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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replicated from early origins, whereas the heavy chain variable (Vh) region is replicated from late origins in S phase (unpublished data); the sequences in between (corresponding to the location of the *Igh*–diversity [Dh], joining [Jh], and constant [Ch] segment [DJC] cluster) are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

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Abbreviations used in this paper: BAC, bacterial artificial chromosome; ChIP, chromatin immunoprecipitation; CMV, cytomegalovirus; DBD, DNA-binding domain; ES, embryonic stem; H3ac, H3 acetylation; H3K4me3, H3 lysine 4 trimethylation; HY, hygromycin phosphotransferase; *Igh*, immunoglobulin heavy chain; RG, red and green; RMCE, Cre recombinase–mediated cassette exchange; SMARD, single-molecule analysis of replicated DNA; TK, thymidine kinase; TTR, temporal transition region; UAS, upstream-activating sequence; WT, wild type.

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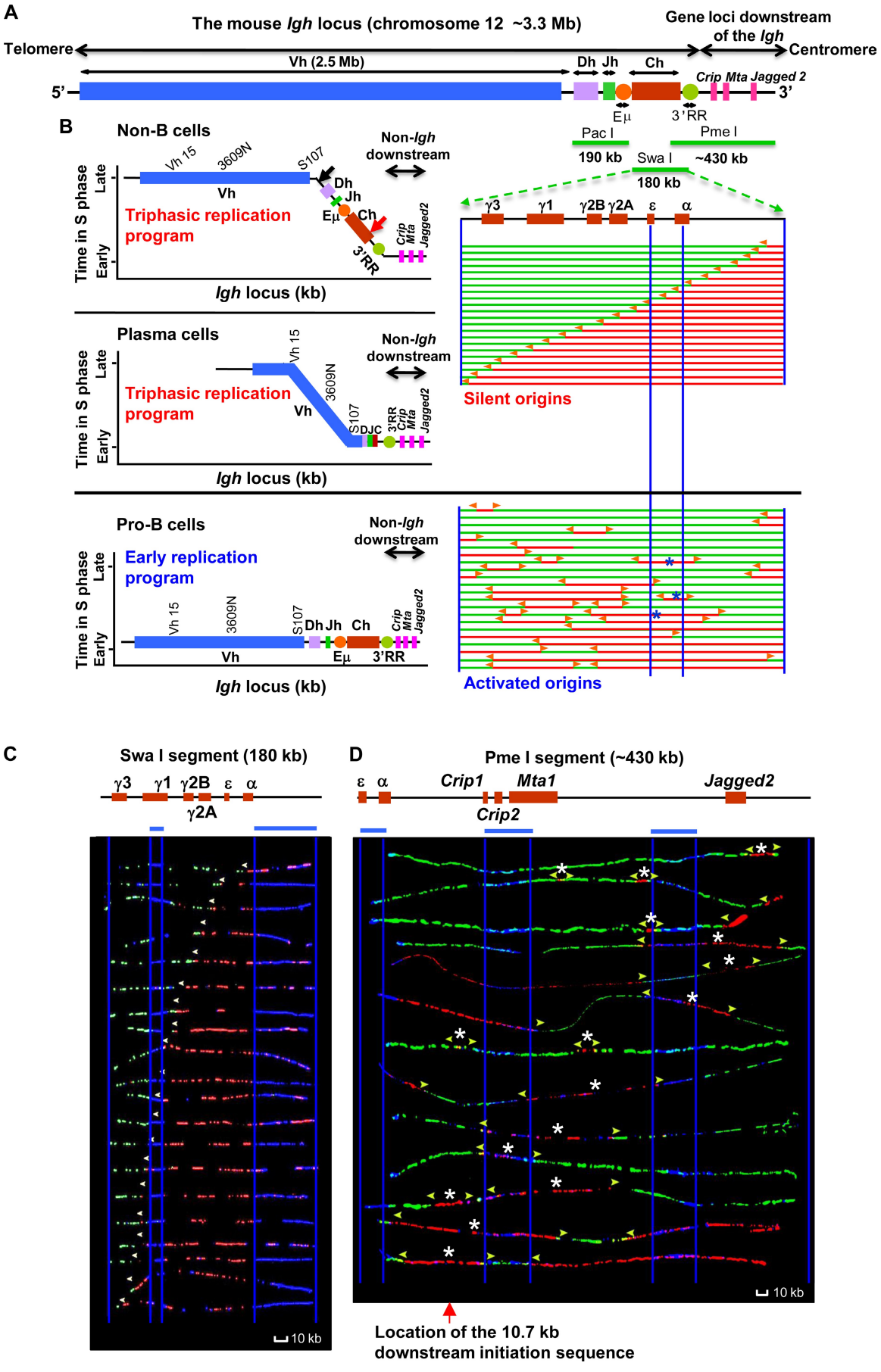
A
The mouse Igh locus (chromosome 12 ~3.3 Mb)
Gene loci downstream of the Igh
Telomere
Centromere
Vh (2.5 Mb)
Dh Jh
Ch
Crip Mta Jagged 2
5'
3'
Eμ
3'RR
Pac I
Pme I
190 kb
Swa I
~430 kb
180 kb
B
Non-B cells
Non-Igh downstream
Vh 15
3609N
S107
Vh
Dh
Jh
Eμ
Ch
3'RR
Crip
Mta
Jagged2
Time in S phase
Late
Early
Triphasic replication program
Igh locus (kb)
γ3
γ1
γ2B
γ2A
ε
α
Plasma cells
Silent origins
DJC
Pro-B cells
Early replication program
Activated origins
C
Swa I segment (180 kb)
γ3 γ1 γ2B ε α
γ2A
10 kb
D
Pme I segment (~430 kb)
ε α
Crip1
Mta1
Jagged2
Crip2
10 kb
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 SwaI 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) SwaI 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 SwaI segment (Norio et al., 2005). In both the SwaI 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 PmeI 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,

