr and Cα [green arrow]. Single-channel images are shown below the merged image. The earliest replication initiation site [double white arrows] is within the 10.7-kb insert. There are two additional initiation sites downstream of the insert. (C) A second SwaI molecule with a replication initiation site was observed out of a total of 56 molecules screened from another clone containing the 10.7-kb insertion. (D) No initiation sites were observed in the 180-kb WT SwaI segment from the unmodified allele in the same cells in which the 10.7-kb sequence had been inserted into the other allele between Ccr and Cα shown in B. 47 out of 120 molecules analyzed are shown (arrowheads).

However, the initiation efficiency in the 10.7-kb sequence is significantly different from that in its endogenous site (P < 0.005; Table S1).

To test whether another reported active origin would exhibit a behavior similar to the 10.7-kb sequence after insertion into the TTR, an exchange cassette containing the human lamin B2 origin was inserted between Ccr and Cα by RMCE (Fig. 7 A). The human lamin B2 origin is one of the few origins with a defined sequence identified in mammalian cells (Abdurashidova et al., 2000; Paixão et al., 2004). The 1.3-kb lamin B2 origin sequence used in this study (positions 3,691–5,038; GenBank accession no. M94363) has been reported to be an active origin when inserted ectopically in both human and mouse cell lines (Paixão et al., 2004; Fu et al., 2006). After the lamin B2 origin was inserted into the IgH TTR, only three molecules with initiation sites were observed from a total of 117 analyzed (Fig. 7 B, top). Most molecules were still replicated by a single replication fork proceeding from 3′ to 5′ as in the WT ES cells (Fig. 7 B, bottom), suggesting that the lamin B2 origin activity was very low when it was inserted in the TTR.

To further test whether the human lamin B2 origin activity was low at another site in the TTR, we also inserted the same cassette containing the lamin B2 origin into the later-replicating end of the IgH TTR between the Vh region and the Dh gene segments by RMCE (the position of the insertion site is indicated by a black arrow in Fig. 1 B, top left). The success of RMCE was confirmed by PCR (Fig. 7 C). Only two origins were observed within 100 kb downstream to the lamin B2 origin in a 190-kb PacI segment (Fig. 7 D, top). Most molecules were replicated by a single replication fork proceeding from 3′ to 5′ in the PacI fragment as in WT ES cells (Fig. 7 D, bottom). These results further support the notion that the initiation efficiency is minimal when ectopic origins are inserted in the IgH TTR in ES cells.

