Bacterial artificial chromosomes establish replication timing and sub-nuclear compartment de novo as extra-chromosomal vectors

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Received August 06, 2017; Revised November 27, 2017; Editorial Decision November 29, 2017; Accepted December 06, 2017

ABSTRACT

The role of DNA sequence in determining replication timing (RT) and chromatin higher order organization remains elusive. To address this question, we have developed an extra-chromosomal replication system (E-BACs) consisting of ~200 kb human bacterial artificial chromosomes (BACs) modified with Epstein-Barr virus (EBV) stable segregation elements. E-BACs were stably maintained as autonomous mini-chromosomes in EBNA1-expressing HeLa or human induced pluripotent stem cells (hiPSCs) and established distinct RT patterns. An E-BAC harboring an early replicating chromosomal region replicated early during S phase, while E-BACs derived from RT transition regions (TTRs) and late replicating regions replicated in mid to late S phase. Analysis of E-BAC interactions with cellular chromatin (4C-seq) revealed that the early replicating E-BAC interacted broadly throughout the genome and preferentially with the early replicating compartment of the nucleus. In contrast, mid- to late-replicating E-BACs interacted with more specific late replicating chromosomal segments, some of which were shared between different E-BACs. Together, we describe a versatile system in which to study the structure and function of chromosomal segments that are stably maintained separately from the influence of cellular chromosome context.

INTRODUCTION

Mammalian DNA replication is temporally and spatially regulated in units termed replication domains (RDs) (1). RDs are 400–800 kb segments of coordinate regulated replication timing (RT) change during cell differentiation (2), and correspond to chromatin structural units defined by chromatin conformation capture as Topologically Associated Domains (TADs) (3–5). Importantly, early and late replicating RDs form two distinct self-interacting compartments inside the nucleus, A and B, such that chromatin that replicates at similar times tends to be in closer proximity in the nucleus (6). A/B compartments occupy distinct positions in the interior or periphery of the nucleus, respectively (7,8), consistent with earlier cytogenetic observations of the distribution of pulse labeled DNA synthesis known as replication foci (9). Functionally, early and late replicating RDs correlate in a general sense with active and inactive chromatin, respectively, and changes in RT during cell fate transitions often coincide with changes in transcriptional activity of the resident genes, although the relationship is complex (10). Early and late replicating regions also exhibit distinct patterns of mutations (11,12), and disruptions in RT appear in several diseases (13,14), which may be linked to aberrant gene expression or genome instability.

Several lines of evidence suggest the importance of DNA sequence in regulating RT, compartmentalization and related large-scale chromatin properties. For example, human chromosome 21 retains a human-like RT pattern when introduced into a transgenic Down syndrome mouse model (15). Tandem integration of multiple BACs into the genome results in their self-assembly into domains of active or repressive chromatin marks with transcriptionally active regions decorated with H3K27me3 located at the exterior and H3K9me3 and HP1 enriched in the interior (16). In addition, a recent study characterizing the spatial arrangement of a 4.26 Mb human artificial chromosome (HAC) (17) in a xenospecific mouse background demonstrated sequence specific conformation of the HAC using 4C and FISH (18). Specifically, gene-rich and SINE-rich euchromatin, gene-poor and LINE/LTR-rich heterochromatin, and gene-depleted and satellite DNA-containing constitutive heterochromatin on the HAC interacted preferentially with chromatin of similar properties in the genome. However, these general sequence properties are not sufficient to dictate RT and cannot explain RT changes during development (1). Hence, the role of DNA sequences in determining large-scale chromatin structure and function and its regulation during development remain challenging questions. Here, we adopted an autonomously replicating system to

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test the sufficiency of isolated sequences in determining RT and sub-nuclear compartment without interference from integration.

