

# Initiation of DNA replication at the human $\beta$ -globin 3' enhancer

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## ABSTRACT

The origin of DNA replication in the human  $\beta$ -globin gene contains an initiation region (IR) and two flanking auxiliary elements. Two replicator modules are located within the upstream auxiliary sequence and the IR core, but the functional sequences in the downstream auxiliary element are unknown. Here, we use a combination of benzoylated-naphthoylated DEAE (BND) cellulose purification and nascent strand abundance assays to show that replication initiation occurs at the  $\beta$ -globin 3' enhancer on human chromosome 11 in the Hu11 hybrid murine erythroleukemia (MEL) cell line. To examine replicator function, 3' enhancer fragments were inserted into an ectopic site in MEL cells via an optimized FRT/EGFP-FLP integration system. These experiments demonstrate that the 1.6 kb downstream auxiliary element is a third replicator module called bGRep-E in erythroid cells. The minimal 260 bp 3' enhancer is required but not sufficient to initiate efficient replication, suggesting cooperation with adjacent sequences. The minimal 3' enhancer also cooperates with elements in an expressing HS3 $\beta$ / $\gamma$ -globin construct to initiate replication. These data indicate that the  $\beta$ -globin replicator has multiple initiation sites in three closely spaced replicator modules. We conclude that a mammalian enhancer can cooperate with adjacent sequences to create an efficient replicator module.

## INTRODUCTION

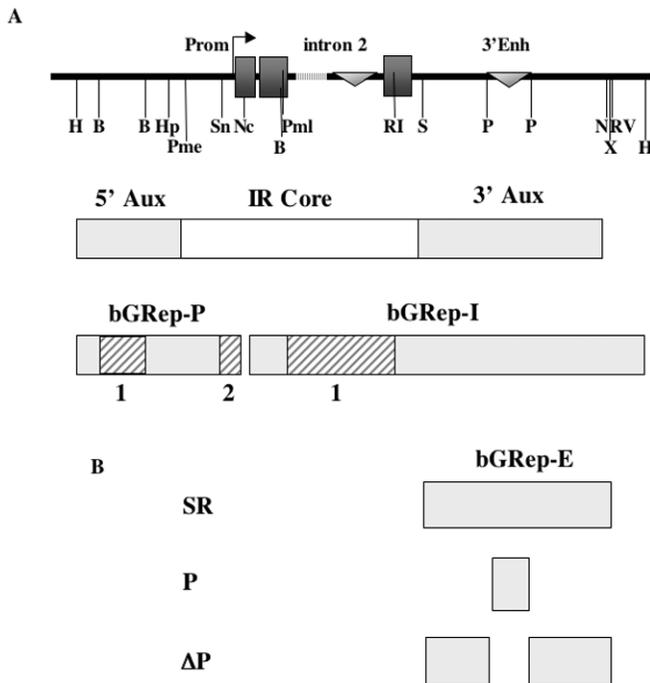
The human  $\beta$ -globin gene contains a well-described origin of bi-directional DNA replication, and its function is interconnected at some level with elements that control transcription of the gene. The origin of replication was first mapped to a 2 kb

region 5' of the  $\beta$ -globin gene by leading strand analysis (1). Using the same method but different probes, the strand transition was subsequently mapped to a replication initiation region (IR) covering the  $\beta$ -globin promoter to intron 2 (2). PCR-based nascent strand abundance assays confirmed that initiation occurs within this IR (Figure 1A) (3,4). However, when the core fragment of the  $\beta$ -globin IR was targeted to an ectopic chromosomal location in CV-1 cells, it did not initiate DNA replication. Instead, initiation activity by the core required either an auxiliary sequence located upstream that colocalizes with the distal promoter (Figure 1A) or an auxiliary sequence located downstream that includes the 3' enhancer (3). These same auxiliary sequences are required for consistent full level expression from  $\beta$ -globin transgenes (5). The role of these auxiliary sequences in  $\beta$ -globin replication and transcription is not understood.

Replicators are defined genetically as DNA sequences that initiate replication at ectopic integration sites. Replicator elements are modular in mammalian origins such as DHFR (6), lamin B2 (7) and c-myc (8). It has been shown recently that binding of transcription factors to DNA *cis*-elements can specify sites of replication initiation (9) and induce c-myc replicator activity (10). Recent studies of  $\beta$ -globin replication initiation at ectopic sites using sub-fragments of the IR revealed that it is composed of at least two modular replicators: (i) bGRep-P is located in the  $\beta$ -globin promoter and (ii) bGRep-I is located in the  $\beta$ -globin transcription unit (Figure 1A) (11). bGRep-P contains two essential regions designated as bGRep-P-1 in the upstream auxiliary element, and bGRep-P-2 in the proximal promoter and therefore, contains part of the original core of the IR. bGRep-I covers the rest of the core IR and the downstream auxiliary sequence. The function of bGRep-I requires the bGRep-I-1 sequence in  $\beta$ -globin intron 2, but the  $\beta$ -globin 3' enhancer is not essential for its activity (11). Hence the identity of the downstream auxiliary sequence and its functional activity on the modular replicators is unknown.

The  $\beta$ -globin 3' enhancer is the only element known to lie in the downstream auxiliary sequence. It contains four GATA-1

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**Figure 1.** Map of the human  $\beta$ -globin gene and replication origin. (A) Solid boxes represent  $\beta$ -globin exons, triangles are  $\beta$ -globin enhancers, the banded line is an AT-rich element. Restriction sites: B-BamHI, H-HindIII, Hp-HpaI, N-NdeI, Nc-NcoI, P-PstI, RI-EcoRI, RV-EcoRV, S-SwaI, Sn-SnaBI, X-XbaI. IR core – initiation region core; 5' and 3' Aux – upstream and downstream auxiliary sequences; bGRep-P and bGRep-I known replicator modules with essential subregions shown as hatched boxes. (B)  $\beta$ -globin replicator fragments used in this study for fine mapping replication initiation in the downstream auxiliary sequence.

binding sites (12), and regulates transcription in a tissue specific and developmentally specific manner (13–16). Deletion of the 3' enhancer in yeast artificial chromosome (YAC) transgenic mice causes a reduction in  $\beta$ -globin gene expression (17). In these mice, the  $\beta$ -globin gene is under the transcriptional control of the locus control region (LCR) composed of at least four DNaseI hypersensitive sites (HS) located 50 kb upstream (18). Stable transfection studies suggested that the 3' enhancer has no role in LCR-mediated induction of the promoter (19,20). However, we have shown that full and consistent expression levels from LCR  $\beta$ -globin transgenes at ectopic, single copy insertion sites requires the simultaneous presence of all four LCR elements, the distal part of the  $\beta$ -globin promoter and the  $\beta$ -globin 3' enhancer (5). Hence, the same auxiliary regions that influence replication initiation at the  $\beta$ -globin IR core also functionally cooperate with the LCR to regulate transcription. The HS3 LCR element can reproducibly activate expression of single copy  $\beta/\gamma$ -globin hybrid transgenes, but this activity requires the simultaneous presence of the AT-rich region in  $\beta$ -globin intron 2 (Figure 1A) and the  $\beta$ -globin 3' enhancer (21,22). These results demonstrate that HS3 functionally interacts with sequences that colocalize with the bGRep-I-1 replicator in  $\beta$ -globin intron 2 and with the 3' enhancer in the downstream auxiliary sequence.

It has been proposed recently that spatial organization of the  $\beta$ -globin locus regulates transcription through a transition

from a chromatin hub (CH) to an active chromatin hub (ACH) (23,24). The CH is formed by physical interactions between the most distant 5' and 3' HS that flank the locus to produce a 180 kb chromatin loop. The CH does not display any tissue or developmental stage specificity and marks the transcriptionally inactive state in which the  $\beta$ -globin origin is late replicating. Globin gene expression and the switch to early replication (25,26) coincides with the formation of the ACH through interaction of the LCR hypersensitive sites with the promoters of transcribed genes in the cluster (27). In definitive erythroid cells, the HS3 element of the LCR is absolutely required for ACH formation and loops to physically interact with sequences near to or within the  $\beta$ -globin gene that are proposed to be either the  $\beta$ -globin distal promoter (60 578–61 323) or 3' enhancer (23). Hence, HS3 may interact with the replicators or their auxiliary sequences to establish or maintain the ACH in definitive erythroid cells.

