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TITLE: Determining the requirement for viral DNA amplification in mediating the reorganization of cellular chromatin during the Epstein-Barr virus lytic cycle

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Determining the requirement for viral DNA amplification in mediating the reorganization of cellular chromatin during the Epstein-Barr virus lytic cycle

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In partial fulfillment of the requirements for the CALS Honors in Research program at the University of Wisconsin - Madison

Table of Contents

Abstract……………………………………………………………………………………..4

List of Abbreviations…………………………………………………………………......5

List of Figures & Tables…………………………………………………………………6

Chapter I: Background and Introduction…………………………………………………7 - 13

Chapter II: Materials and Methods……………………………………………………...14 - 18

Chapter III: EBV and ROCC………………………………………………………….....19 - 41

References…………………………………………………………………………….42 - 44

ABSTRACT

(Determining the requirement for viral DNA amplification in mediating the reorganization of cellular chromatin during the Epstein-Barr virus lytic cycle)

We have uncovered how Epstein-Barr virus (EBV) induces the reorganization of cellular chromatin (ROCC), where host chromatin is compacted and marginated within the nucleus. We tested the role of EBV lytic DNA amplification in driving ROCC and learned that inhibiting it supports chromatin compaction but blocks margination. We favor two steps for EBV's ROCC: EBV first mediates a cellular response leading to global chromatin compaction, and second, viral DNA synthesis drives margination of cellular DNA. We asked if the histone-associated simian virus 40 (SV40) DNA synthesis could substitute for EBV's histone-free viral DNA synthesis and found that EBV's ROCC is incompatible with SV40 DNA replication. We conclude that, during its lytic phase, EBV blocks DNA synthesis in which histones are loaded onto newly synthesized DNA, in favor of its own histone-free lytic DNA amplification.

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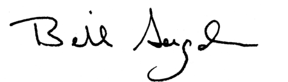

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04-25-23

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List of Abbreviations

TAg	Large T antigen
SV40	Simian virus 40
EBV	Epstein-Barr virus
polyA	polyadenylation signal
GFP	green fluorescent protein
oriLyt	origin of lytic replication
FBS	fetal bovine serum
ROCC	reorganization of cellular chromatin
HSV	herpes simplex virus
DPBS	Dulbecco’s phosphate buffered saline
PFA	paraformaldehyde
EdU	(5-ethyl-2’-deoxyuridine) thymidine nucleoside analog
DAPI	(4’,6-diamidino-2-phenylindole) fluorescent DNA stain

List of Figures & Tables

Chapter I:

Figure I.1…………………….....Spatial distributions of cellular chromatin and EBV DNA in EBV positive iD98HR1 cells during the EBV lytic cycle

Table I.1………………………Requirement of EBV genes/processes for ROCC

Chapter III:

Figure III.1……………………Construction of SVLuc plasmid

Figure III.2……………………SVLuc replication in lytically-induced EBV+ cells

Figure III.3……………………SV40 TAg alone does not induce or inhibit ROCC in iD98HR1-ZER cells

Figure III.4……………………ROCC+ cells support high levels of SV40 TAg expression

Figure III.5……………………SV40 DNA amplification is not detectable in ROCC+ cells

Figure III.6……………………EBV's ROCC is incompatible with histone-bound DNA replication in lytic cells

Supplemental Figures:

Figure SIII.1…………………..Detectable lytic DNA synthesis is dispensable for chromatin condensation but required for its margination

Figure SIII.2…………………..Representative images of cells not displaying ROCC or displaying ROCC Type I or Type II

Figure SIII.3…………………..Dose-dependent expression of firefly luciferase detected with a monoclonal luciferase antibody

Chapter I:

Background and Introduction – ROCC and DNA amplification

*Portions of this chapter are derived and/or copied from the February 2023 research article “How Epstein-Barr virus induces the reorganization of cellular chromatin” by Quincy Rosemarie, Elijah Kirschstein, and Bill Sugden published in *mBio*

Introduction

Human tumor viruses are infectious pathogens that account for ~10-15% of all human cancers (1). Epstein-Barr virus (EBV), a gamma-herpesvirus, is a human tumor virus that was first discovered in 1964 and isolated from tumor cells from a Burkitt's lymphoma patient (2). EBV has since been found to cause infectious mononucleosis and be associated with several human cancers including Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma, and gastric carcinoma [reviewed in (3)]. EBV has been extensively characterized since its discovery in 1964, but there are still many unanswered questions about its life cycle and its interactions with host cells during infection. As a result, it is necessary to have a more complete understanding of EBV infection and pathogenesis to inform the development of effective therapeutics and vaccines.