Epstein-Barr Viruses (EBV) is a chronic intra-cellular parasite in infected individuals, stably maintaining its circular 165 kb genome extra-chromosomally in host cells during latency (19,20). A 1.8 kb region of the EBV genome containing a replication origin termed OriP, which is composed of Dyad Symmetry (DS) and Family of Repeats (FR) elements, and can support stable retention of circular plasmids in human cells (20–23). The ability of EBV to stably maintain its circular genome is granted by the FR element, Epstein-Barr Virus Nucleus Antigen 1 (EBNA1) (24,25). EBNA1 binds to the FR during mitosis in a fashion that permits FR containing plasmids to be transmitted approximately equally to daughter cells (26). The other component of EBV OriP, the DS element, functions as a replication origin in small circular plasmids, but in the full length EBV genome replication initiation does not occur at the DS but instead occurs at many sites distributed throughout the EBV genome (27–30), and, in fact, the DS is dispensable for full-length EBV replication (31,32). A previous study has shown that any DNA longer than ~10 kb harbored on an FR-containing plasmid and introduced into an EBNA1-expressing human cell line can replicate efficiently and autonomously (33), and that replication proceeds semi-conservatively, once per cell cycle and initiates at multiple sites, including within prokaryotic DNA segments (34,35). In one study, three plasmids carrying 14–20 kb fragments of human DNA were found to replicate at specific times during S phase, but not necessarily at the same time as in the chromosome (22). It was speculated that the segments may not have been long enough to establish proper RT. Later work showed that Bacterial Artificial Chromosomes (BACs) containing the FR can be stably retained in EBNA1-expressing human or mouse cells, thus this system was proposed as a potential gene therapy application for transgene expression (36–39). In fact, a beta-globin gene carried by this vector system was reported to be transcribed and correctly spliced in murine fibroblasts (37). Therefore, we sought to investigate whether these extrachromosomal autonomous replicating units could serve as useful tools to dissect the role of DNA sequences in RT regulation, avoiding interference from neighboring regions that is experienced with ectopic integration approaches. Our results show that ~200 kb segments can establish RT and sub-nuclear compartment de novo on E-BAC vectors.

MATERIALS AND METHODS

Cell culture

HeLa S3 cells were cultured in 10% cosmic calf serum supplemented Dulbecco’s Modified Eagle Medium (DMEM). HeLa-EBNA1 and E-BAC transfected cells were grown in 500 mg/ml G418 and 0.2 mg/ml hygromycin B, respectively. Human iPSK 3 or K3-EBNA1 were grown in StemPro (Thermo Fisher, A100701) culture media on GelTrex (Thermo Fisher, A14133) coated dishes. HeLa-EBNA1 and K3-EBNA1 was made by electroporating AttI linearized p2091 plasmid (gift from Bill Sugden lab). One G418 resistant colony was expanded for the study, and K3-EBNA1 was maintained under 50 ug/ml G418 selection.

Recombineering and E-BAC transfection

FR cassette was prepared by digesting pDY-HA1-HA2-neo plasmid with BsiWI and EcoRV. Plasmid pDY-HA1-HA2-neo was made by replacing AmpR gene at Asel-SspI sites with HA1-neoR fragment and inserting HA2 fragment into NruI site of pDY-plasmid (gift from Michele P. Calos Lab). The two homology arms (HAs) are 527 and 535 bp sequences from the sacB gene on pBACe3.6 vector. BACs were ordered from BACPAC Resource Center (www.bACPAC.chorI.org/), fingerprinted using restriction mapping, and electroporated to sw105 [(cro-bioA) < araC-PBADfIpe] using Bio-Rad Gene Pulser using conditions of 1.2 kV, 25 μF and 200 Ω. Sw105 carrying BACs were inoculated at 42°C for exactly 15 min to induce the expression of exo, bet, and gam recombination proteins, after which competent cells were prepared and FR cassette was introduced by electroporation. Cells were grown on low-salt LB broth with 25 μg/ml kanamycin at 32°C. E-BACs were confirmed by colony PCR (Supplementary methods), purified using Qiagen large-construct kit, and 2 ug was transfected to HeLa-EBNA1 using Qiagen effectene according to manufacturer’s specifications (Lonza P3 Primary Cell Kit, V4XP-3032). GFP positive cells were enriched by FACS, and expanded before performing Gardella gel and repli-seq.