Here, we report a modified replication initiation method that fine maps a peak of high-level nascent strand abundance to the  $\beta$ -globin 3' enhancer in Hu11 cells, showing that a novel site of initiation of replication is located at or near this fragment. To functionally test the ability of the 3' enhancer to initiate replication at an ectopic location, we inserted a FLP recombination target (FRT) acceptor site into murine erythroleukemia (MEL) cells that express erythroid *trans*-acting factors including GATA-1. Using the site-specific FRT/EGFP-FLP integration system to introduce constructs containing the 3' enhancer, we show that the SR fragment initiates DNA replication in a region that encompasses the 3' enhancer. The minimal 3' enhancer alone does not initiate replication, suggesting that additional sequences are required. However, the minimal 3' enhancer can initiate replication when combined with the  $\beta$ -globin promoter/intron 2 and HS3, showing that the function of the larger downstream auxiliary sequences can be compensated by the promoter/intron 2 and/or HS3. This is the same functional interaction of elements that is required for  $\beta$ -globin gene expression in transgenic mice. These data demonstrate that the downstream auxiliary sequence contains a bGRep-E replicator module, indicating that the human  $\beta$ -globin replicator has multiple initiation sites in three closely spaced replicator modules rather than a single discrete initiation region. In a minimal LCR  $\beta$ -globin cassette, these replicator modules can jointly direct a replication initiation pattern which qualitatively resembles that observed at the endogenous human  $\beta$ -globin gene. Moreover, our findings show that a mammalian enhancer can cooperate with adjacent sequences to create an efficient replicator module, and support the concept that transcription factor recruitment to *cis*-elements can specify sites of replication initiation.

## MATERIALS AND METHODS

### Cells and culture conditions

MEL cells were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), L-glutamine 100  $\mu$ M, penicillin 100  $\mu$ g/ml and streptomycin 292  $\mu$ g/ml (Gibco-BRL). The Hu11 cell line was maintained in  $\alpha$ -MEM medium supplemented as above and HAT supplement (Gibco-BRL).

## Plasmids

The pOG33 plasmid contains a  $\beta$ -gal cassette interrupted by an FRT and a Neomycin resistance (Neo) gene (28). pEGFP-FLP-C1 plasmid (29) was the generous gift of D.E. Sabath. The SFV plasmid contains a single FRT site, the Hygromycin resistance (Hygro) gene and several unique restriction sites (NotI, BamHI) for cloning  $\beta$ -globin sequences. The SR (SwaI-EcoRV)  $\beta$ -globin fragment was cloned with BamHI linkers into the BamHI site of SFV vector. The P fragment was subcloned as an NheI fragment from BGT50 (21) into the NheI site of partially digested SFV vector. The  $\Delta$ P fragment was generated by subcloning the SR construct BamHI fragment into pGem7Zf which lacks PstI sites. The 3' enhancer was deleted by PstI digestion and religation, and this  $\Delta$ P fragment was reinserted into the BamHI site of the SFV vector. The BGT50 fragment was cloned by ClaI digestion and NotI linker ligation into the SFV vector NotI site.

## Transfection

MEL cells were electroporated with linearized pOG33 plasmid by double pulses at 25  $\mu$ F, 700 V using a Bio-Rad Gene-Pulser. After 48 h cells were plated at different densities ( $10^3$ ,  $10^4$  per 200  $\mu$ l in 96-well plates) for selection in medium containing

1 mg/ml of geneticin (Gibco-BRL). After two weeks of culturing, geneticin resistant single clones were expanded, and analyzed by Southern blot analysis. Transient transfection of the chosen MEL/FRT clone was performed with circular plasmids as follows: (i) 100  $\mu$ g pEGFP-FLP-C1 or (ii) 100  $\mu$ g SFV based  $\beta$ -globin vectors together with 100  $\mu$ g pEGFP-FLP-C1 by a single pulse at 260 V and capacitance of 960  $\mu$ F. In the first case, transient EGFP expressing cells were sorted by Flow cytometry 24 h after transfection. In the second case, cells were plated 48 h after transfection at different densities ( $10^3$ ,  $10^4$  per 200  $\mu$ l in 96-well plates) for selection in medium containing 500  $\mu$ g/ml of Hygromycin (Gibco-BRL).

## Flow cytometry

EGFP-positive cells were sorted with a FACStar<sup>+</sup> (Becton Dickinson) Flow cytometer using 488 nm argon laser excitation and 530 nm bandpass filter. Flow cytometric analysis of  $\beta$ -galactosidase ( $\beta$ -gal) activity (FACS-GAL) was performed as described (30). Cells were subjected to an analytical Flow cytometry on a FACScan (Becton Dickinson) using 488 nm argon laser excitation and a 525 nm bandpass filter to detect FITC fluorescence emission and 620 nm bandpass filter to detect PI fluorescence emission.

**Table 1.** Primers used for real-time PCR analysis

Primer pair	Primer sequence coordinates	Product size (bp)	Primer sequence
U01317			
9 (5'control)	52332–52352 F <sup>a</sup> 52692–52662 R <sup>b</sup>	360	GTAACCAGATCTCCCAATGTG ATATGTGGATCTGGAGCTCAG
3	62202–62221 F 62597–62578 R	395	CCTGAGGAGAAGTCTGCCGT CAGTGCAGCTCACTCAGTGT
4	62570–62590 F 62770–62749 R	200	CCTTTGCCACACTGAGTGAGC CCTGAGACTCCACACTGATGC
Oct	62875–62900 F 63115–63093 R	240	TGCCTTAACATTGTGTATAACAAAAG TACCCTGATTTGGTCAATATGTG
SATB	62954–62973 F 63228–63208 R	274	CCTAGTACACTACTATTTGG CATTGTATCATTATGGCCCTG
5	63438–63462 F 63817–63792 R	379	CCCTTTTGCTAATCATGTTTCATACC TTTATGTTTTAAATGCACCTGACCTCC
6	63863–63886 F 64071–64049 R	208	GAGATGCAGGATAAGCAAATGGG AAACTCCATGAAAAGAAGGTGAGGC
7	64308–64329 F 64547–64525 R	239	GTCTCCCGGAACATCACTCTT GCACCATAAGGGACATGATAAGG
UE	64053–64076 F 64570–64289 R	259	TTTGCTTATCCTGCATCTCTCAGC TGCTAATAACTGCAGATTCGGG <sup>c</sup>
10 (3'control)	72883–72904 F 73248–73228 R	365	AGTTCATGTCCTTTGTAGGGAC GCTCTACGGATGTGTGAGATC
$\beta/\gamma$ hybrid OR2/3	62153–62173 F ( $\beta$ -promoter) 39640–39621 R ( $A\gamma$ -intron1)	207	CAACTGTGTTCACTAGCAACC CACAAATCCTGAGAAGCGAC
7*	41306–41328 F (3' of $A\gamma$ -Exon3) 64547–64525 R ( $\beta$ - 3'Enh)	310	GGCTGGATTGATTGCAGCTGAGT GCACCATAAGGGACATGATAAGG
Mouse mito	357–376 F 215–196 R	180	GACATCTGGTTCTTACTTCA GTTTTGGGGTTTGGCATT
LacZ	3525–3549 F	350	TTTTGCATCGAGCTGGGTAATAAGC TTGACCACTACCATCAATCCGG
J01636	3875–3854 R		CGGCGAGTACTTCTACACAGCC
Hygro2	1599–1620 F	260	CTTGATGGAGCAGCAGACGC
U40398	1859–1839 R		TCACGCCATGTAGTGTATTGACC
Hygro	2132–2154 F	259	GTTTATCGGCACCTTGCATCG
U40398	2391–2371 R		AGCGACAGAGAAGATTACAATGC
BstR	229–251 F	357	GAGTGAATGAGTTCTTCAATCG
S81409	586–564 R		

<sup>a</sup>F-forward primer.