EBV infects B cells, establishing either a latent or lytic infection (4). During latent infection, EBV persists as a plasmid in memory B cells for the life of the host, replicating once per cell cycle while silencing ~90% of its viral gene expression to evade host immune detection (5). EBV lytic infection involves the activation of a "latent-lytic switch" controlled primarily by immediate early viral proteins, BZLF1 and BRLF1, that function as transcriptional activators to initiate viral gene transcription. During this lytic phase, EBV establishes what is called a productive infection where EBV DNA is amplified > 100 fold and progeny virus particles are assembled and released from lysed cells (6,7). EBV DNA amplification is peculiar in that newly synthesized viral DNA excludes histones whereas replicated cellular DNA requires histones (8). The observation that EBV DNA replicates in the absence of histones indicates that dramatic changes likely occur in nuclear chromatin organization and virus-host interactions, some of which have been previously described (9-12).

EBV is known to change the architecture of cellular chromatin during the lytic cycle by induction of BZLF1 expression. ATAC-seq, an assay for chromatin accessibility, showed that high levels of BZLF1 expression create areas of open chromatin (euchromatin) in areas where BZLF1 binds to DNA; however, across the nucleus, a genome-wide increase of closed chromatin (heterochromatin) occurs upon high BZLF1 expression (9,10). Simultaneously, during the lytic cycle of infection, replication of EBV DNA occurs in distinct replication compartments that act as amplification factories in the nucleus of infected cells. These amplification factories synthesize EBV DNA and reorganize cellular chromatin, thus excluding cellular histones and growing both in number and size as the lytic phase progresses (Figure I.1) (11,12). We have named this characteristic of the EBV lytic cycle the "reorganization of cellular chromatin (ROCC), and it is a distinct process that EBV, a herpesvirus, shares with four other virus families including baculoviruses, geminiviruses, parvoviruses, and adenoviruses (13-16).

ROCC, as it occurs in the EBV lytic cycle, simply describes the compaction and margination of host cellular chromatin to the periphery of the nucleus. Recently, genes encoded by EBV have been determined to be required or not to support the formation of ROCC (see Table I.1 for a complete list). A subset of EBV early genes including, but not limited to, BMRF1, BAFL5, and BALF2 along with EBV's *oriLyt,* the origin of lytic replication, are required for ROCC to occur during EBV's lytic cycle; however, late genes are not required for ROCC (17). Past work has focused on the contributions of the viral proteins to induce this phenotype in lytic cells, but it remains unclear what is the extent and type of DNA amplification that can or cannot support the margination of cellular chromatin during ROCC as well as the contributions of cellular factors in mediating this phenomenon.

The observed requirement of ROCC for all EBV genes essential for viral DNA amplification made it likely that blocking DNA synthesis would block ROCC as well. We tested this hypothesis with two DNA synthesis inhibitors (GCV/PAA) and uncovered a surprising result. When GCV (chain terminator) or PAA (pyrophosphate analogue) were added to cells induced to enter the EBV lytic cycle, cellular chromatin condensed but was not marginated to the nuclear periphery, resulting in a different ROCC phenotype (17). As a result, we envision a model with two ROCC phenotypes: ROCC Type I (chromatin condensation) and ROCC Type II (chromatin condensation and margination). With this notion in mind, we sought to ask whether a different kind of viral DNA amplification lacking EBV's *oriLyt* could substitute for EBV-mediated DNA amplification to promote ROCC Type II.

To answer this question, we used Simian Virus 40 (SV40), a small polyomavirus, which amplifies and encapsidates its genomes in the nucleus. We hypothesized that SV40 DNA amplification alone cannot induce ROCC, because it is known that histones are rapidly loaded onto newly synthesized SV40 DNA, contrasting with EBV DNA amplification that occurs independently of histones (18-19). In addition to testing the ability of SV40 to induce ROCC on its own, we also determined the ability of SV40 and EBV to be co-replicated in the same cell to test if SV40 can inhibit ROCC. We found that SV40 does not induce ROCC on its own, and that, in fact, the EBV lytic cycle along with its DNA replication and induction of ROCC is incompatible with SV40 DNA replication. Therefore, we could not determine if SV40-mediated DNA amplification could substitute for *oriLyt*-mediated DNA amplification in mediating ROCC Type II. However, from these experiments, we derived a mechanistic insight into ROCC: EBV likely inhibits SV40 DNA synthesis in a manner similar to its inhibition of cellular DNA synthesis in favor of its own histone-free DNA amplification.