Gardella gel and episome isolation

Horizontal Gardella gel was prepared based on (40). Cells were resuspended in sample buffer A (15% Ficoll, 40ug/ml RNaseA, 0.01% bromophenol blue in TBE), loaded in each well, and laid over by lysis buffer (5% Ficoll, 1% SDS, freshly added 1mg/ml pronase, Roche 10165921001, 0.05% xylene cyanol in TBE). Electrophoresis was carried out at 35 V for 5 h, followed by 109 V for 32.5 h in 1× TBE (Tris-borate–EDTA buffer). DNA was fragmented by staining with 1 ug/ml ethidium bromide for exactly 30 min with constant agitation, followed by UV irradiation at 60 mJ of energy (UVP CL-1000 UV crosslinker). DNA was then denatured in 0.4 N NaOH, 1.5 M NaCl for 15 min, neutralized using 87.66 g of NaCl, 60.5 g Tris–base, 1 L ddH2O, pH 7, and transferred to GE healthcare Amersham Hybond–N+ membrane overnight in Capillary blotting apparatus in 20× SSC (300 mM Tri-Na-citrate, 3 M 175.32 g NaCl, pH 7). DNA was covalently linked to the membrane by UV radiation. After pre-hybridized at 65°C with gentle agitation for 30 min, the membrane is hybridized in Church and Gilbert buffer at 65°C overnight, followed by stringency Wash in following conditions: 2× SSC, 0.1% SDS for 5 min at RT, twice; 1× SSC, 0.1% SDS for 15 min at RT; and 0.1× SSC, 0.1% SDS for 10 min at 65°C, twice. Probe was 32P dATP labeled PCR fragment from pBACe3.6

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Genome-wide replication timing profiling

Genome-wide RT profiles were constructed as previously described (45, 46). Briefly, cells were pulse-labeled with BrdU for 2 h and separated into early and late S-phase fractions by Propidium iodide staining and flow cytometry. BrdU-substituted DNA from 40k early and late S-phase cells was immunoprecipitated by anti-BrdU antibody (Becton Dickinson 347580), and sequencing libraries were prepared by NEBNext Ultra DNA Library Prep Kit for Illumina (E7370). Sequencing was performed on Illumina HiSeq 2500 using 50 bp single end. Reads of quality scores above 30 were mapped to customized hg19 reference genome with BAC backbone using bowtie2. Read counts were binned into 6 kb non-overlapping windows, and log2 ratio of read counts between early and late fraction are plotted in R. All profiles were quantile normalized to yield the same density distribution.

E-BAC copy number

E-BAC copy numbers were calculated using the following formula using read numbers in repli-seq data:

\[ \frac{\text{Mean}(\text{Read number}_{\text{BAC region}})}{\text{Mean}(\text{Read number}_{\text{BAC upstream 150 kb}} + \text{Read number}_{\text{BAC downstream 150 kb}})} \times N_{\text{ploidy}} - N_{\text{ploidy}}. \]

\( N_{\text{ploidy}} \) refers to the copy number of BAC corresponding chromosomal regions. Since the HeLa genome has a high degree of copy number variation (CNV) we used a combination of our total repli-seq reads and prior quantification of CNV in HeLa cells (41) to determine copy number. For Sox17-3 and Sox17-1 BAC, \( N_{\text{ploidy}} = 3 \); for BAC5, \( N_{\text{ploidy}} = 4 \). We also compensated for the deletion in BAC5 clone 1 and clone 2 by removing the deleted region from the mean (read number_{BAC region}). \( N_{\text{ploidy}} \) for K3-EBNA1 cells is two.

4C-seq

4C-seq was performed as previously described (42) with minor modifications. 10 million cells were fixed in 1% formaldehyde, and chromatin was digested using HindIII before proximity ligation, followed by DpnII after reverse crosslinking. 4C libraries were prepared using inverse-PCR primers that contain illumina adapters and anneal to the pBAC3.6 bacterial sequences (forward primer: 5’-ATAAACTACCGATTAAGC; reverse primer: 5’-GGAGCCACTATCGACTACGC). Libraries were sequenced with 3–7 million mappable reads using 50 bp single-end sequencing with Illumina HiSeq 2500. Reads were mapped to hg19 build using BWA. Each profile was then normalized to RPM, binned to 10 kb windows, and smoothed using rolling mean smoothing across 15 windows. 4C-seq strong interaction sites (SIs, 39-400RPM) or moderate interactions sites (MIs, 20-39RPM) are considered shared between two datasets if they overlap by 20 kb upstream or downstream. Shared sites were counted using bedtools Intersect function.