<sup>b</sup>R-Reverse primer.

<sup>c</sup>Bold letters correspond to sequence 3' of the junction across  $\Delta$ PstI.

## DNA analysis

Southern blot hybridization. Analysis was performed using standard methods with 10 µg of genomic DNA and hybridization with random primed radiolabeled probes for the 2.9 kb PvuII fragment of the LacZ gene from pOG33 plasmid, the 1.3 kb XbaI fragment of Neo gene from pOG33 plasmid or the 0.47 kb HindIII fragment of the blasticidin resistance (BstR) gene from the SFV plasmid.

## BND nascent strand abundance assay

To minimize breakage of genomic DNA and protect RNA from degradation, cell lysis was performed carefully in a large volume ( $4 \times 10^7$  cells in 200 ml) of TEN buffer containing high concentration of EDTA 100 mM, SDS 0.5% and proteinase K 200 µg/ml at 52°C overnight. DNA was extracted by gentle phenol/chloroform treatment for 6 h and precipitated with 7.5 M NH<sub>4</sub>Ac and isopropanol overnight at 4°C.

Affinity chromatography using BND cellulose was performed to enrich for single-stranded DNA (ssDNA). Two ml of BND cellulose was combined with 50 ml of genomic DNA solution in NET buffer (1 M NaCl, 1 mM EDTA, 10 mM Tris and pH 8.0), incubated for 1 h at room temperature, spun down at 2000 r.p.m. for 2 min and washed with 50 ml of NET buffer 3–5 times. The ssDNA bound BND cellulose was used to pack a column and ssDNA was eluted with 1.8% caffeine in NET buffer at 50°C. The eluate was spun down briefly to remove particles of BND cellulose. DNA with ethanol was precipitated overnight at –20°C and the DNA pellet washed twice with 70% ethanol. DNA was dissolved in H<sub>2</sub>O and subjected to T4 polynucleotide kinase treatment, and then λ-exonuclease treatment to remove the broken double-stranded DNA (dsDNA) as described (31). To control for the efficiency of both enzymatic reactions dephosphorylated pUC18/EcoRI plasmid DNA was added. To size select nascent ssDNA 1.2% low melt alkaline gel electrophoresis was performed. The gel was neutralized, and the marker lane was cut out and stained with EtBr and the ssDNA lane cut into several fractions. The 0.8–1 kb fraction was extracted from the gel piece with β-agarase (Bio-Labs) for real-time PCR analysis.

Primers are shown in Table 1. Only primer pairs with efficient real-time PCR profiles were used, based on standard curve slopes for Hu11 or site-specifically integrated MEL cell genomic DNA. A slope of –3.3 indicates perfectly logarithmic amplification. Analyses were restricted to primer pairs with the perfect slope  $\pm 0.3$ , and primer sets with slopes outside this range were excluded.

## Real-time PCR analysis

SYBR Green based real-time PCR was performed on an ABI 7700 using fractions of nascent DNA and a number of primer pairs. For each primer pair a calibration curve was generated using diluted genomic DNA (from Hu11 or MEL acceptor cell lines with integrated globin plasmids) corresponding to 1–10 000 copies of the target sequence. Every PCR was performed in duplicate. Each reaction had a volume of 20 µl and contained 10 µl of 2× SYBR Green reagent from Applied Biosystems (reaction buffer, SYBR Green Dye, Gold Taq Polymerase, UNG nuclease), 0.2 µM of each primer, DNA, H<sub>2</sub>O. The cycling conditions were 2 min at 50°C,

10 min at 94°C, [30 s at 94°C, 1 min at 65°C (or 60, or 69)]  $\times$  55 cycles. SYBR Green detection was followed by agarose gel electrophoresis and/or Melting Curve analysis using Dissociation Curves software, to ensure that a single fragment was amplified during the reaction. The specificity of amplification for each primer pair was verified by the absence of PCR product in MEL genomic DNA.

## RESULTS

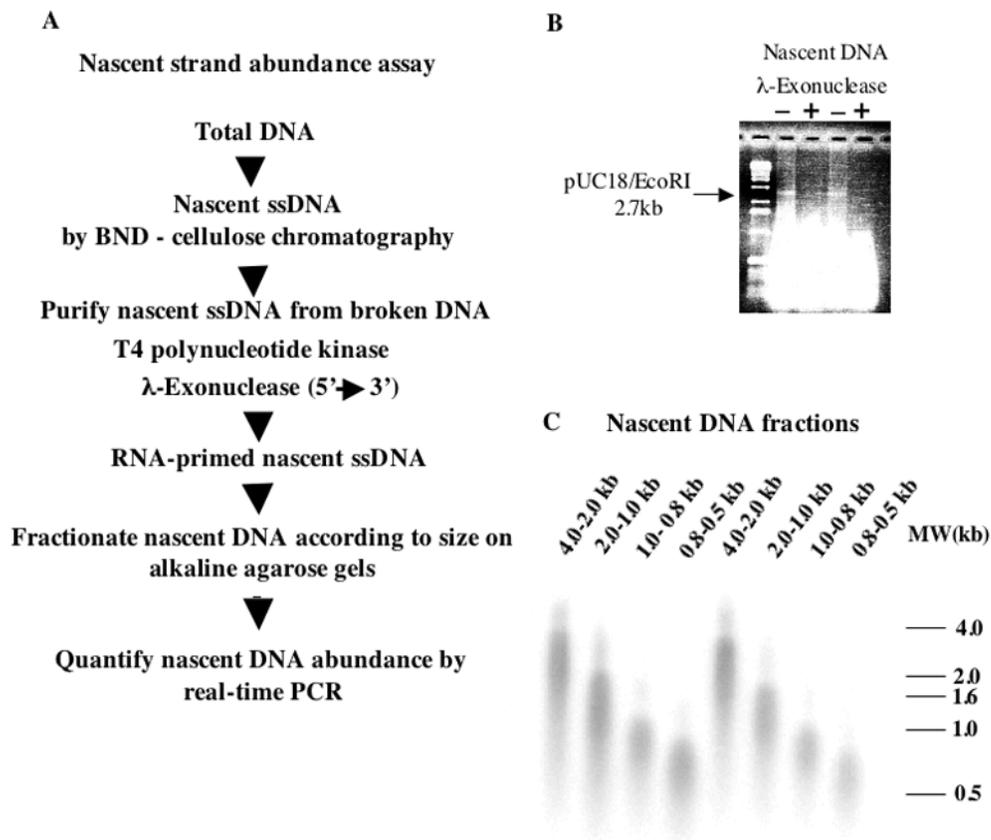
### BND nascent strand abundance assay

We combined the strengths of BND cellulose and λ-exonuclease procedures to optimize the nascent strand abundance assay to achieve high resolution replication initiation mapping in the human β-globin gene (Figure 2A). The BND cellulose chromatography step on total genomic DNA purifies single-stranded nucleic acids including nascent ssDNA, RNA and broken dsDNA with single-strand ends (32–34). To enrich for nascent DNA which retains an RNA primer at its 5' end, the DNA preparation was treated with T4 polynucleotide kinase to phosphorylate dsDNA and then specifically digested with λ-exonuclease (31,34). The quality of these enzymatic reactions was assessed by adding linearized plasmid DNA to the nascent DNA samples (35). As shown in Figure 2B, the control plasmid DNA was completely digested after phosphorylation and λ-exonuclease treatment, indicating that broken dsDNA has been removed from the nascent DNA samples. After this step, RNA primed nascent DNA was size fractionated through alkaline agarose gel electrophoresis and gel slices of defined molecular weight purified using β-agarase. The DNA fractionation was verified by Southern blot analysis of fractions probed with labeled genomic DNA from Hu11 cells (Figure 2C). The 0.8–1 kb nascent DNA fraction was chosen for nascent strand abundance assays because it is larger than Okazaki fragments.