Discussion

Numerous studies have indicated that DNA replication is associated with various factors, including cis-regulatory elements, transcriptional status, histone modification, high order chromatin structure, and nuclear compartmentalization (Gilbert, 2002; Aladjem, 2007). Our results indicated that neither insertion of a transcription unit nor tethering of a potent transcriptional activator Gal4-VP16 was sufficient to activate potential origins in the IgH TTR in mouse ES cells, even though a high level of transcription or elevated histone H3ac and H3K4me3 within a region of a few kilobases was induced. Moreover, very few initiation events were observed when two active origins in mouse cells (a 10.7-kb sequence from the IgH downstream initiation zone and the human lamin B2 origin) were inserted in the TTR. These data suggest that the IgH TTR has unique properties that define a repressive compartment for replication initiation.
Figure 7. **Initiation at the human lamin B2 origin was low in the Igh TTR.** (A) PCR confirmation of the insertion of human lamin B2 replication origin between Cc and Cα. The success of insertion was confirmed by the presence of human lamin B origin (top) and loss of HYTK between Cc and Cα (bottom). WT ES cells were used as a negative control. (B, top) Three molecules with replication initiation sites [double white arrows] were observed in the 180-kb SwaI segment after insertion of the human lamin B2 replication origin sequence. (bottom) Other SwaI molecules replicated from 3' to 5'. 22 from a total of 117 molecules are shown (arrowheads). (C) PCR confirmation of the insertion of human lamin B2 replication origin sequence in another region of the Igh TTR (between Vh and Dh), which replicates later than the region between Cc and Cα. The success of insertion was confirmed by the presence of human lamin B origin (top) and loss of HYTK between Vh and Dh (bottom). WT ES cells were used as a negative control. (D, top) Initiation sites (double white arrows) were observed in the 190-kb PacI segment after the human lamin B2 replication origin was inserted between the Vh and Dh region. The map of the 190-kb PacI fragment is shown above the molecules. The position of the insertion site is indicated by a green arrow. The PacI segment is 18-kb 3' to the insertion site. It contains all of the Dh and Jh genes and some part of the Ch genes, including Cγ5 and Cγ3 (Fig. 1A). We could not analyze an Igh segment containing the inserted lamin B2 replication origin because an appropriate restriction enzyme was not available. (bottom) Other PacI molecules replicated from 3' to 5'. 21 out of a total of 48 molecules examined are shown (arrowheads).
Transcription, transcription factors, histone modifications, and origin activation
Aspects of the relationship between transcription and replication timing have been well documented. Tissue-specific genes often replicate early in S phase when they are expressed but late when they are silent (Gilbert, 2002; Norio, 2006). Genome-wide analyses identified a strong correlation between early replication and high transcriptional potential (MacAlpine et al., 2004; Woodfine et al., 2004; Farkash-Amar et al., 2008; Hiratani et al., 2008). Transcriptional status also correlates with replication origin activation, but whether there is a causal relationship is still unclear. In this study, transcription of two different cassettes (HYTK and Gal4EGFP) inserted into the Igh TTR did not activate neighboring silent origins (Fig. 1 B, blue asterisks indicate origin positions). This suggests that transcriptional activity in a short region (a few kilobases), even at a very high level, is not sufficient to activate the developmentally regulated origins in the TTR. However, unlike germline transcription, which occurs at particular sites spaced over a several hundred–kb region in the Igh locus during B cell development, the transcription of the HYTK or EGFP gene occurs within a very small region. In Drosophila melanogaster, early replication domains (>100 kb) were associated with transcription over large genomic regions rather than at individual genes (MacAlpine et al., 2004). Thus, our results do not rule out the possibility that high levels of transcription from multiple sites across extended regions, such as the germline transcription at the Igh locus in pro– and pre–B cells, might play a role in the activation of origins.

Epigenetic status, such as histone modifications, also affects origin activation. Often, histone marks for active chromatin are associated with higher origin activity (Pasero et al., 2002; Aggarwal and Calvi, 2004; Ghosh et al., 2004). In addition, recruitment of histone acetyltransferase to the vicinity of late origins is required to alter the replication program in mammalian cells. This suggests that transcriptional activity might play a role in the activation of origins.

Histone modifications, and origin activation
Sequence specificity and replication origins
The role of specific sequences in origin selection and activation in mammalian cells is still not fully understood. In prokaryotic organisms and lower eukaryotes, origins are defined by specific sequences, whereas in mammalian cells, origins are not defined absolutely by sequence. However, it has been reported that the Dhfr origin β in CHO cells is functional at ectopic sites in mammalian cells (Gray et al., 2007). In our study, no replication initiation was detected in 830 RG SwaI molecules that did not have origins inserted (WT ES, 408; ES with HYTK, 110; ES with Gal4-EGFP, 192; WT allele from ES cells with 10.7-kb insert on the other allele, 120), but a very low number of initiation events were seen in ES cells with known origins inserted into the Igh TTR. Although we observed a small number of molecules with initiation sites, the difference between the two groups is statistically significant (P < 0.001; Table S2). We cannot exclude the possibility that there might be a very low number of initiation events in the TTR, as the number of SwaI molecules we observed (830) is not indefinite; however, even if there is one initiation event in the TTR, the efficiency is so low that the conclusion of our statistical analysis remains the same. These data suggest that some cis-regulatory elements are required for replication initiation, but more factors are needed to initiate replication efficiently in mammalian cells.