Figure 1. **TTR and the replication program of the mouse *Igh* locus.** (A) The genetic map of the mouse *Igh* locus (not to scale). It contains >150 Vh grouped in 15 families (>2.5 Mb), 10 Dh segments, four Jh segments, eight Ch region genes, and two important regulatory regions (Eμ and 3′RR). The region containing all of the Dh, Jh, and Ch is called the *Igh*–DJC cluster. Several widely expressed genes (*Crip*, *Mta*, and *Jagged 2*) downstream of the *Igh* locus are also shown in the map. The restriction segments (green) below the map show regions of the *Igh* locus that are used in this study for SMARD analysis. (B) Developmentally regulated activation of origins in the *Igh* TTR. The x axis indicates the relative positions of the *Igh* sequences, and the y axis indicates the time of replication during S phase. In non–B cells (e.g., ES cells, top left), replication of the *Igh* locus is triphasic. Downstream genes replicate early, Vh genes replicate late, and the region in between (DJC gene cluster) replicates increasingly later, generating a TTR. Two sites in the TTR, one in the early part (red arrow) and one in the late part (black arrow), were selected to insert a different DNA sequence to modify the TTR. In late stages of B cell development (mature B and plasma cells; middle left), replication of the *Igh* locus is also triphasic. These cells have a TTR similar in size to the TTR in non–B cells; however, the sequences in the TTR are different because of the V(D)J rearrangement. Many Vh region families (e.g., 3609N) that replicate late in non–B cells now become part of the TTR and replicate earlier in plasma cells. In pro– and pre–B cells (bottom left), the whole *Igh* locus replicates early. A change in replication timing is achieved by activation of origins in the *Igh*–DJC cluster (the SwaI and PacI segment) during development. When the whole *Igh* locus replicated early in pro–B cells, multiple origins silent in non–B cells (top right) were activated. (bottom right) The developmentally regulated origins (double arrows) in the diagram are illustrated according to our previous SMARD data (Norio et al., 2005). In pro–B cells, initiation sites (blue asterisks) were detected near Cε and Cα genes where the RMCE insertion site examined in this study is located. (C) Developmentally regulated origins are silent in the *Igh* TTR of WT mouse ES cells. The positions of blue FISH probes are indicated below each map. The SwaI segment includes most of the Ch genes in the *Igh* locus and an ∼50-kb region downstream of the *Igh* gene. 20 examples from 408 molecules are shown (arrowheads). (D) Multiple origins are activated in the downstream sequence of the *Igh* locus. The PmeI segment (∼430 kb) includes 3′ of the *Igh* locus and >300 kb of the downstream region. Multiple initiation sites (white asterisks) are observed in the PmeI segment, forming a replication initiation zone. A 10.7-kb fragment (located ∼80 kb from Cα; red arrow) containing a preferred initiation site was selected, cloned, and inserted into the *Igh* TTR by RMCE. 13 examples from 25 molecules examined are shown (arrowheads).