### Replication initiation profile in Hu11 cells

The method was validated by analysis of the replication initiation profile in the human β-globin locus using nascent DNA from the Hu11 cell line, a MEL cell line bearing human chromosome 11 (Figure 3). For quantitative real-time PCR using SYBR Green detection we used several primer pairs that cover the β-globin gene sequences extending from the promoter to the 3' enhancer. For all primer pairs, a standard curve was generated using dilutions of known concentrations of Hu11 genomic DNA, and correct amplification was determined by gel electrophoresis of the PCR products and melting temperature analysis. As a control, we used primer pairs located 10 kb upstream and 9 kb downstream of the β-globin gene that did not detect any replication initiation activity as expected (4) (Figure 3B, primer pairs 9 and 10). All values presented are the mean copy number of duplicate samples. Primer pair 3 specific for the human β-globin transcriptional start site detected abundant nascent DNA near the β-globin proximal promoter in agreement with the previously published data on the known bGRep-P-2 (3,4). These findings validate the accuracy of our method.

Interestingly, we observed reduced abundance in the β-globin intron 2 region using the Oct primer pair, with a second



**Figure 2.** Replication initiation assay. (A) Schematic representation of the methodology used for the nascent strand abundance assay. (B) Quality assessment of enzymatic reactions by adding linearized plasmid DNA to the nascent DNA samples. Plasmid DNA has been completely digested after phosphorylation and  $\lambda$ -exonuclease treatment, which indicates that broken DNA has been removed from the nascent DNA samples. (C) Southern blot analysis of the nascent DNA fractions probed with labeled genomic DNA from Hu11 cells to verify DNA fractionation. The 0.8–1 kb nascent DNA fraction was subjected to abundance analysis by real-time PCR.

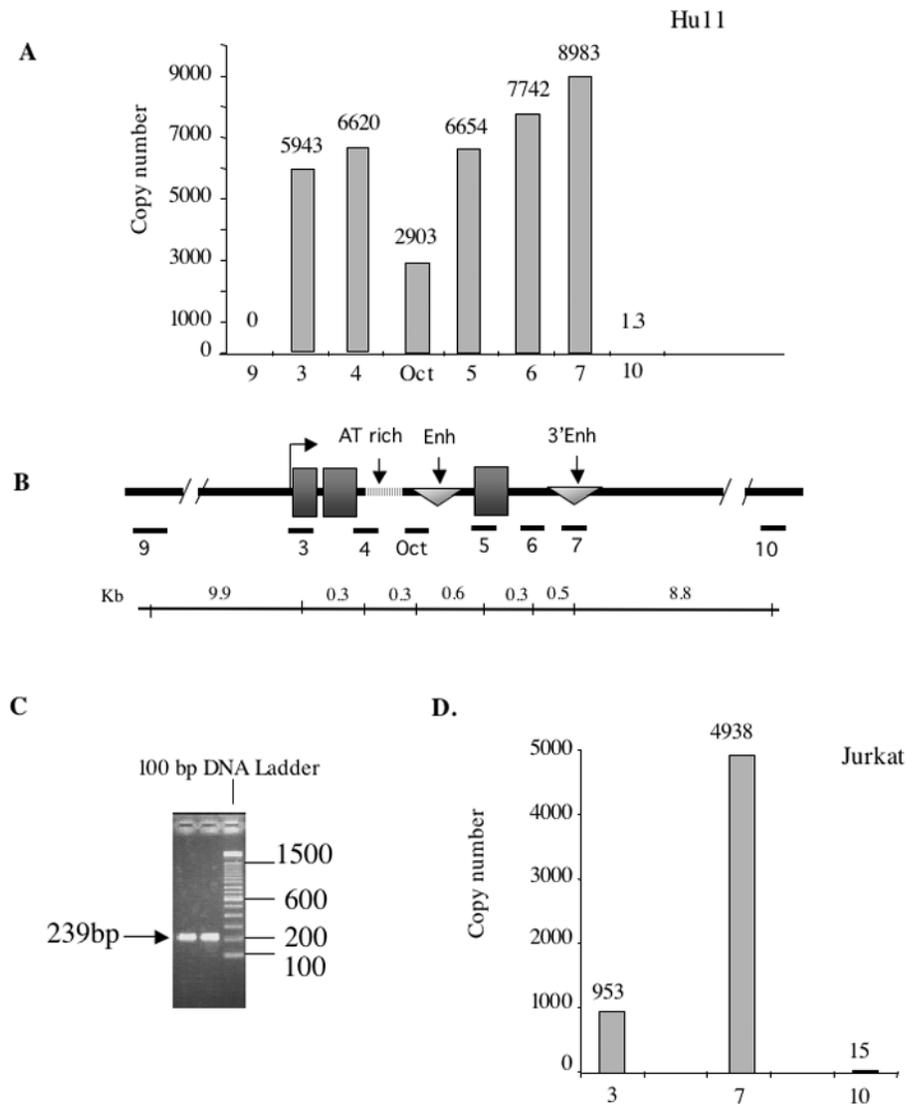
peak of amplification rising from primer pair 5 to 7 at the 3' enhancer region. Each primer pair did not produce any product from genomic MEL DNA that lacks human  $\beta$ -globin sequence, and the correct size of the primer pair 7 product detected in Hu11 nascent DNA is shown in Figure 3C. This result suggests that the replication initiation zone extends into the downstream auxiliary element (3) and peaks near the 3' enhancer. The 3' boundary of the replication initiation zone is difficult to define because we have been unable to identify any efficient primer pairs in this downstream region for real-time PCR analysis due in part to repetitive DNA sequences. Analysis of nascent DNA obtained by the sucrose gradient method (11,36) from the Jurkat human T-cell leukemia cell line revealed that these cells also initiate DNA replication near the 3' enhancer (Figure 3D).

#### MEL acceptor cell line for ectopic integration

To characterize the ability of downstream auxiliary sequences to function as a replicator at an ectopic integration site, we established an acceptor MEL cell line that contains a single FRT site for site-specific integration (Figure 4A). The MEL acceptor cell line was obtained in two steps. First, MEL cells were transfected by electroporation with linearized pOG33 plasmid which contains an SV40-FRT-Neo-FRT-LacZ

cassette. After two weeks selection in G418, single cell clones were selected and expanded. To identify clones containing a single integration site of intact vector DNA, Southern blot analysis of genomic DNA digested with BamHI or HindIII was performed. Digestion of genomic DNA with BamHI, which cuts twice in the vector DNA and hybridization with the LacZ probe detected only a single band of the expected 5.2 kb size in 3 of 44 tested clones (data not shown). HindIII digestion, which cuts the vector DNA once and hybridization with Neo or LacZ probes also produced a single band with the same three clones, indicating single copy integration of pOG33 DNA. One of these clones was chosen for the second step which involved transient transfection to express FLP recombinase.

FLP-mediated recombination at the FRT sites will excise the Neo sequence and bring the LacZ gene under the control of the SV40 promoter, with only a single FRT site remaining for use as an integration target (Figure 4A). Due to the low efficiency of transient transfection in MEL cells (29,37,38) and low efficiency FLP-mediated recombination (29), conventional CMV-FLP transient expression failed in our hands. Therefore, we used pEGFP-FLP-C1 in which the FLP coding sequence is cloned in frame 3' to the EGFP coding sequence (29). Transient expression of EGFP-FLP produced 0.5% EGFP+ cells that were sorted by FACS 48 h after transfection