Interestingly, introduction of two different constitutively active replication origins into the TTR led to low frequency initiation in flanking regions rather than in the introduced origins (Fig. 6, B and C; and Fig. 7, B and D). One possible explanation is that when the origin sequence was inserted into the Igh locus, cis-elements within the sequence may have influenced the loop organization of the chromatin so that activity of the neighboring origins was also affected. This is consistent with the observation that many origins are activated at similar times in an early replication initiation zone downstream of the Igh locus, but different cells may use different origins to replicate the same genomic region (Fig. 1 C).

The TTR represents a repressive compartment for replication initiation
Our results demonstrated that local changes in transcription and histone modifications were insufficient to activate silent origins in the TTR. In addition, although insertion of known origins resulted in replication initiation, the initiation efficiency within the TTR was significantly lower than in pro–B cells (P < 0.001; Table S3). Collectively, these findings indicate that the TTR represents a repressive compartment to prevent replication initiation. Further evidence supporting this idea comes from our previous finding that when the DNA sequence for the entire Igh TTR (>400 kb) was deleted in a plasma cell line, the adjacent Vh region families were converted to a new TTR of similar size, whereas the rest of the Vh region remained late replicating (Fig. 1 B, middle left; Zhou et al., 2002b). This suggests that when sequences are placed in this compartment, they are instructed to become a TTR. It is possible that the unique higher order chromatin structure of the TTR (including organization of chromatin loops) and its nuclear compartmentalization (including association with DNA replication factories) play important roles in the regulation of its internal origin activity (Sadoni et al., 2004; Kitamura et al., 2006; Meister et al., 2006; Hiratani et al., 2008; Misteli, 2008; Göndör and Ohlsson, 2009).

The Igh locus undergoes changes in higher order chromatin structure during development. The distances separating...
Materials and methods

ES cell culture

The DS2A ES cell line (from 129/Sv) was maintained on gelatinized plates with DME supplemented with 10\% FBS or equivalent leukemia inhibitory factor (Millipore) and 10\% ES cell–qualified fetal bovine serum (Invitrogen). ES cells containing the HyTK cassette were maintained in medium supplemented with 150 µg/ml hygromycin.

SMARD

SMARD experiments were performed as described previously (Norio and Schildkraut, 2001). In brief, cells in exponential phase were pulsed with 30 µM IdU (5′-ido-2′-deoxyuridine; MP Biomedicals) for 4 h followed by 30 µM CldU (5′-chloro-2′-deoxyuridine; MP Biomedicals) for another 4 h. Cells were embedded in 0.5% low melting agarose (Invitrogen) agarose and lysed with proteinase K. Lysates were then digested with rare-cut restriction enzymes and DNA was separated by pulsed-field gel electrophoresis. Part of the lane was hybridized with a unique DNA probe by Southern blotting to locate the position of the DNA segment of interest. The corresponding gel slice was excised, melted, and digested with agarase. The resulting DNA in solution was stretched onto silanized microscope slides. The slides were hybridized to a mixture of biotinylated DNA probes to identify the target DNA molecules and their orientation using FISH. FISH signals were detected by five alternating layers of Alexa Fluor 350–conjugated avidin (Invitrogen) and biotinylated anti–avidin D (Vector Laboratories). The two reagents were used for immunostaining; mouse anti-β-actin (BD) for IdU, rat anti-β-gal (Accurate Chemical and Scientific Corporation) for CldU, Alexa Fluor 568 goat anti–mouse IgG (Invitrogen) for red fluorescence, and Alexa Fluor 488 goat anti–rat IgG (Invitrogen) for green fluorescence.

Image acquisition and processing

Images were collected using a fluorescence microscope (Axioskop 2 mot plus; Carl Zeiss, Inc.) and a cooled charge-coupled device camera (CoolSNAP HQ; Photometrics). The images were processed using Photoshop (Adobe) and aligned according to the physical map with Claris-Draw (Dekorra Optics LLC) or Illustrator (Adobe).