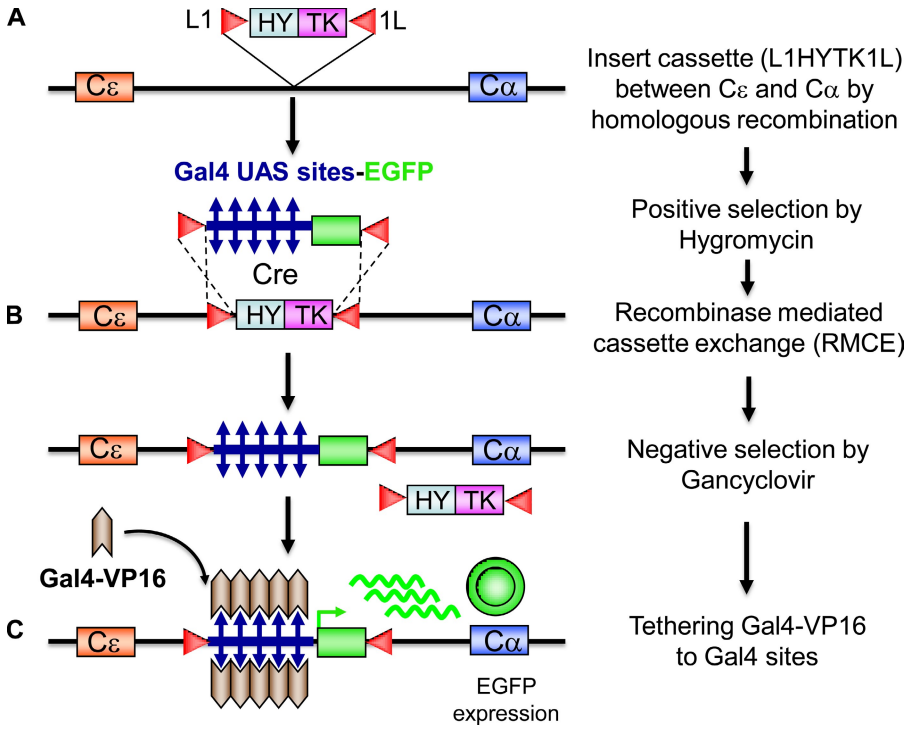

Figure 2. **Strategy to systematically modify the genetic and epigenetic status of the *Igh* locus in vivo.** (A) First, an exchangeable cassette (HYTK) flanked by two inverted Lox511 sites (L1 and 1L, red triangles) was inserted between Cε and Cα at the *Igh* locus by homologous recombination. The HYTK 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.

asterisks) were observed in this segment, forming an initiation zone of several hundred kilobases. An active initiation region is located ~70–90 kb downstream of Cα. 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. It can be divided into three steps. First, an exchangeable cassette (L1HYTK1L) was introduced into the *Igh* locus by homologous recombination in mouse ES cells (Fig. 2 A). 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 conferring hygromycin resistance and herpes simplex virus TK driven by a cytomegalovirus (CMV) promoter. Hygromycin was used to select clones with successful homologous recombination (positive selection). Second, another cassette containing sequences of interest flanked by the same inverted L1 sites was exchanged with the L1HYTK1L cassette by Cre recombinase–mediated cassette exchange (RMCE) through site-specific recombination between the loxP sites (Fig. 2 B). Clones with successful cassette exchange (TK lost) were selected for gancyclovir treatment, as cells without exchange were sensitive and eliminated (negative selection). For some experiments, an additional step, expression of fusion proteins containing a DNA-binding domain, was required to achieve locus-specific modification. Specifically, when repeats of the Gal4 upstream-activating sequence (UAS) were exchanged with the HYTK cassette, fusion proteins containing the Gal4 DNA-binding domain (DBD) were expressed. This resulted in recruitment of the fusion protein to the Gal4 UAS at the target locus (Fig. 2 C). Using this system, we investigated differential regulation of replication initiation under physiological conditions in the native chromatin context.

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 would be detected by SMARD. The segment between Cε 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 Cε 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).

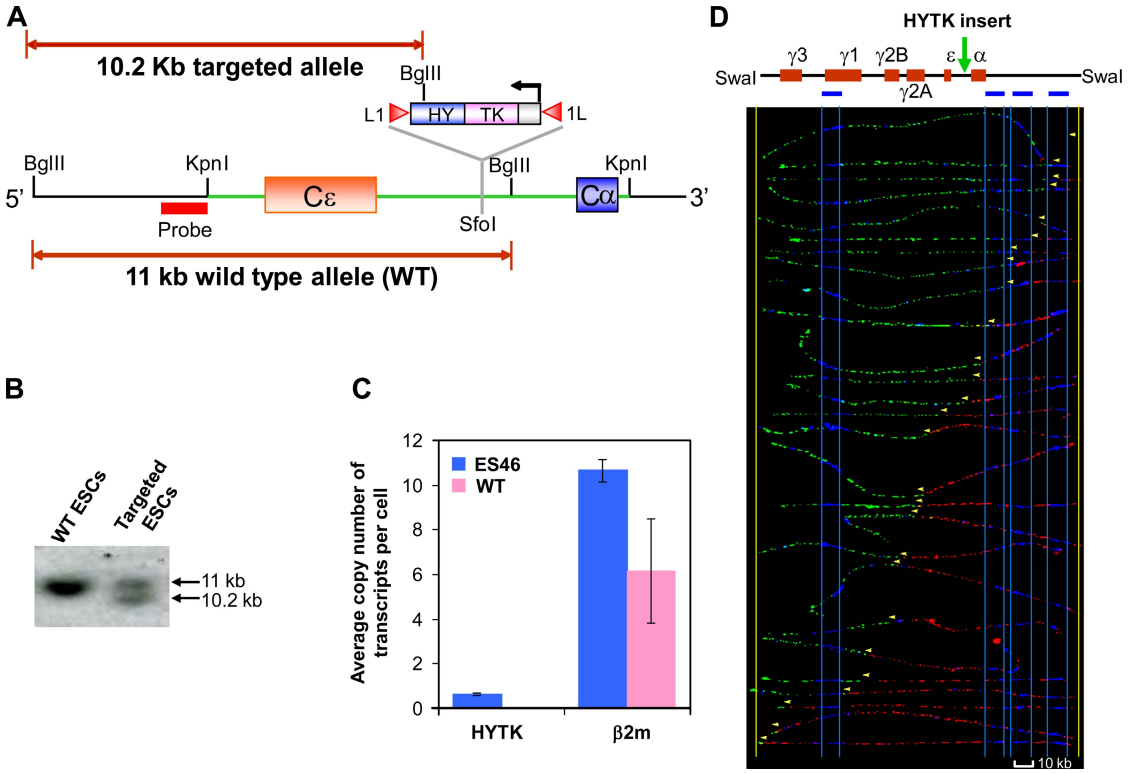

Figure 3. **Low level expression of the HYTK transcription unit did not activate origins.** (A) The map of the targeted allele of the *Igh* locus after insertion of the L1HYTK1L cassette. The gene-targeting vector contains an 8.8-kb KpnI segment (green) of the *Igh* locus, including the Cε (orange box) and Iα exon (blue box) of the Cα 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 Cε and Cα. 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 Cε and Cα. 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 Cε and Cα. 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.