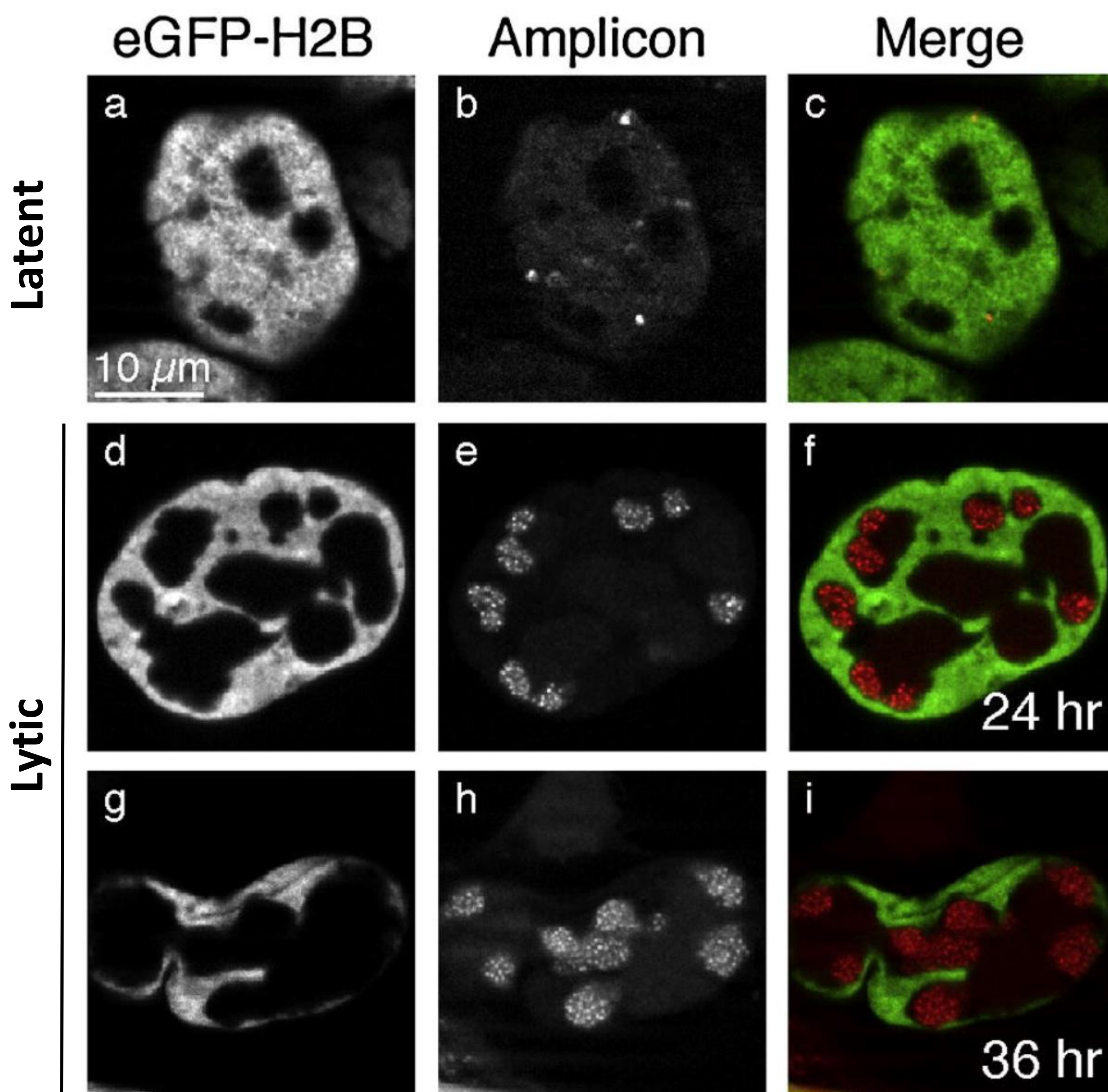
eGFP-H2B
Amplicon
Merge
Latent
Lytic
a
b
c
10 μm
d
e
f
24 hr
g
h
i
36 hr

Figure I.1. Spatial distributions of cellular chromatin (eGFP-H2B) **and EBV DNA** (EBV amplicon) **in EBV-positive iD98HR1 cells during the EBV life cycle. (a-c)** Typical distribution of cellular chromatin during latent EBV infection. **(d-f)** Representative cells in which the lytic cycle was induced with tamoxifen and fixed with paraformaldehyde 24 hrs post-induction. The cellular chromatin appears visibly compacted and begins to move to the nuclear periphery as EBV DNA amplification factories appear in the chromatin voids. Note: The voids in the nucleus that are not occupied by chromatin or the EBV amplicon harbor the colorless, endogenous EBV DNA (P3HR1 strain) which is present in iD98HR1 cells. **(g-i)** Similar images to d-f except at 36 hrs. post-induction. Figure adapted from Chiu et al. 2013.