RESULTS

Characterization of the E-BAC system

Figure 1 illustrates the E-BAC system. HeLa cells stably expressing EBV EBNAA1 were transfected with the E-BACs harboring an FR cassette inserted by recombineering into the BACs listed in Figure 2A (and Supplementary Figures S1 and S2). Hygromycin resistant colonies were collected as well as the pool of remaining resistant cells, which are analyzed as indicated for autonomous replication and copy number before repli-seq and 4C-seq are performed.

Figure 1. E-BAC experimental design. FR cassette contains the EBV family of repeats (FR) and selectable markers for recombineering in E. coli (Em7, KmR) and for selection in mammalian cells (TK, HygR). The FR cassette is flanked by sequences homologous to the pBAC3.6 vector sequences, and inserted into candidate BACs by recombineering (see Materials and Methods). The resulting E-BAC is then transfected into a cell line stably expressing the EBV EBNAA1 protein (example: HeLa-EBNA1). After selection for the E-BAC, drug resistant colonies were collected as well as the pool of remaining resistant cells, which are analyzed as indicated for autonomous replication and copy number before repli-seq and 4C-seq are performed.
of the E-BACs were measured by repli-seq (Marchal et al. accepted), and chromatin compartments were evaluated by 4C-seq (42) using a bait within the pBAC3.6 plasmid vector sequences to assay genome-wide interaction partners (Figure 1). 4C is also a very sensitive detection method for integration events, since huge numbers of 4C interaction partners will derive from sequences close to any integration site.

Figure 2 summarizes the three chromosomal segments contained within the BACs chosen to represent an early replicating constant timing region (CTR), the early replicating border of the Timing transition region (TTR) adjacent to the same CTR, and a late CTR (Figure 2) (44). In repli-seq data CTRs are interpreted to be domains of coordinate activation of multiple adjacent replicons, activated stochastically so that different cells are using different cohorts of origins, which averages out to a region of relatively constant replication timing in the population repli-seq data (45). TTRs are regions of suppressed origin activity, where forks travel mostly unidirectionally for many hours (45). The sox17-3 BAC corresponds to a segment within an early CTR on chromosome 8 that contains the gene sox17 (Figure 2B), which is actively transcribed in HeLa cells. The native locus in HeLa is depleted of repressive histone marks, such as H3K9m3 and H3K27m3, which may be required for targeting to the nuclear periphery (46). The AT percentages (47) of the three BACs vary slightly (58%-62%), and architectural protein binding sites such as CTCF and Rad21 are present at single copy, suggesting that those sequences were accepted. Moreover, architectural protein binding sites are present at single copy, suggesting that those sequences were accepted.

E-BAC replication timing

We performed repli-seq to assay RT of the E-BACs (Figure 4A). Briefly, cells were pulse labeled with BrdU for 2 h, and FACS (Fluorescence Activated Cell Sorting) sorted into early vs late S phase by DNA content. BrdU substituted DNA was then immunoprecipitated, amplified, and sequenced with ~10 million mappable reads per fraction (Figure 4A). We plotted the log2 ratios of early versus late coverage for 6 kb non-overlapping bins. Results revealed that the Sox17-3 E-BAC (derived from an early replicating CTR) replicated early in S phase in both the pool and the two clones, including Subclone #1 that showed a high degree of variation in read coverage (Figure 4B). However, the Sox17-1 BAC replicated in mid-late S phase, de-
sox17-1 (P = 0.00062) and BAC5 (P = 0.00024), whereas RT of sox17-1 and BAC5 are not statistically different (P = 0.11). However, these RT values are not quantitatively the same as the endogenous locus. Statistically significant differences were found between Sox17-3 (E-BAC earlier; P = 0.0063) and Sox17-1 (E-BAC later; P = 0.0094) but not E-BAC5 (P = 0.41) and their corresponding chromosomal loci. As a control, we also performed repli-seq analysis of Raji cells (48), an EBV transformed human lymphoid cell line that carries more than 50 copies of the latent virus, as well as HeLa-EBNA1 cells transfected with a BAC containing the entire EBV genome (49) (gift from Wolfgang Hammerschmidt lab), and found that EBV replicates in early-middle S phase in both of these contexts (Figure 4C, D and Supplementary Figure S9). These control experiments suggest that RT of our E-BACs is not dictated by the presence of EBV FR sequences. Altogether, these results provide evidence that the ~200 kb DNA segments contained within each BAC are sufficient to establish RT patterns de novo that are qualitatively consistent with endogenous loci and different from the RT of native EBV. The E-BAC derived from an early CTR replicates early while the E-BACs from a TTR or a late CTR replicate with a mid-late pattern and with more RT variability.