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 Cε and Cα (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

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 only (Fig. S2, C and D). Similar to HYTK, the expression of EGFP gene was also expressed at a low level. Only ∼3% of the ES cells expressed EGFP (EGFP$^+$) 1 mo after the Gal4EGFP cassette was inserted between Cε and Cα as measured by FACS, which is lower than most clones with the Gal4EGFP cassette randomly inserted into the genome (unpublished data).

We used Gal4 DBD 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 DBD fused with three different proteins with the potential to

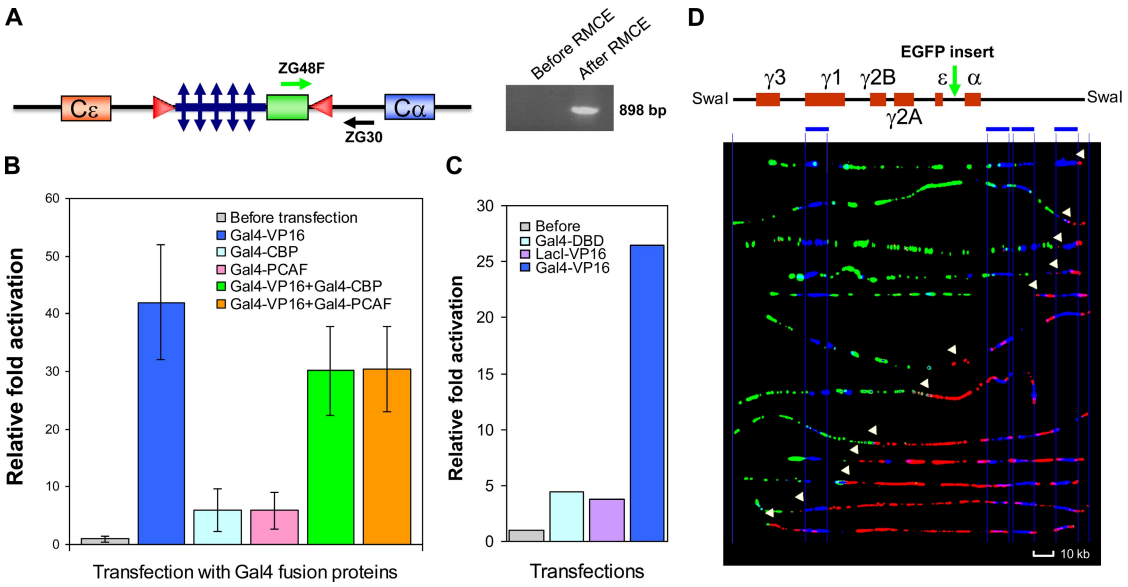

Figure 4. **Transient activation of EGFP reporter gene by Gal4-VP16 did not lead to replication initiation.** (A) Insertion of the Gal4EGFP cassette into the *Igh* locus by RMCE. (left) A map of the Gal4EGFP cassette after insertion between Cε and Cα is shown. (right) The PCR result using an EGFP-specific primer (green arrow) and an *Igh*-specific primer (black arrow) is shown. The left lane shows ES cells before RMCE, and the right lane shows ES cells after RMCE with Gal4EGFP cassette in 5′ to 3′ orientation. (B) Transient expression of Gal4-VP16–activated EGFP expression in ES cells with the Gal4EGFP cassette. The relative activation of the EGFP reporter gene by different fusion proteins was measured by FACS. Values have been normalized for transfection efficiency. (C) Expression of LacI-VP16 fusion or Gal4 DBD alone did not increase the expression level of the EGFP reporter gene. These data are representative 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 SwaI 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.

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 LacI-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 A and Fig. S3 A). The mean copy number of EGFP transcripts from the sorted EGFP$^+$ ES cells was significantly higher (>120-fold) than a housekeeping gene, β2 microglobulin, as measured by real-time quantitative RT-PCR (Fig. 5 B). After being sorted by FACS, the brightest EGFP$^+$ ES cells (Fig. S3, B and C) were subjected to SMARD analysis. As in WT ES cells, all of the molecules had replication forks proceeding from 3′ to 5′. No replication initiation was observed in 158 RG SwaI molecules analyzed (Fig. 5 E). These results showed that localized transcription was not sufficient to activate origins nearby in the *Igh* TTR even at a high level.

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).