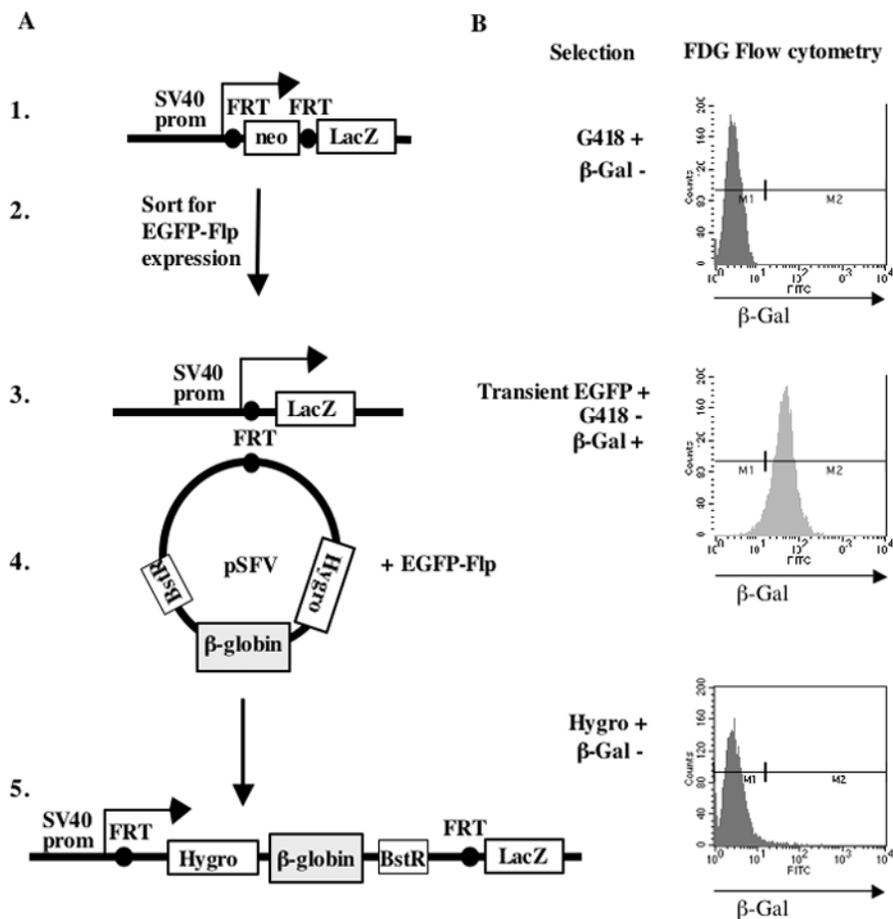


**Figure 3.** Replication initiation profile of the endogenous  $\beta$ -globin gene. (A) Real-time PCR analysis of nascent DNA from Hu11 cells. Primer pairs cover the entire  $\beta$ -globin gene including the promoter and 3' enhancer. Control primer pairs localized 10 kb upstream and 9 kb downstream of the  $\beta$ -globin gene did not detect any replication initiation activity (primer pairs 9 and 10). Primer pair 3 revealed nascent DNA abundance near the  $\beta$ -globin core promoter as expected (3,4). Nascent strand abundance peaks at primer pair 7 in the 3' enhancer suggesting the presence of another replicator. Copy number is the absolute value obtained from a sample dilution that produces linear amplification. (B) Structure of the  $\beta$ -globin gene with the distance between chosen primer pairs shown under the map. (C) Electrophoresis of final PCR products amplified with primer pair 7. (D) Real-time PCR data of replication initiation activity at the  $\beta$ -globin gene in nascent DNA obtained by the sucrose gradient method from the Jurkat human T-cell leukemia cell line. Primer pair 7 revealed abundant amplification at the 3' enhancer, indicating initiation of replication.

and plated by limiting dilution to obtain independent colonies. To identify clones that recombined the FRT sites and therefore, expressed  $\beta$ -gal, fluorescein di- $\beta$ -D-galactopyranoside (FDG) Flow cytometry was performed (30) (Figure 4B). Prior to EGFP-FLP transfection the MEL clone was FDG negative, while successful deletion of Neo was revealed as FDG+ subclones that were G418 sensitive. Recombination was verified by Southern blot analysis with EcoRI digestion and hybridization with the LacZ probe giving a 5.5 kb band (Figure 5B left). Thus, sorting for EGFP-FLP+ cells enabled identification of a MEL acceptor cell line bearing a single FRT site. The target FRT site will accept different  $\beta$ -globin gene fragments inserted by subsequent FLP-mediated recombination to study replication at a fixed ectopic site.

#### Site-specific insertion into the ectopic acceptor site

Three  $\beta$ -globin constructs were generated to test the ability of each fragment to initiate DNA replication at the ectopic site in the acceptor MEL cell line (Figure 1B). Site-specific insertion into the FRT site was performed by co-transfection of the SFV vector bearing each specific  $\beta$ -globin fragment and pEGFP-FLP-C1 by electroporation (Figure 4A). After two days, cells were serially diluted in Hygromycin selection medium at different densities to obtain single Hygromycin resistant clones. Two weeks later independent clones were expanded and subjected to FDG Flow cytometry to identify clones that lost  $\beta$ -gal expression and therefore, contained integrated  $\beta$ -globin sequences at the FRT site (Figure 4B). Intactness of  $\beta$ -globin



**Figure 4.** Site-specific integration of globin sequences into acceptor MEL cells. (A) Schematic representation of the steps involved in obtaining the MEL acceptor cell line with the single FRT site and integration of the globin sequences at this site. (1) Stable transfection of vector DNA containing two FRT sites. (2) Transient expression of EGFP-FLP to excise the Neo gene by FLP-mediated recombination at FRT sites. (3) Structure of the MEL acceptor cell line bearing a single FRT site. (4) Transient co-transfection of SFV/globin cassette and EGFP-FLP vector to integrate globin sequence at the FRT site. (5) Structure of the integrated globin cassette at the FRT site in MEL cells. (B) Selection strategy. (1) Stable transfection of MEL cells resulted in G418 resistant clones. Due to interruption of the SV40 promoter and LacZ sequence by the Neo gene these clones were  $\beta$ -gal negative as detected by FDG Flow cytometry. (2) Transient expression of EGFP-FLP protein allowed enrichment for cells expressing FLP recombinase by Flow sorting of EGFP-positive cells. (3) FLP-mediated recombination at FRT sites excised the Neo sequence and brought LacZ gene under the control of the SV40 promoter.  $\beta$ -gal positive clones were selected by FDG staining and Flow cytometry. (4) Hygromycin resistant clones, in which integration of SFV/globin cassettes occurred, were selected, expanded and stained with FDG to select for clones with FLP-mediated integration at the FRT site.

sequences was verified by Southern blot analysis using the LacZ probe (Figure 5B left). Digestion of genomic DNA with EcoRI, which cuts the integrated SFV vector DNA once in Hygro and cuts MEL acceptor genomic DNA 3' of LacZ, detected a single band of 5.5 kb in the MEL acceptor cell line and the expected size of 4.6 kb in MEL/BGT50 cell line (an additional EcoRI site is present in the  $\beta/\gamma$ -globin hybrid sequence) and 7.3 kb in four different MEL/SR clones. Single site integration of the  $\beta$ -globin plasmids was verified by Southern blot analysis of genomic DNA digested with EcoRI and hybridization with the BstR probe (Figure 5B right). Detection of a single band of the expected size demonstrated that integration of the transgene occurred at the FRT site with no randomly integrated vector DNA. Identical analysis was performed on all the  $\beta$ -globin constructs inserted into the specific FRT target site (Figure 5C).