Table I.1: Requirement of EBV genes/processes for ROCC

Gene/process	Description	Required for ROCC	Not required for ROCC	Unknown if required for ROCC
EBV lytic DNA synthesis	N/A	✓		
BALF5	DNA polymerase	✓		
BALF2	ss-DNA binding protein	✓		
BBLF2/3	Primase-associated factor	✓		
BBLF4	Helicase	✓		
BSLF1	Primase	✓		
BMLF1	RNA processing and export	✓		
BMRF1	Polymerase processivity factor	✓		
OriLyt	Origin of lytic replication	✓		
Complete set of EBV early genes	N/A		✓	
EBV late genes	N/A		✓	
EBV lytic DNA amplification	N/A	✓		
Any viral DNA amplification	N/A		✓	
Cellular genes	Ex. Cohesins/condensins, repressive histone marks, chromatin remodelers, kinesins, etc.			✓

Chapter II:
Materials and Methods

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Recombinant DNA Plasmids

p184Mertz is an SV40 plasmid containing the SV40 origin of replication, the SV40 early promoter driving large TAg, and an ampicillin resistance gene that was used as a vector to mediate SV40 replication (Figure SII.1a). p4241 is a plasmid that contains the firefly luciferase gene and an SV40 polyA sequence (Figure SII.1b). In order to use firefly luciferase expression as a surrogate signal for SV40 DNA amplification, the firefly luciferase gene, and the SV40 polyA sequence were amplified via PCR from p4241 using sequence-specific primers (IDT DNA). The final SV40 construct (SVLuc) was created by ligating the p4241 PCR product insert cut with *XmaI* (5') and *StuI* (3') into the p184Mertz vector at the *NgoMIV* (5') and *EcoRV* (3') restriction sites (Figure SII.1c). The final SVLuc plasmid was verified by restriction digest and sequencing. p3802 is an SV40 large T antigen plasmid that expresses GFP.

Cell Culture

Human embryonic kidney (HEK) 293 cells, NIH3T3 mouse cells, and iD98HR1ZER H2B-GFP cells containing the P3HR1 EBV strain and histone H2B fused to GFP (12) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum (FBS). HEK 293/2089 WT EBV cells and 293 cells carrying a full *oriLyt* deletion from Eric Johannsen (20) (293/2089 WT EBV Δo*riLyt*) were cultured in DMEM supplemented with 10% FBS with 200 μg/ml hygromycin B [MP Biomedicals] and iD98HR1ZER H2B-GFP cells with 1 μg/ml puromycin [MP Biomedicals] were maintained similarly. Cells were grown and kept in a 37°C humidified chamber with 5% CO_2.

Transfections

Adherent cells were plated in 6-well plates or on MaTek plates (MaTek Corporation) and grown to ~70% confluency. The cells were transfected with an appropriate mixture of Opti-MEM (Invitrogen), Lipofectamine 2000 (Invitrogen), and DNA. The cells were washed once with DMEM and incubated with the transfection mixture in serum-free media for four hours at 37^oC. Induction of the EBV lytic cycle for HEK293 cells was mediated by BZLF1 expression from a reporter plasmid introduced during transfection while the EBV lytic cycle in D98HR1ZER cells was induced with 200 nM 4-OHT. After the incubation, DMEM was removed and fresh D10F media was added to the cells.

Primers

Primer sequences for SVLuc cloning and quantitative PCR are shown in Table II.1.

Luciferase Assays

Cells were plated in 6-well plates and grown to ~70% confluency. 48 hours after cell plating, the cells were transfected with appropriate DNA-lipofectamine 2000 mixtures. For initial assays which focused on optimization and verification of SVLuc replication, the cells were assayed for OneGlo (Promega) firefly luciferase signal and CellTiterGlo (Promega) with cells counted at 24, 48, and 72 hours post-transfection. For experimental assays, firefly luciferase signals and cell numbers were assayed at 0 (immediately after 4-hour transfection incubation), 24, 48, and 72 hours post-transfection.

Click chemistry and immunofluorescence

Cells grown on 22x22mm coverslips were washed twice with 1X DPBS and fixed with 4% PFA solution in 1X DPBS (10 minutes incubation at room temperature). The cells were then washed three times with 1X DPBS and permeabilized with 0.2% Triton-X solution in 1X DPBS (10 minutes incubation at room temperature) and washed three times with 1X DPBS. For EdU detection, coverslips were incubated in freshly prepared click chemistry solution (4mM CuSO4, 50mM ascorbic acid, 5µM Cy5-Azide (Sigma-Aldrich, 777323) in 1X DPBS). Click reactions were incubated for 30 minutes, in a dark humidified chamber, at room temperature. Coverslips were then washed three times with 1X DPBS. For immunofluorescence, samples were blocked with a 2% BSA solution, incubated for 30 minutes with gentle rocking. Primary antibodies were prepared in 2% BSA solution, and the coverslips were incubated with this solution for 1 hour at room temperature, following which they were washed three times with 1X DPBS. Secondary antibodies were prepared in 2% BSA solution, and coverslips were incubated with this solution for 1 hour at room temperature, following which they were washed three times with 1X DPBS. All antibody incubations were done in a dark, humidified chamber. Coverslips were then carefully dried and mounted with VectaShield (Vector Laboratories) + 1.5ug/mL DAPI (Invitrogen). Immunofluorescence images were acquired using a Nikon A1RS HD confocal microscope. The sources and concentrations of antibodies used were as follows: rabbit anti-firefly luciferase (Abcam, EPR17790, 1:250) mouse anti-BMRF1 (EMD Millipore, MAB8186, 1:1000), and rabbit anti-SV40 Large T antigen (GeneTex, GTX134378, 1:250). Secondary antibodies: Alexa Fluor 546 goat anti-mouse (Molecular Probes, A11018, 1:1000), Alexa Fluor 568 goat anti-mouse (Molecular Probes, A11019, 1:1000), and Alexa Fluor 647 donkey anti-rabbit (Invitrogen, A31573, 1:500).