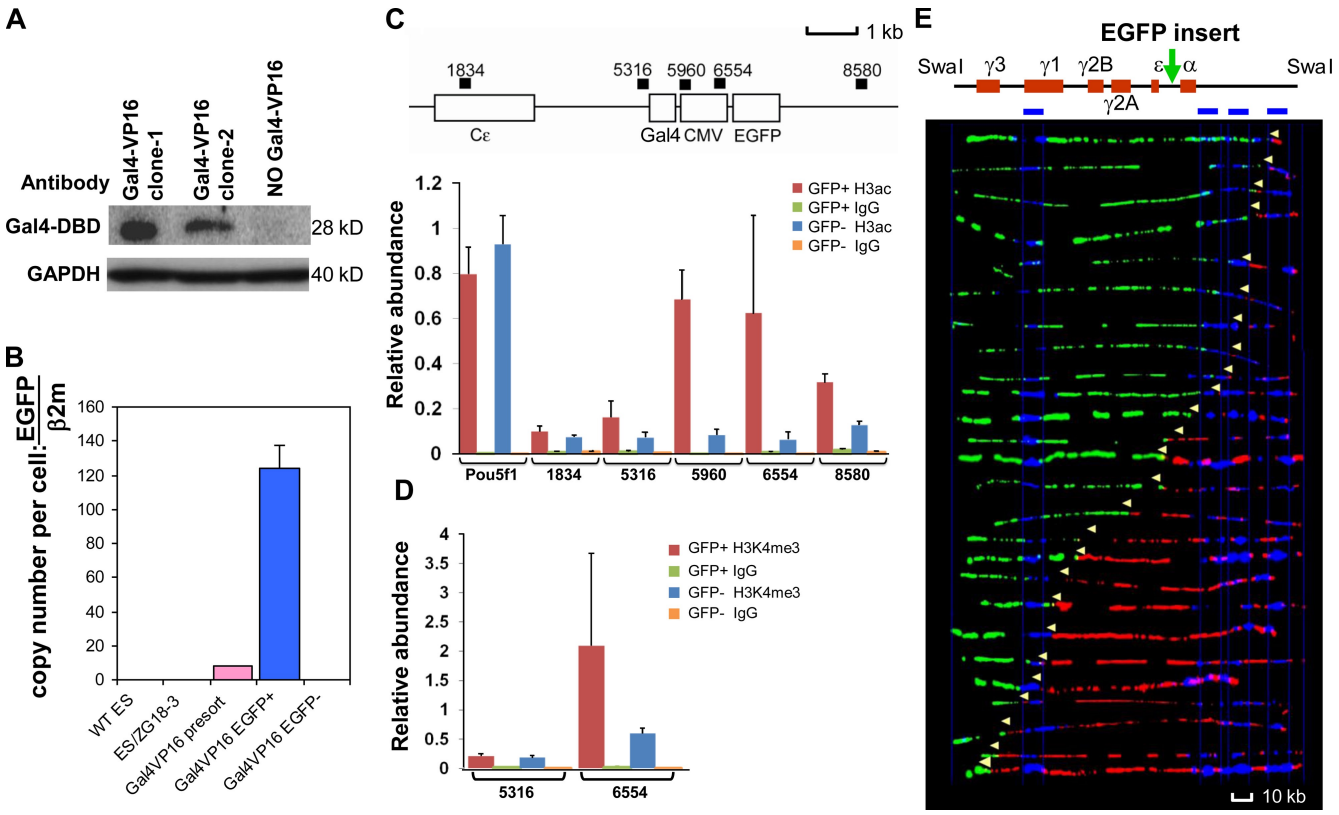

Figure 5. **Increased levels of EGFP transcription or H3ac and H3K4me3 did not result in detectable initiation sites.** (A) Western blot showing stable expression of Gal4-VP16 in ES cells with the Gal4EGFP cassette (ES/ZG18-3). (lanes 1 and 2) Expression of Gal4-VP16 was detected in two G418-resistant clones. Clone 1 with a higher Gal4-VP16 expression was used for the following experiments. (lane 3) No band was detected in the ES cells before Gal4-VP16 expression. GAPDH was used as a loading control. (B) The transcription of the EGFP reporter gene was activated to a very high level in FACS-sorted EGFP$^+$ cells after stable expression of Gal4-VP16. The mean copy number of EGFP transcripts per cell (y axis) was measured by real-time quantitative RT-PCR in different cells (x axis). The mean copy number of EGFP in the FACS-sorted EGFP$^+$ population was >120-fold higher than that of the housekeeping gene β2 microglobulin (β2m). (C) Significant increase in H3ac after Gal4-VP16 expression. (top) The positions of the ChIP primers (black boxes) on *Igh* locus are indicated. The relative abundance of histone H3ac (compared with the input control) in the EGFP$^+$ cells at different numbered positions around the Gal4 UAS site was determined for different cell populations. *Pou5f1* (*Oct4*) gene was used as a positive control. (D) H3K4me3 was enriched near the Gal4 UAS sequence in GFP$^+$ cells versus GFP$^-$ cells. (E) No initiation sites were detected from the 180-kb SwaI fragment of *Igh* in ES cells after stable expression of Gal4-VP16. 26 of a total of 158 molecules analyzed are shown (arrowheads). Error bars indicate mean ± SD of several repeated experiments.