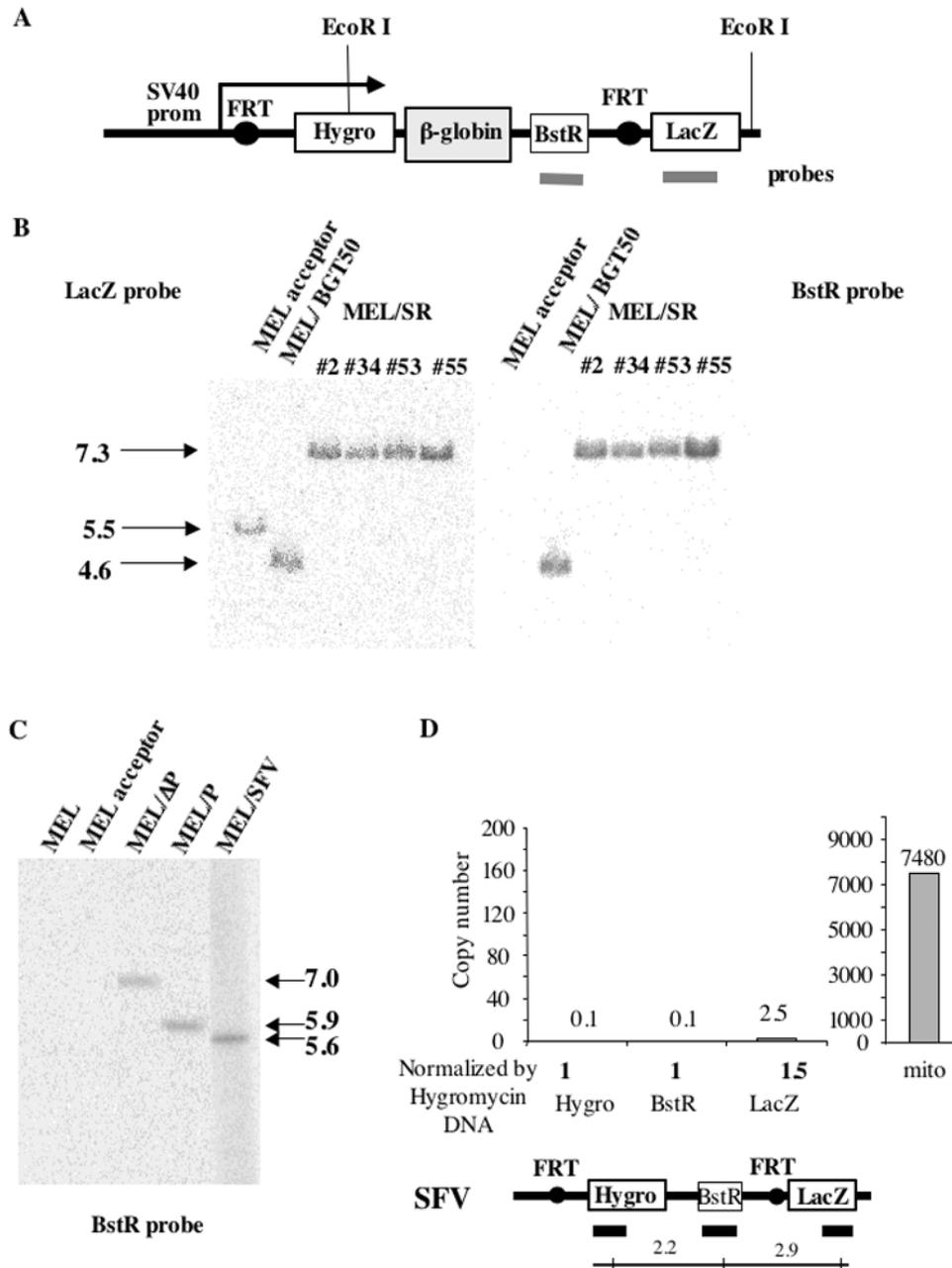
#### MEL acceptor site has no background replication initiation

To estimate background replication initiation activity at the chromosomal location, we integrated an empty SFV

vector into the site. Nascent DNA was purified from MEL/SFV cells and real-time PCR with primer pairs specific for Hygro, BstR and LacZ marker genes revealed no replication initiation activity at these sequences (Figure 5D). All three primer pairs produced PCR product of the right size on genomic DNA from the MEL/SFV line (data not shown). In contrast, PCR product was detected for mouse mitochondrial DNA demonstrating that nascent DNA was present. These results demonstrate that non-specific replication initiation does not occur at the MEL acceptor cell line target site.

#### Replication initiation by downstream auxiliary sequences

To investigate the ability of  $\beta$ -globin downstream auxiliary sequences to initiate replication at the ectopic site, we subcloned the SmaI-EcoRV fragment (63 755–65 428) that contains the 3' enhancer and surrounding sequences into the SFV vector and integrated this SR construct (Figure 1B) by FRT/EGFP-FLP recombination into the MEL acceptor cell line. To assess replicator function, two independent nascent



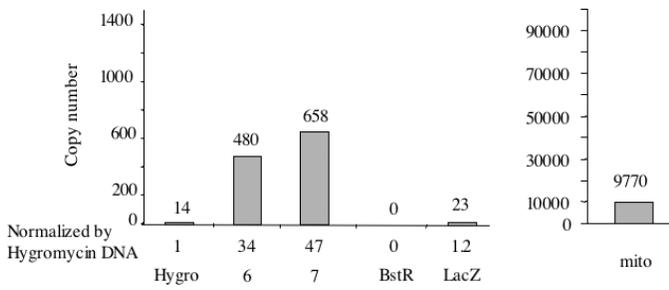
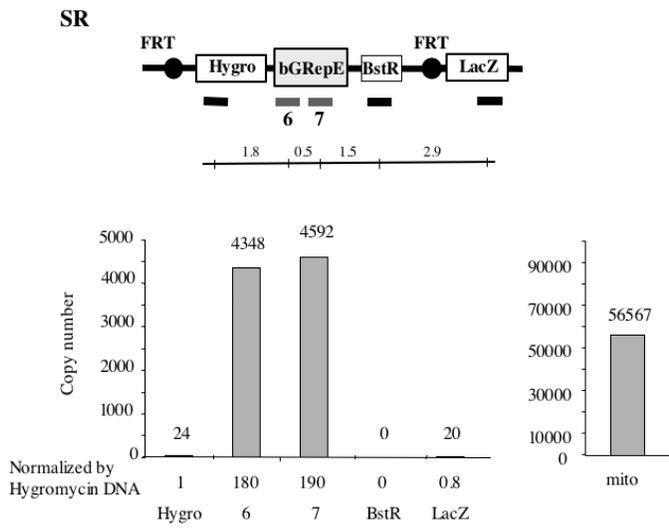
**Figure 5.** Single copy intact integration of globin sequences at the fixed FRT site. (A) Structure of targeted MEL cells. Genomic DNA was digested with EcoRI. Southern Blots were hybridized with labeled LacZ or BstR DNA probes (grey lines). (B) Digestion of genomic DNA with EcoRI (which cuts the integrated vector DNA once and cuts MEL acceptor DNA 3' of LacZ) hybridized with LacZ probe (left) confirming intactness of the integrated constructs. Hybridization with BstR probe detected no band in the MEL acceptor cell line as expected and a single band of the expected size indicating single site integration of the β-globin sequences at the FRT site and the absence of randomly integrated vector DNA. (C) Southern analysis for single site integration in additional constructs produces the correct size bands after hybridization with the BstR probe. (D) Real-time PCR analysis of nascent DNA from MEL/SFV cells indicating that there is no background replication initiation activity at LacZ, BstR and Hygro marker gene sequences. Abundance of amplification with primers specific to mitochondrial DNA indicates the quality of the nascent DNA preparations.

strand preparations were purified from MEL/SR cells and real-time PCR analysis performed (Figure 6A). As expected, the Hygro, BstR and LacZ controls were all negative and mitochondrial nascent DNA was detected. In contrast, DNA amplification with primer pair 7 specific for the human 3' enhancer detected abundant nascent DNA that exceeded the background amplification by the Hygro primers by 47–190-fold. Amplification with primer pair 6 located 5' of the

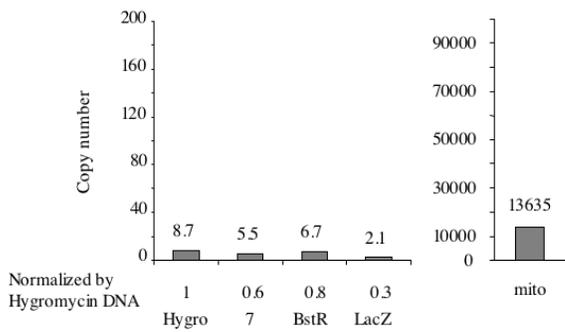
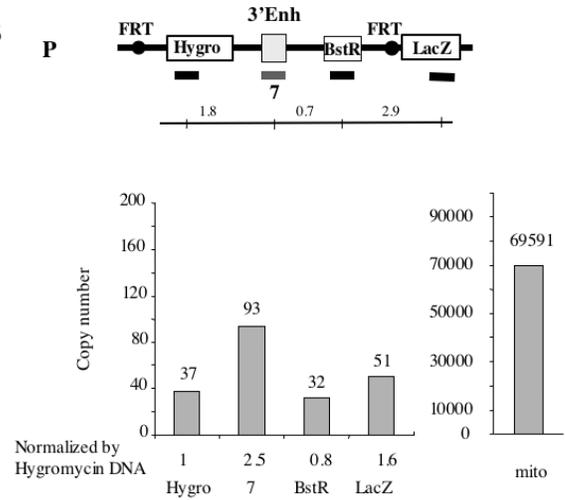
3' enhancer was 34–180 higher than the background. These data demonstrate that the SR fragment initiates DNA replication at the ectopic site in a region that encompasses the 3' enhancer, and supports the existence of a bGRep-E replicator module that can function independently in erythroid cells.