SV40 co-transfection assay

iD98/HR1 cells were plated on coverslips in 6-well plates and treated with 100 µg/ml of PAA (21), 24 hours prior to being mock-transfected or transfected with isolated SV40 DNA. The transfection mix was incubated with the cells for 4 hours in the 37°C cell incubator (humidified, with 5% CO2), following which cells were washed with 2 ml of DMEM with 10% FBS (D10F) before another 2 ml of D10F was added as growth media. The cells were grown for 1 hour, and then 100 µg/ml PAA was added back to the media. The cells were grown for another two hours, and then were induced to enter EBV's lytic phase by the addition of 200 nM 4-OHT. 48 hours after transfection, the cells were incubated with 10 µM EdU for 1 hour, and then fixed for click chemistry and immunofluorescence assays.

Chapter III:
EBV and ROCC

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Introduction:

The EBV origin of lytic replication, *oriLyt*, is required for the initiation of EBV DNA synthesis in its lytic phase (22). EBV's *oriLyt* promotes >100 fold amplification of viral DNA during the lytic cycle with the support of EBV's core lytic replication genes (22). As described previously, the *oriLyt*-mediated amplification of EBV DNA occurs in the absence of histones, concurrent with the margination of histone-occupied cellular chromatin to the periphery of the nucleus. Although it is unclear how the cellular chromatin condenses prior to margination, the observation that all EBV early genes required for DNA synthesis are necessary to produce ROCC, makes it clear that EBV-mediated DNA amplification plays an essential role in mediating the margination of chromatin during ROCC.

Our work with the DNA synthesis inhibitors GCV and PAA revealed that detectable EBV DNA synthesis was not necessary to drive the global chromatin compaction that characterizes ROCC Type I (17). This conclusion was based on the absence of EdU (staining of newly synthesized viral DNA) in EBV lytic, ROCC+ cells upon treatment with either GCV or PAA (Figure SIII.1A & B). These results indicated that, following the formation of ROCC Type I, extensive amplification of EBV genomes leads to the margination phenotype of ROCC Type II (Figure SIII.1A & B). We have tested our model of EBV's lytic DNA amplification mediating the margination of chromatin, thus driving ROCC type I into ROCC type II, by asking if a different kind of viral DNA amplification, which lacks EBV's oriLyt, could substitute for EBV's oriLyt-mediated DNA amplification in promoting ROCC type II. We chose SV40, a polyomavirus, that was not previously known to reorganize cellular chromatin. To test our hypothesis, we substituted DNA amplification from the SV40 origin of replication during the EBV lytic cycle to replace *oriLyt*-mediated EBV DNA amplification.

Using a plasmid to mediate SV40 DNA amplification, we first showed that SV40 DNA is replicated in the HEK293 human cells used in subsequent experiments. After verifying its ability to replicate, we introduced the SV40 DNA into cells induced for the EBV lytic cycle and discovered that SV40 DNA amplification is not sufficient to mediate ROCC on its own, but in cells that normally support ROCC, SV40 DNA amplification does not inhibit ROCC formation. These observations were consistent with our hypothesis that SV40 DNA amplification cannot induce ROCC in the context of an EBV lytic environment because of its requirement for histones on newly synthesized viral DNA. We then asked if SV40 and EBV can co-replicate in the same cell which would be required if SV40 DNA amplification could support ROCC. Interestingly, herpes simplex virus (HSV) can induce extensive amplification of plasmids containing SV40 DNA which made it likely that SV40 DNA can be replicated in EBV-infected cells (23, 24). However, in cells that exhibit EBV DNA amplification that causes ROCC, it was unclear if SV40 DNA amplification could occur. Due to the evidence for co-replication of herpesviruses and SV40 at a population level, we hypothesized that SV40 DNA would be amplified detectably in EBV lytic cells that support EBV ROCC. We were able to demonstrate that SV40 DNA amplification does occur at a population level in cells that can support EBV ROCC; however, SV40 DNA amplification was only detected in cells not supporting EBV's lytic cycle when examined individually. Therefore, SV40 DNA amplification was incompatible with the EBV lytic cycle.