**E-BAC compartment analysis**

To determine whether E-BACs become compartmentalized in the nucleus, we performed 4C-seq (42) using a bait on the pBAC3.6 plasmid backbone in common to each BAC. Three to seven million mappable reads were obtained for each profile. Since the presence of integrated BACs is easily detected as an extremely high interaction frequency with sequences flanking the ectopic integration site, we first scanned the genome for such high frequency interaction profiles. Results confirmed that most of the E-BACs remain extra-chromosomal, as the strongly enriched reads were restricted to the genomic sequences contained within each E-BAC. One exception was the BAC5 pool, which had a clear signature of integration at a specific site on chr14 (supplementary Figure S9). These control experiments suggested that RT of our E-BACs is not dictated by the presence of EBV FR sequences. Altogether, these results provide evidence that the ~200 kb DNA segments contained within each BAC are sufficient to establish RT patterns de novo that are qualitatively consistent with endogenous loci and different from the RT of native EBV. The E-BAC derived from an early CTR replicates early while the E-BACs from a TTR or a late CTR replicate with a mid-late pattern and with more RT variability.

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Figure 4. E-BAC replication timing. (A) Experimental flowchart for Repli-seq. Exponentially growing cells were labeled with BrdU for 2 h, sorted for DNA content into early versus late S phase. DNA was isolated, sheared and the nascent DNA synthesized either early or late during S phase was immunoprecipitated with an anti-BrdU antibody. BrdU-substituted DNA was sequenced and the log2 ratio of sequences in early versus late S phase fractions was calculated in 6 kb non-overlapping windows. To determine copy number, the reads from early and late fractions were summed to acquire whole genome sequencing information and the total number of reads per 6kb window was determined (see Figure 3B and Materials and Methods). (B) E-BAC repli-seq profiles. The Y-axis for each data point is the log2 ratio for reads within a 6 kb window. HeLa-EBNA1 cells without E-BAC transfection were used as control (gray data points). Vertical pink dashed lines indicate the human chromosomal map positions of the segments contained in each BAC. RT of the 21 kb bacterial sequences of each E-BAC (pBACe3.6 E-BAC backbone) was plotted separately and indicated as horizontal lines (average of all 6kb windows) next to the chromosomal regions. (C, D) Replication timing of the EBV genome in Raji cells (C) and the EBV BAC in HeLa-EBNA1/EBV (D) (red data points). The datapoints for HeLa-EBNA1 EBV BAC are more dispersed because there are fewer total reads due to the substantially lower copy number of the EBV sequences in this cell line (Supplementary Figure S9). Data from 15 Mb of chr8 is plotted for comparison (grey data points).
Figure 5. E-BAC compartment analysis. 4C-seq was performed using a bait on the pBACe3.6 plasmid backbone. (A) Exemplary 4C-seq and repli-seq profiles for chr12 are plotted as a black or yellow histogram, respectively. Strong Interaction sites (SIs) with reads between 39 to 400 RPM, and Moderate Interaction site (MIs) with reads between 20 and 39 RPM, are shaded by red or yellow dashed vertical lines, respectively, demonstrating how the strong and moderate interaction sites were defined for B and C. The red and pink horizontal lines indicate the y-axis positions of the SI and MI cut-offs. (B) Boxplots demonstrate RT distribution of E-BAC Strong Interaction sites (SI, red box), Moderate Interaction sites (MI, pink box) and the rest of genome as control (R, gray box) for all three pools and Sox17-3 subclone 5 (Sox17-3 #5), Sox17-1 clones 3 and 9 (Sox17-1 #3 and #9). (C) Venn diagram shows the number of shared SI or MI sites (50kb windows) in 4C-seq profiles of Sox17-3, Sox17-1 and BAC5 pool.