To determine whether the minimal enhancer sequence is sufficient to initiate replication, we subcloned the 260 bp

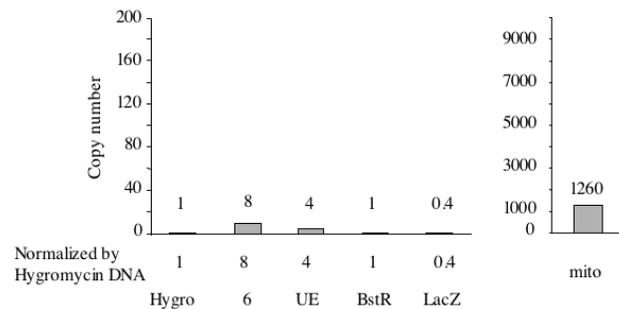
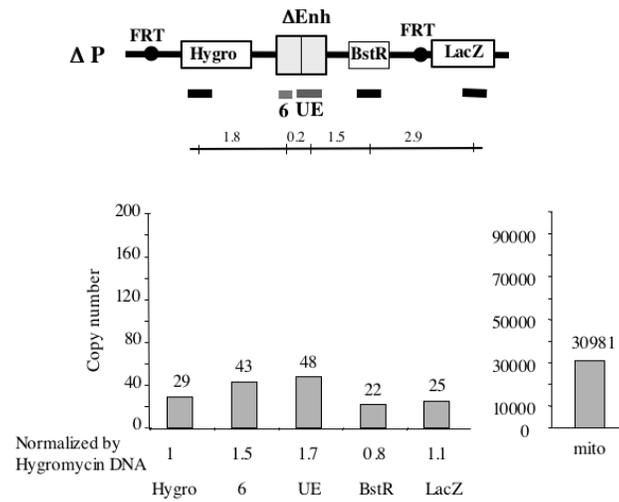
A



B



C



PstI fragment (64 302–64 561) into the SFV vector and integrated this P construct (Figure 1B) into the ectopic FRT site in the MEL acceptor cell line. Analysis of two independent nascent DNA preparations from MEL/P cells showed that mitochondrial nascent DNA was detectable (Figure 6B). However, <100 copies of nascent DNA was detected at the 3' enhancer using primer pair 7 that was 0.6–2.5-fold the level of background amplification by the Hygro primers. These results indicate that the minimal 3' enhancer does not efficiently initiate replication and suggest that additional sequences in the larger SR fragment are required to increase the efficiency of the replication module.

To investigate whether replication could be initiated on adjacent sequences in the absence of the 3' enhancer, an internal deletion was made between the PstI sites of the SR construct and this  $\Delta$ P construct was integrated into the ectopic site (Figure 1B). Real-time PCR analysis on duplicate nascent DNA preparations from MEL/ $\Delta$ P cells using primer pairs UE and 6 that span the PstI deletion junction or are 5' of the deletion, respectively, detected <50 copies of nascent DNA (Figure 6C). These data show that initiation does not efficiently occur on sequences that surround the 3' enhancer when it is deleted. This demonstrates that the 3' enhancer element is essential for bGRep-E replicator function, either as a required *cis*-acting element in the module or by acting as a spacer between other essential elements.

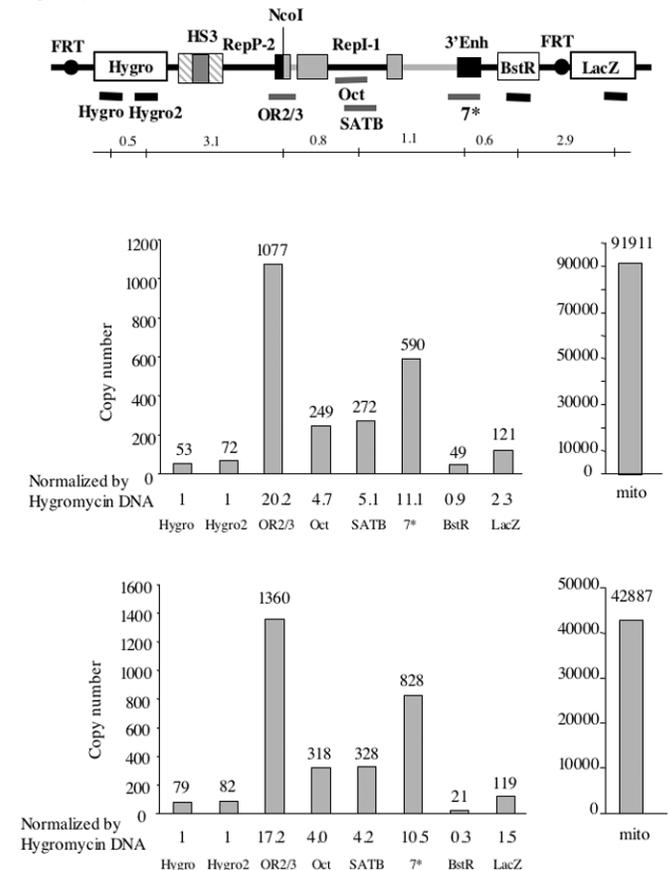
### Replication initiation at the minimal 3' enhancer in an HS3 $\beta$ / $\gamma$ -globin construct

Because the minimal 3' enhancer functionally interacts with the HS3 LCR element to activate single copy transgene expression (21), we wished to determine whether this functional interaction is accompanied by replication initiation at the minimal 3' enhancer in erythroid cells. The HS3 $\beta$ / $\gamma$ -globin hybrid construct called BGT50 (21) was selected for this analysis. BGT50 contains A $\gamma$ -globin coding sequences regulated by the  $\beta$ -globin promoter (+815 to –54 bp), the  $\beta$ -globin intron 2 and the minimal  $\beta$ -globin 3' enhancer. These transcriptional control elements in the promoter and intron 2 overlap with the known replicators, and are labeled on the BGT50 map as shown in Figure 7 as bGRep-P-2 and bGRep-I-1, respectively. In this construct, the minimal 3' enhancer is spaced 0.3 kb closer to the other replication modules than normal.

To determine whether replication initiation occurs at the minimal  $\beta$ -globin 3' enhancer in this cassette, we subcloned the BGT50 construct into the SFV vector and integrated it into the MEL acceptor cells. Real-time RT-PCR analysis of MEL/BGT50  $\gamma$ -globin RNA revealed that the BGT50 hybrid construct is transcriptionally active at the ectopic site in MEL cells (Figure S1). Results of the real-time PCR analysis of nascent DNA preparations of MEL/BGT50 cells are summarized in

Figure 7. Real-time PCR with primer pair OR2/3 specific for the bGRep-P-2 fragment extending from the  $\beta$ -globin promoter to  $\gamma$ -globin intron 1 revealed nascent DNA abundance 17–20-fold greater than with Hygro primers. Amplification with primer pair 7\* which covers the entire 3' enhancer and its junction with  $\gamma$ -globin 3' sequences detected nascent DNA at the enhancer that exceeded background amplification by the Hygro primers by 11-fold. Detection of <100 nascent copies with the Hygro primer pair and an additional Hygro2 primer pair confirms that peak abundance occurs near OR2/3. In addition, the detection of ~250–300 nascent copies with

### BGT 50



**Figure 7.** Initiation at the minimal 3' enhancer in an HS3  $\beta$ / $\gamma$  globin cassette. Genomic structure of MEL/BGT50 cells and real-time PCR quantification data of two independent MEL/BGT50 nascent DNA preparations showing two peaks of DNA replication initiation at the 3' enhancer (primer pair 7\*) and near the globin proximal promoter (primer pair OR2/3). The distance between chosen primer pairs is shown under the map. These data suggest that downstream auxiliary sequences in the bGRep-E can be compensated for by HS3 or the proximal promoter (bGRep-P-2) or intron 2 (bGRep-I-1).