This observed incompatibility led us to ask if inhibiting EBV DNA synthesis completely with PAA would allow SV40 DNA synthesis to occur so we could observe any changes in the distribution of ROCC phenotypes in these cells. Our previous work indicated that inhibiting EBV's DNA amplification would allow us to detect SV40 DNA synthesis with EdU, but EBV's

distribution of ROCC phenotypes would remain unchanged +/- SV40 DNA synthesis. We tested this hypothesis and found that, while SV40 DNA synthesis did occur in these cells, the distribution of cells displaying no ROCC or Type I or Type II was statistically similar in PAA-treated cells +/- SV40 DNA synthesis.

Results:

SVLuc can be replicated in 293/2089 EBV Δ*oriLyt* cells

In order to test if EBV-mediated DNA amplification was specifically required for ROCC Type II (chromatin condensation & margination), we needed to design and test a biologically comparable model of DNA amplification with a different virus. As a result, we designed a plasmid, referred to hereafter as SVLuc, which contained the SV40 origin of replication, SV40 large TAg, and the firefly luciferase gene as a proxy for visualization of SV40 DNA replication (Figure III.1). Since SV40's natural infection host is monkeys, specifically rhesus macaque monkeys, it was necessary to determine the ability of SVLuc to replicate in human HEK293 cells which were the cellular system of interest.

All cells used for these luciferase assays were grown for 48 hours followed by transfection of DNA for four hours. After transfection, ~10^4 cells were assayed for luciferase production, which was normalized to cell number at 0, 24, 48, and 72 hours post-transfection. First, we verified by a luciferase plate assay that SVLuc is incapable of extensive replication in non-permissive NIH3T3 mouse cells in agreement with previous work (25); moreover, we found that SVLuc can be replicated in human 293 cells at ~10-fold higher levels than in NIH3T3 cells (unpublished data). The SVLuc plasmid contains SV40 large TAg *in cis* as described previously, but in order to achieve higher levels of SV40 replication from the SVLuc plasmid, we sought to provide additional SV40 large TAg *in trans*. To do this, we first used a HEK293 cell derivative,

293FT, which produces large amounts of SV40 large TAg to increase production of luciferase and found that the increase in luciferase production, while measurable, was not exponential as expected (unpublished data). Next, we chose a small plasmid (p3802) that contains SV40 large TAg fused to green fluorescent protein (GFP), and introduced this plasmid, along with SVLuc, into 293 cells by transfection. We found that this SV40 large TAg-expressing plasmid facilitated an exponential increase in luciferase production 72 hours post-transfection of ~160-fold relative to control samples without the firefly luciferase gene (unpublished data). Finally, we needed to verify that SVLuc could replicate in another 293 cell derivative, 293/2089 EBV Δ*oriLyt* cells. These cells contain the B95.8 strain of EBV, but have a full deletion in *oriLyt* which is the origin of lytic replication for EBV, so that these cells are incapable of supporting the EBV DNA amplification required for ROCC. We demonstrated that SVLuc was capable of extensive SV40 replication measured by luciferase intensity in 293 Δ*oriLyt* cells at 72 hours post-transfection (Figure III.2). Two unexpected observations arose from this finding: 1) SVLuc luciferase intensity was decreased in induced 293 Δ*oriLyt* cells relative to uninduced cells and 2) the addition of *oriLyt* to induced 293 Δ*oriLyt* cells led to an exponential increase in luciferase intensity compared to induced cells that lacked *oriLyt*. Combined together, these findings led us to conclude that it is likely that SV40 DNA replication from the SVLuc plasmid can occur at a population level in lytically-induced EBV+ cells that have *oriLyt*.

SV40 large TAg does not induce or inhibit ROCC in cells induced for the EBV lytic cycle

The addition of a plasmid encoding large amounts of SV40 large TAg in excess of that produced by the SVLuc plasmid prompted us to ask whether SV40 large TAg alone was capable of either inducing ROCC without EBV's *oriLyt* or inhibiting ROCC in cells with *oriLyt* that can support ROCC. Since SV40 large TAg functions to promote replication of the SV40 genome, a

histone-associated genome, we hypothesized that SV40 large TAg would not be able to induce ROCC nor inhibit it in lytically-induced EBV+ cells.

First, we cultured iD98HR1-ZER cells with an inducible Z-ER (EBV immediate early protein BZLF1 fused to the estrogen receptor) that contain the P3HR1 strain of EBV, transfected them with the plasmid encoding SV40 large TAg only fused to an IRES-GFP sequence, and at 48 hours post-transfection, we scored and counted cells for ROCC and took representative images of each condition (Figure III.3). Representative images for classification of non-ROCC and ROCC Type II (complete ROCC) cells are described (Figure SIII.2) (17). Induced cells without SV40 TAg exhibited similar ROCC percentages to those of cells induced with SV40 TAg (Figure III.3) indicating that SV40 TAg does not induce or inhibit EBV's ROCC.