Primers 0.1-kb 3′ to the 14× Gal4 UAS sites detected an approximately sevenfold increase in pan H3ac in the EGFP$^+$ cells when compared with the EGFP$^-$ cells (Fig. 5 C). However, as 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 Cε and Cα 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 Cε and Cα 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 Cε and Cα 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.

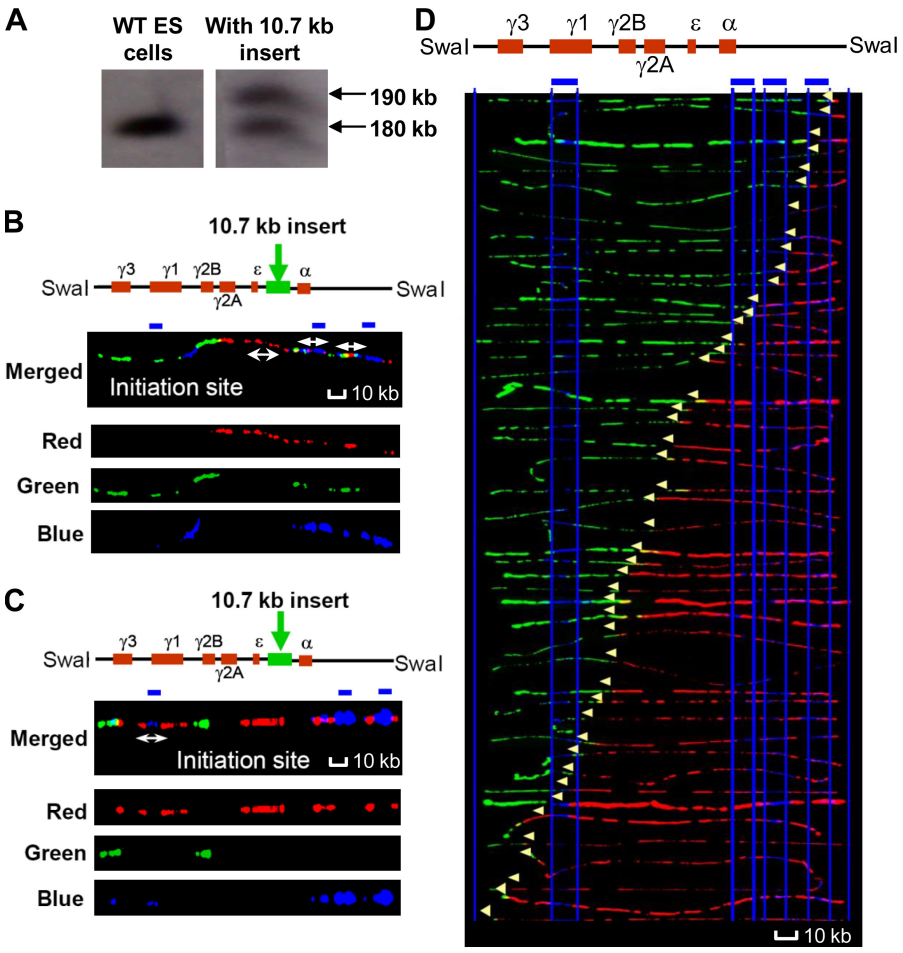

Figure 6. **Initiation from the 10.7-kb origin sequence downstream of the *Igh* locus was minimal after insertion into the TTR.** (A) Demonstration of the insertion of the 10.7-kb initiation zone sequence between Cε and Cα by Southern blotting (SwaI digestion) after pulsed-field gel electrophoresis. 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 Cε 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 Cε 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 Cε 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.