A large percentage of specific interaction sites (Figure 5C). This overlap was highly significant as measured by Jaccard similarity index, which was 12.9% for SIs and 19.2% for MIs between Sox17-1 and BAC5, in comparison to 0–5.2% between sox17-3 and BAC5 and 1.9% to 7.4% between sox17-3 and sox17-1. These sites are not specific to EBNA1 binding sites in the genome (EBNA1 ChIP in Raji cells) (51), and it will be interesting to explore the mechanistic underpinnings of these common interaction sites. Altogether, 4C analysis demonstrates that E-BACs establish de novo interactions with chromatin in the sub-nuclear compartment that is appropriate for their respective replication times.
A late E-BAC maintains late replication in human induced pluripotent cells

To test whether the E-BAC system works in other cell types, we made stable EBNA1 expressing human induced pluripotent cells (hiPSCs), using hiPSC line K3 (Supplementary Figure S7), and introduced E-BAC sox17-1. Because the TK promoter, driving hygR in our E-BACs, is a weak promoter in pluripotent stem cells, we replaced the TK-hygR gene in the FR cassette (Figure 1) with CAG-GFP and FACS enriched GFP positive cells instead of drug selection. Gardella gel (Figure 6A) and 4C-seq results (Supplementary Figure S8A) confirmed that the sox17-1 E-BAC is maintained extrachromosomally. Since we found that the pool had only 1–2 copies of the E-BAC, we picked a GFP-positive colony, which was then expanded, BrdU-labeled, and sorted additionally for bright GFP-expressing cells by FACS immediately prior to repli-seq. This yielded a population of cells with 6 copies per cell on average (Figure 6B). Both pool and clones demonstrated unaltered pluripotency based on their genome-wide RT profiles, which are a strong cell type specific marker of pluripotency as they change rapidly upon differentiation (Supplementary Figure S8B and C). Repli-seq results revealed that sox17-1 E-BAC maintained late replication in both the pool and the subclone, consistent with that of the endogenous locus (Figure 6C). These results suggest that RT can be maintained on extrachromosomal vectors in pluripotent cells and the E-BAC system can be used to study chromosome structure and function in stem cells.

DISCUSSION

We have developed an extra-chromosomal vector system that enables functional dissection of genomic DNA. We have shown that E-BACs can be stably maintained as extrachromosomal replicating units in EBNA1-expressing cells and the 200 kb of DNA they harbor is sufficient to dictate their RT and genome compartmentalization. An E-BAC derived from an early replicating region maintains early replication, and preferentially interacts with early replicating regions. In contrast, E-BACs corresponding to a TTR or a late CTR replicate in mid- to late-S phase and interact with the late replicating chromatin compartment, consistent with our previous finding that TTRs share chromatin properties with late regions (52,53). A previous report has shown that double minute chromosomes replicate at a similar time as their genomic counterparts (54), but this study could not distinguish whether RT was transferred epigenetically from the original chromatin or derived from the underlying DNA sequences. Our results suggest that ~200 kb DNA sequences can harbor sufficient information to direct appropriate RT and compartmentalization. Altogether, we describe a versatile system derived from EBV with which BACs of interests can be stably maintained as autonomous replicating units in EBNA1 expressing human cells including pluripotent stem cells.