**Figure 6.** The downstream auxiliary sequence initiates replication at the ectopic site. (A) Genomic structure of MEL/SR cells and real-time PCR quantification data of two independent MEL/SR nascent DNA preparations. A peak of initiation at the 3' enhancer (primer pair 7) suggests the existence of a third replicator module called bGRep-E. Abundance is presented as mean number of molecules (copy number) in duplicate samples for each nascent preparation obtained using a dilution of the sample that produces linear amplification. The distance between chosen primer pairs is shown under the map. (B) Genomic structure of MEL/P cells and real-time PCR quantification data of two independent MEL/P nascent DNA preparations. Absence of specific amplification with primer pair 7 despite the high abundance of the mitochondrial DNA in the two nascent DNA preparations, indicates that the minimal 260 bp 3' enhancer sequence is not sufficient to initiate efficient replication. (C) Genomic structure of MEL/ $\Delta$ P cells and real-time PCR analysis on duplicate nascent DNA preparations using primers 5' of the PstI deletion that detected only background amplification. These data suggest that deletion of the minimal 3' enhancer abolishes initiation of replication on surrounding sequences.

the Oct and an additional SATB primer pair sets (specific for bGRep-I-1) confirms the reduced initiation levels seen in this region in the Hu11 cells (Figure 3A). More importantly, these two primer pairs allow a clear delineation of the second peak of abundance near primer pair 7\* at the enhancer. These data show that the peak of abundance is in the 3' enhancer and not due to long nascent DNA derived from bGRep-I-1 although it is less abundant than in the context of endogenous  $\beta$ -globin sequences (Figure 3A). The three modules present in the BGT50 cassette (bGRep-P-2, bGRep-I-1 and the 3' enhancer) are sufficient to initiate DNA replication in a pattern that qualitatively resembles the endogenous human  $\beta$ -globin gene in Hu11 cells but it is not clear whether all three modules are required to obtain this pattern. As the 3' enhancer is separated by neutral  $\gamma$ -globin sequences from bGRep-I-1 which initiates lower level replication detected by two primer pairs, these findings strongly indicate that the 3' enhancer can cooperate with adjacent  $\beta$ -globin elements to initiate the second peak of replication.

## DISCUSSION

Initiation of DNA replication at the human  $\beta$ -globin gene is controlled by a modular arrangement of two known replicators that are influenced by auxiliary sequences whose roles have not been defined. We have examined the function of the downstream auxiliary sequence at the endogenous human  $\beta$ -globin gene, and at an ectopic integration site in erythroid cells, using a BND nascent strand abundance assay. This assay attempts to immediately enrich for nascent ssDNA through BND cellulose chromatography and eliminates contaminating broken dsDNA with  $\lambda$ -exonuclease treatment, prior to size fractionation on alkaline agarose gels. Abundance of specific sequences in the nascent DNA is detected by highly specific and quantitative real-time PCR and therefore, allows fine mapping of replication initiation. We applied this method to map initiation of DNA replication at the  $\beta$ -globin locus of Hu11 cells and determined two major peaks. One peak is co-localized with the well-characterized site of replication initiation near the  $\beta$ -globin promoter. Another peak was detected downstream of the gene, indicating replication initiation within the downstream auxiliary sequence.

The downstream auxiliary sequence contains an erythroid-specific 3' enhancer that may influence replication initiation in erythroid cells. Therefore, we examined replication initiation by downstream auxiliary sequences at an ectopic site in erythroid cells. For this purpose, we created an acceptor MEL cell line using an improved EGFP-FLP expression system to sort transient EGFP-FLP+ cells enriched for the recombined acceptor with a single FRT site. Subsequent FLP-mediated integration delivered  $\beta$ -globin fragments into this acceptor. The SR fragment containing the entire downstream auxiliary sequence was able to initiate replication at the 3' enhancer at this FRT site, suggesting the existence of a third replicator module called bGRep-E. In contrast, a minimal 3' enhancer fragment in the P construct was not sufficient to initiate efficient replication at the ectopic site, indicating that other sequences in bGRep-E are required for initiation to occur. Internal deletion of the minimal 3' enhancer from bGRep-E in the  $\Delta$ P construct abolished replication initiation on

sequences that surround the 3' enhancer, suggesting that the 3' enhancer has an essential role as a *cis*-acting replicator element or acts as a functionally important spacer between other elements. We conclude that the downstream auxiliary sequence can serve as an independent replicator module in erythroid cells, and that the minimal 3' enhancer is an important component of this bGRep-E activity.

Prior work at an ectopic site indicates that the downstream auxiliary sequence is not efficiently used as a replicator in non-erythroid CV-1 cells (3), and that the 3' enhancer is not required for activity by the bGRep-I replicator (11). However, high-level nascent strands at the 3' enhancer (64.3 kb) are detectable in CV-1 cells that contain the entire bGRep-I fragment (11), indicating that initiation at the 3' enhancer may occur in non-erythroid cells in the presence of bGRep-I. Taken together with our data, we propose that the downstream auxiliary sequence is composed of the bGRep-E module that can independently function as a replicator in erythroid cells, but requires the bGRep-I module or other  $\beta$ -globin IR core elements for initiation in non-erythroid cells. The 3' enhancer is a major contributor to bGRep-E activity in erythroid cells, but requires additional sequences in the SR fragment to initiate high levels of nascent DNA strands.

We have previously shown (5,21) that the minimal 3' enhancer has functional interactions with HS3 and  $\beta$ -globin intron 2 to activate gene expression in transgenic mice. Therefore, we also tested its ability to initiate DNA replication when linked to these regions but not the rest of the downstream auxiliary sequences. Two peaks of initiation were detected at the minimal 3' enhancer and the  $\beta$ -globin proximal promoter in the BGT50 cassette at the ectopic site in MEL cells, similar to the pattern identified in Hu11 cells. The use of neutral  $\gamma$ -globin spacers between the  $\beta$ -globin replicator modules in the BGT50 construct and detection of nascent DNA with additional primer pairs, supports the conclusion that the 3' enhancer cooperates with adjacent  $\beta$ -globin elements to initiate the second peak of replication. These results demonstrate that the rest of the downstream auxiliary sequences are not required for initiation at the 3' enhancer and can be compensated for by HS3 or the proximal promoter (bGRep-P-2) or intron 2 elements (bGRep-I-1). Furthermore, they indicate that the  $\beta$ -globin replicator has multiple initiation sites in three closely spaced replicator modules. Despite its narrow initiation zone, the  $\beta$ -globin replicator shares this feature of multiple modules with other mammalian replicators that have broad initiation zones. Thus, narrow or broad initiation zone sizes may reflect the spacing between replicator modules, rather than being the consequence of two conceptually different replication initiation mechanisms.

Together our results suggest that a replicator module called bGRep-E is located in the downstream auxiliary sequences, can be independently active at ectopic sites in erythroid cells and utilizes the 3' enhancer and other flanking sequences for its activity. Overall, our results suggest that the  $\beta$ -globin auxiliary sequences are replicator modules that require interactions with a core replicator to function in non-erythroid cells and may serve to broaden the initiation zone. These findings support the concept that transcriptional regulatory elements can stimulate replication initiation by showing that a mammalian enhancer can cooperate with adjacent sequences to create an efficient

replicator module. They also raise the interesting possibility that one role of the 3' enhancer and its associated bGREP-E replicator activity may be to stabilize the  $\beta$ -globin ACH by physically interacting with HS3 in replication and/or transcription factories (39) and thereby facilitate transcription in definitive erythroid cells.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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*Conflict of interest statement.* None declared.

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