Vector construction

An 8.8-kb unique KpnI DNA segment containing Cε and the ε exon of Cε was cloned from bacterial artificial chromosome (BAC) CT7.34H16 into pGem3Zf (+) cloning vector to generate pZG01 (11.6 kb). The gene-targeting vector (pZGEG2) was constructed by inserting the 3.1-kb L1HYTK1 sequence as an XhoI–SalI segment into the SfiI site in pZG01 by blunt end ligation. The Gal4EGFP exchange vector (pZG1B) was constructed by inserting (by blunt end ligation) a HindIII–Sall segment containing 14× Gal4 UAS sites into the Clal site upstream of the CMV promoter from an exchange vector containing the EGFP gene driven by a CMV promoter. The 10.7-kb sequence from the downstream initiation zone of the IgH locus was cloned by PCR with high fidelity DNA polymerase (PfuUltra; Agilent Technologies) from BALB/c mice. The PCR product was cloned into the pCR-XL-TOPO vector (Invitrogen) to generate the exchange vector (pZG34). The exchange vector containing human lamin B2 origin (pZG1B) was constructed by insertion of the human lamin B2 origin sequence (1.3-kb NsiI site) into the Sall site of the pZG1B vector (provided by A. Belmont, University of Illinois, Urbana, IL), and expression vectors for Gal4-PCAF and Gal4-CBP (provided by T. Kouzarides, University of Cambridge, Cambridge, England, UK).

RMCE

ES cells were cultured in the presence of 150 µg/ml hygromycin (Invitrogen) 2–3 d before the RMCE procedure. ES cells were trypsinized and aliquoted at 2–4 × 10^5 cells/15-ml tube and spun down just before electroporation. Cells were electroporated using a mouse ES cell nucleofection kit (Amazexa) along with 4–6 µg plasmid DNA (e.g., 4 µg exchange vector with 1 µg pCMV-Cre vector). ES cells were plated into warm medium in a 6-well plate after electroporation. The medium was changed the next day. The ES cells were cultured for at least 3 d before serial dilution into 6-well plates. The next day, gancyclovir was added to 10 µM final concentration to each well. Medium was changed daily the first week. Because TK phosphorylates gancyclovir and generates a cytopotoxic product, cells without exchange are killed by gancyclovir. Survived colonies displaying good morphology and distinct separation from other colonies were picked into 96-well plates ~2 wk after gancyclovir selection. ES cells were passaged into duplicate 24-well plates for genotyping and frozen storage at −140°C.
Real-time quantitative RT-PCR
Real-time quantitative RTPCR was performed using a real-time RTPCR kit (QuantOne SYBR; QIAGEN) and a LightCycler Roche). 300 ng RNA was used in each reaction (20 µl). The final concentration for each primer was 1 µM. The RTPCR program was 50°C for 20 min for reverse transcription, 95°C for 1 min to activate Taq polymerase, and 30 cycles of PCR amplification at 94°C for 15 s, 60°C for 20 s, and 72°C for 30 s.

ChIP
ChIP was performed with 2 x10^6 ES cells using the ChIP assay kit (Milli-

Online supplemental material
Fig. S1 illustrates the experimental protocols for SMARD and diagrams of replication patterns. Fig. S2 shows Southern blot confirmation of the stable Gal4-VP16 expression by RT-PCR, the resulting elevation of EGFP reporter gene expression, and H3K9 acetylation. Fig. S3 demonstrates the stable Gal4-VP16 expression by RTPCR, the resulting elevation of EGFP reporter gene expression, and H3K9 acetylation. Fig. S4 shows the confirmation of the insertion of the 10.7kb sequence between Ca and Cp by Southern blotting and long-range PCR. Many molecules lacking initiation sites after insertion of the 10.7kb sequence are also shown. Tables S1–S3 summarize the statistical analysis of SMARD experiments by Fisher’s exact test. Table S4 lists the primer sequences used in this study. Online supplemental material is available at http://www.jcb .org/cgi/content/full/jcb.200905144/DC1.

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