We also cultured 293/2089 EBV Δ*oriLyt* cells, transfected them, and at 48 hours post-transfection, we observed cells for ROCC and took representative images of each condition. We observed 0 cells that were double positive for TAg and ROCC in the absence of EBV's *oriLyt* (Figure III.4) . However, when we transcomplemented EBV's *oriLyt* in an expression vector so it was present *in trans* along with SV40 TAg, we found that the ROCC phenotype was rescued and appeared similar to that of induced cells with *oriLyt* that lack TAg. Taken together, these results confirmed that SV40 TAg on its own is not capable of inducing or inhibiting ROCC and showed that SV40 TAg can be highly expressed in EBV lytic cells that are ROCC+.

The amplification of SV40 DNA as indicated by its luciferase expression is not detected in lytically-induced EBV+/ROCC+ cells

Since SV40 TAg alone does not affect ROCC, we sought to answer our initial question: can SV40 DNA amplification support ROCC in EBV lytic cells? Since SV40 DNA amplification

was labeled indirectly by GFP staining for SV40 TAg in previous experiments, it was unclear whether SV40 DNA amplification was occurring in ROCC+ cells as expected in Fig. III.3 & III.4. The presence of SV40 TAg from p3802 suggested that SV40 DNA amplification was likely to occur; however, staining for firefly luciferase was a direct proxy for the amplification of SV40. Therefore, we used luciferase expression from the SVLuc plasmid as an immunofluorescence marker to identify SV40 DNA amplification in lytically-induced EBV+ cells that were ROCC+ at a single-cell level. Dose-dependent expression of the firefly luciferase antibody was tested for detection by immunofluorescence (Figure SIII.3).

To do this experiment, we cultured 293/2089 EBV Δ*oriLyt* cells, transfected them, and at 48 hours post-transfection, performed immunofluorescence to detect SV40 TAg, firefly luciferase, and the early EBV lytic protein BMRF1 as a marker for cells that had entered the lytic cycle. These experiments resulted in two important observations: 1) Almost all detectable SV40 DNA amplification occurred in EBV-infected cells not supporting the lytic phase (negative for BMRF1), and 2) SV40 DNA amplification was excluded from ROCC+ cells (Figure III.5). As a result, these experiments led us to conclude that detectable SV40 DNA amplification does not occur in lytically-induced EBV+ cells, including ROCC+ cells, and thus cannot support ROCC in the absence of *oriLyt*-mediated EBV DNA amplification.

EBV's ROCC is incompatible with the DNA replication of SV40.

iD98/HR1 cells were plated and treated with 100 mg/mL PAA 24 hours prior to mock or SV40 transfection, and then the cells were induced to enter EBV's lytic phase (Fig. III.6A). At 48 hours post-transfection, the cells were incubated for 1 hour with EdU. The cells were scored for their expression of EBV's BMRF1, indicating entry into EBV's lytic phase, and SV40's large T antigen, indicating expression from SV40's viral DNA. The proportions of cells that express

BMRF1 alone (BMRF1+ only), T antigen alone (TAg+ only), or both (BMRF1+/TAg+) were measured (Fig. III.6B). Control experiments showed that, in the presence of PAA, all cells that entered the lytic phase, as noted by their expression of BMRF1, failed to synthesize EBV DNA and were EdU negative (Fig. SIII.1). Moreover, control samples that were pretreated with PAA, mock transfected, and induced to enter EBV's lytic phase did not incorporate EdU (0% EdU+ cells, n > 100 cells counted). We therefore used EdU incorporation to identify cells supporting SV40 replication, in those cells that were transfected with SV40 viral DNA. From this experiment, we found that we could not answer our initial question: whether SV40's DNA amplification could substitute for EBV's oriLyt-mediated DNA amplification in driving ROCC type I into ROCC type II, as it appears that EBV's ROCC and SV40's DNA amplification are incompatible (Fig. III.6C). Of all the cells that had entered EBV's lytic phase and were transfected with SV40 DNA (BMRF1+/TAg+), only those that had no ROCC were found to support SV40 replication (EdU+). In addition, the proportion of total EdU+ cells was reduced in the BMRF1+/TAg+ population compared to cells transfected with SV40 DNA alone (TAg+ only) (Fig. III.6D). In contrast, the distribution of ROCC+ cells in these BMRF1+/TAg+ double-positive cells was not significantly different from that of BMRF1+ cells (Fig. III.6E). Thus, EBV's ROCC and SV40's DNA amplification are incompatible, with the co-transfections favoring EBV's ROCC. In both the BMRF1+-only and BMRF1+/TAg+ populations of this experiment, there is a higher proportion of cells supporting ROCC type I than those supporting ROCC type II (Fig. III.6E), an apparent discrepancy with the population distribution of ROCC types in previous experiments (17). This difference can be explained by one condition of the experiments involving transfections of SV40 viral DNA. When EBV-positive cells are induced into the lytic phase, they enter the lytic phase at the beginning of their next S phase (12).