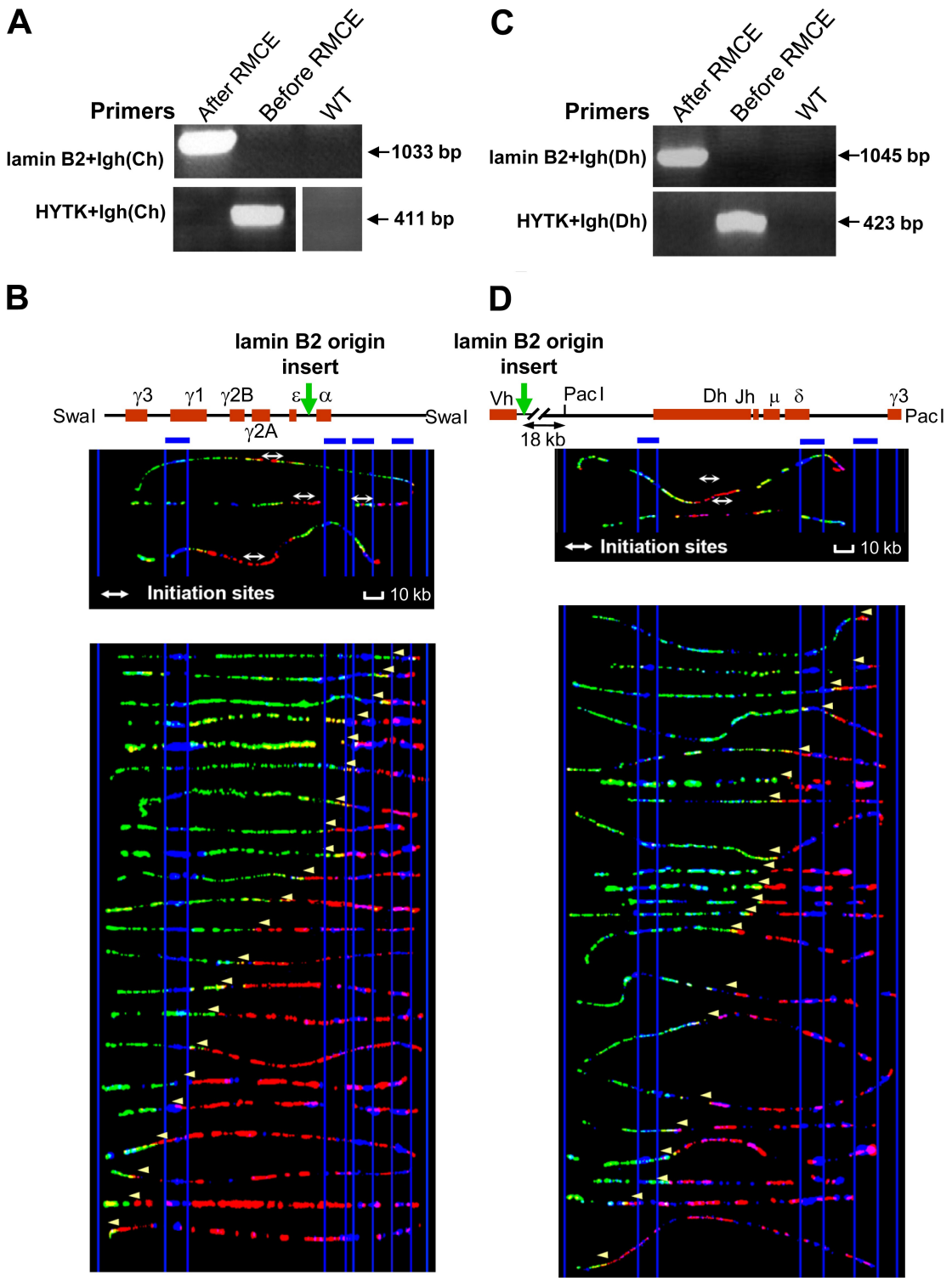

Figure 7. **Initiation at the human lamin B2 origin was low in the *Igh* TTR.** (A) PCR confirmation of the insertion of human lamin B2 replication origin between Cε and Cα. The success of insertion was confirmed by the presence of human lamin B origin (top) and loss of HYTK between Cε and Cα (bottom). 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 Cε 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 Cμ and Cγ3 (Fig. 1 A). 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 induced earlier initiation (Vogelauer et al., 2002; Goren et al., 2008). However, the association of histone acetylation with origin activity is not universal. For example, half of the active origins identified in 1% of the human genome (Encyclopedia of DNA Elements regions) in HeLa cells lack the histone marks for open chromatin (Cadoret et al., 2008).

In this study, high levels of histone H3ac and H3K4me3 were induced near the insertion site after Gal4-VP16 binding. Our ChIP results demonstrated that histone modifications induced by Gal4-VP16 were between Cε and Cα in the SwaI segment where origins apparently are located at random positions in pro–B cells (Fig. 1 B, bottom right; Norio et al., 2005). However, these local modifications did not activate any potential origins. This suggests that more global changes are required to alter the replication program in mammalian cells. It is possible that in a large region with active transcription and widespread histone acetylation, an open chromatin structure might permit accessibility over a long range so that silent origins can be activated.

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

Ch region genes and distances separating Vh region genes differ between pre– and pro–B cells (Jhunjhunwala et al., 2008), suggesting that the chromatin loops of the *Igh* locus reorganize before V(D)J rearrangement to facilitate the joining of the Vh, Dh, and Jh region genes (Sayegh et al., 2005). Because changes in the loop organization of the *Igh* locus occur concurrently with the activation of silent origins in the TTR, these changes may affect the selection of origins within the TTR and its replication timing. Although the insertions we made may be too small or lack sufficient elements to generate changes in the loop organization, it is possible that larger insertions containing multiple loop-organizing cis-elements could reorganize the higher order chromatin structure of the *Igh* locus and efficiently activate silent origins.

The nuclear location and compartment of the *Igh* locus may also contribute to the developmental activation of origins in the TTR. The location of the *Igh* locus changes during development (Kosak et al., 2002). In non–B lineage cells, the *Igh* locus is near the nuclear periphery, where it replicates in a triphasic pattern with no origins activated in the TTR. In pro– and pre–B cells, the *Igh* locus is positioned away from nuclear periphery, and the whole locus replicates early in S phase with multiple initiation sites activated (Zhou et al., 2002b). It is possible that when its nuclear position changes during development, the *Igh* locus is placed into a different functional nuclear compartment, such as a different replication factory, which specifies early replication and a particular initiation zone (Meister et al., 2006). An important question to answer is whether silent origins can be activated if the *Igh* locus is artificially moved away from the nuclear periphery in non–B cells.