The RT of full length EBV has been somewhat controversial, with studies reporting either early (55–57) or late replication (54,58), even between different groups working with Raji cells. Our results find that EBV replicates during early-mid S phase in Raji cells, as well as HeLa EBNA1. We do not know the source of the discrepancy, but we find that genome-wide analyses, where the RT of each genomic window validates the RT of the adjacent genomic window,
are more reproducible and reliable than the single site Q-PCR assays done in the past. Moreover, our results in both HeLa EBNA1 and Raji cells are consistent with a previous report of latent EBV’s association with regions of de-condensed, presumably early replicating, chromatin in primary human B lymphocytes (50). This early-mid replication is likely contributed by EBV genomic sequences other than the FR carried on our vectors, as small FR plasmids replicate in late S phase in 293 cells (34). In any case, since our three E-BACs replicate at different times, one of which is significantly earlier than the replication time of full length EBV viral genomes, it is unlikely that E-BAC replication timing and genome compartment are dictated by the supplied EBV viral retention system (EBNA1 + FR). Moreover, our 4C-seq results with different E-BACs show differential compartmentalization of early vs. late replicating E-BACs, suggesting that the genome DNA segments carried on E-BAC vectors can override both the natural RT of EBV and any chromatin interaction preferences. Presumably, cis-elements within those genomic segments control the RT and compartmentalization of the E-BACs.

Transcription has long been correlated with early replication, but causal relationships between the two have not been established (10). All of our E-BAC transfected HeLa-EBNA1 cells were grown under hygromycin B selection requiring that the hygR gene be expressed. However, the E-BACs manifested distinct RT and compartment patterns. Similarly, the sox17-1 E-BAC steadily expresses GFP in hiPSCs, yet it was clearly late replicating. Therefore, our results support the conclusion from many other correlative studies that gene transcription is not sufficient to dictate early replication or sub-nuclear position (59).

Our results also raise several interesting questions. First, what are the sequence features underlying early versus TTR/late sequences that give rise to higher order properties such as RT and sub-nuclear localization? Second, how do those higher properties relate to other 2D chromatin features such as nucleosome occupancy and histone modifications? It is as yet unknown whether the same chromatin marks are deposited on E-BACs as compared to their genomic counterparts, and if those marks are cause or effect of compartmentalization. Third, previous studies have characterized sequences from the beta-globin locus that are sufficient for dictating early replication of an ectopic chromosomal insertion sites in avian and mammalian cells (60,61). Whether such discrete functional elements exist on each BAC and what their role may be in replication remain to be examined. Our results demonstrate that E-BACs show promise to address these questions by providing a relatively facile method to study large-scale chromosome structure and function.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

**ACKNOWLEDGEMENTS**

We thank Bill Sudgen, Wolfgang Hammerschmidt, Michele P. Calos and Aloys Schepers for sharing plasmids/BACs generously and gave constructive advices on the project. We thank Paul M. Lieberman for sharing Raji cells, Vishnu Dileep for suggestions on 4C-seq data analysis, Carley Huffstetter for modifying the figures, Ruth Dider for florescent-activated cell sorting, Peiyao Zhao, Ariana Sky and Robinson Blake for helping with BAC isolation, Rajkumari Gayatri for help with Gardella gels, Denis Avery, Brian Washburn, Cheryl Pye and Kristina Poduch for vector cloning, Fanxiu Zhu for helpful discussions.

The genome sequence described/used in this research was derived from a HeLa cell line. Henrietta Lacks, and the HeLa cell line that was established from her tumor cells without her knowledge or consent in 1951, have made significant contributions to scientific progress and advances in human health. We are grateful to Henrietta Lacks, now deceased, and to her surviving family members for their contributions to biomedical research. The data generated from this research were submitted to the database of Genotypes and Phenotypes (dbGaP), as a substudy under accession number phs000640.

**Authors Contributions:** J.S. conducted the majority of the experiments and analyzed the data. D.B. performed the 4C-seq experiments. M.G. performed the experiments in hiPSCs. J.S. and D.M.G. wrote the manuscript.

**FUNDING**

National Institutes of Health (NIH) [GM083337, GM085354 to D.M.G.]. Funding for open access charge: NIH [GM083337, GM085354 to D.M.G.].

**Conflict of interest statement.** None declared.

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**AVAILABILITY**

The HeLa repli-seq and 4C-seq data from this study have been submitted to the database of Genotypes and Phenotypes (dbGaP), as a substudy under accession number phs000640. The K3 repli-seq and 4C-seq data from this study have been submitted to the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) with accession numbers GSE102704.
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