TTRs are widely distributed across the mammalian genome (Hiratani et al., 2008; Desprat et al., 2009). In this study, we have observed, in a systematic manner, the effect of modifications of the *Igh* TTR on replication initiation and fork progression. Other studies have shown that VP16 can activate origins in SV40, *Saccharomyces cerevisiae*, and HeLa cells (Cheng et al., 1992; Li et al., 1998; Ghosh et al., 2004), but we find that in the TTR, it does not. Furthermore, initiation by two known origins is very inefficient when they are inserted in the TTR. These results suggest that TTRs have special properties, such as unique higher chromatin structure, which can repress internal origin activation and maintain the boundaries between early- and late-replicating domains. Alternatively, origin activity within TTRs might be regulated by the association with replication factories. Perhaps only origins located in a functional replication factory are activated. Similar to insulators for transcription, TTRs may function as a barrier between the early and late replication domains to prevent deleterious early activation of origins, which should be fired later in S phase, thus maintaining proper genome organization and protecting genome integrity.

Materials and methods

ES cell culture

The DS2A ES cell line (from 129/Sv) was maintained on gelatinized plates with DME supplemented with 10^3 U/ml 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′-iodo-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 (InCert agarose; FMC) gel and lysed with detergent. Proteins were degraded by proteinase K treatment. Plugs were 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 multiple biotinylated DNA probes to identify the target DNA molecules and their orientation by FISH. FISH probes were labeled by biotin nick translation mix (Roche). FISH signals were detected by five alternating layers of Alexa Fluor 350–conjugated avidin (Invitrogen) and biotinylated anti–avidin D (Vector Laboratories). The two incorporated, halogenated nucleosides were visualized by indirect immunostaining. The following reagents were used for immunostaining: mouse anti-BrdU (BD) for IdU, rat anti-BrdU IgG2a (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.) with a Plan Apochromatic 100× 1.4 NA oil objective and a charge-coupled device camera (CoolSNAP HQ; Photometrics) using IPLab software (BD) at room temperature. Images were processed using Photoshop (Adobe) and aligned according to the physical map with CorelDraw (Dekorra Optics LLC) or Illustrator (Adobe).

Vector construction

An 8.8-kb unique KpnI DNA segment containing Cε and the Iα exon of Cα was cloned from bacterial artificial chromosome (BAC) CT7-34H6 into pGEM3Zf(+) cloning vector to generate pZG01 (11.6 kb). The gene-targeting vector (pZG02) was constructed by inserting the 3.1-kb L1HYTK1L cassette as an XhoI–SbfI segment into the SfoI site in pZG01 by blunt end ligation. The Gal4EGFP exchange vector (pZG18) was constructed by inserting (by blunt end ligation) a HindIII–SalI segment containing 14× Gal4 UAS sites into the ClaI 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 BAC 199M11 (GenBank accession no. AF450245), and the inverted Lox511 sites were added to the ends of the primers. 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 (pZG36) was constructed by insertion of the human lamin B2 origin sequence (1.3-kb NotI segment; provided by M. Aladjem, National Cancer Institute/National Institutes of Health, Bethesda, MD) in the StuI site upstream of the Gal4 UAS repeats in pZG18 by blunt end ligation.

The following vectors were also used in this study: expression vectors for Gal4-VP16 and Gal4 DBD (provided by D. Reinberg, New York University School of Medicine, New York, NY; Vaquero et al., 2004), an expression vector for LacI-VP16 (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 × 10^6 cells/15-ml tube and spun down just before electroporation. Cells were electroporated using a mouse ES cell nucleofection kit (Amaxa) 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 5 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 cytotoxic 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 RT-PCR was performed using a real-time RT-PCR kit (QuantiTect 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 RT-PCR program was 50°C for 20 min for reverse transcription, 95°C for 15 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 ×10^6 ES cells using the ChIP assay kit (Millipore). Real-time PCR was performed in triplicate using SYBR green PCR master mix (Applied Biosystems) on a real-time PCR system machine (7300; Applied Biosystems). The following antibodies were used: anti–acetyl histone H3 (Millipore), anti–acetyl histone H3K9 (Millipore), anti-H3K4me3 (Abcam), and IgG (Bethyl Laboratories, Inc.).

PCR, RNA extraction, Southern blotting, and Western blotting were conducted according to standard procedures. The antibodies used for Western blotting were antibody against Gal4 DBD (Millipore) and GAPDH antibody (Abcam). All primers used in this study are listed in Table S4.

Online supplemental material

Fig. S1 illustrates the experimental protocols for SMARD and diagrams of replication patterns. Fig. S2 shows Southern blot confirmation of RMCE with the Gal4EGFP cassette between Cε and Cα. Fig. S3 demonstrates the stable Gal4-VP16 expression by RT-PCR, the resulting elevation of EGFP reporter gene expression, and H3K9 acetylation. Fig. S4 shows the confirmation of the insertion of the 10.7-kb sequence between Cε and Cα by Southern blotting and long-range PCR. Many molecules lacking initiation sites after insertion of the 10.7-kb 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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