Following transfection, cells tend to have a delayed growth/cycle, likely resulting in a later entry into the lytic phase, thus affecting the proportion of ROCC+ cells at 48 hpi. Taking this delay into account, the proportions of cells supporting ROCC type I and type II in the experiment involving SV40 transfections are consistent with those previously seen in iD98/HR1 cells induced to enter the lytic phase in the presence of PAA (17). Our findings from the co-transfection of SV40 viral DNA into EBV lytic cells led us to an additional mechanistic insight: EBV inhibits cellular DNA synthesis during its lytic phase, and we now found that its ROCC is incompatible with SV40 DNA synthesis. Both cellular and SV40 DNA synthesis require cellular DNA polymerases and also are histone associated. Polyomaviruses, such as SV40, as well as papillomaviruses, encapsidate histone-bound genomes and do not induce ROCC (26, 27). In contrast, all five families of DNA viruses that induce ROCC lack cellular histones in their virions (28-32). In EBV, cellular DNA synthesis does not occur once viral DNA synthesis begins. Thus, it is likely that the mechanism that underlies the failure of cellular DNA synthesis extends to that of SV40 through a shared mechanism involving the inhibition of histone-associated DNA synthesis.

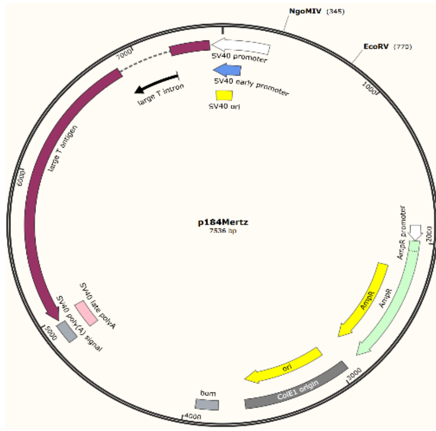
Discussion:

Five families of viruses induce the reorganization of cellular chromatin that we have characterized in EBV. The conservation of this ROCC phenotype suggests that it plays a major role in facilitating the productive infection of these viruses, and illumination of EBV's induction of ROCC sheds light on how other viruses coordinate this process. Here, we tested one condition of a model for EBV's ROCC that we had previously established: initial compaction of cellular DNA is followed by extensive margination of cellular DNA to the nuclear periphery after significant EBV DNA synthesis. We used SV40, a virus known not to induce ROCC, to test

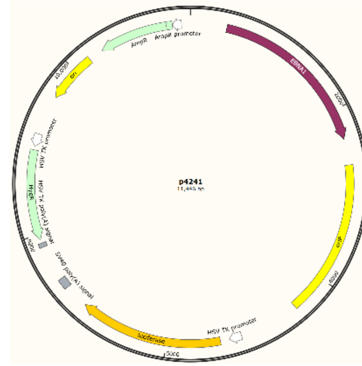
whether any viral DNA amplification could be used to elicit ROCC. First, we determined that the SV40 protein, TAg, was incapable of inducing ROCC on its own. Next, we observed that when cells entered EBV's lytic phase and displayed either Type I ROCC (compaction) or Type II ROCC (compaction and margination), SV40 DNA synthesis was not detected and thus was determined to be incompatible with EBV's ROCC. SV40 DNA synthesis requires histones while EBV DNA synthesis does not; therefore, we conclude that EBV inhibits SV40 DNA synthesis in the same way that it inhibits cellular DNA synthesis.

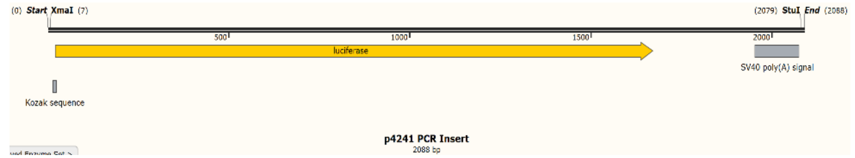
It is clear, however, that this model does not explain all cases of ROCC in the context of the EBV lytic cycle, and furthermore, we have not directly proven that Type I ROCC is a precursor to Type II ROCC. We have observed a small fraction of ROCC Type II+ positive cells that can display this phenotype in the absence of substantial EBV DNA amplification. In addition to our hypothesis that the initiation of viral DNA synthesis triggers global chromatin condensation, this exception further suggests a role for cellular factors in EBV's ROCC including potentially cellular motor proteins, histone deacetylases, and other proteins implicated in chromatin compaction such as condensins. In fact, EBV may even "trick" cellular proteins to assist in the margination of chromatin during ROCC when EBV DNA synthesis does not occur. In order to further understand this model, we hope to identify cellular factors that help promote ROCC in EBV and the other virus families that induce this phenotype.

A



B





C

Figure III.1. Construction of SVLuc plasmid. Parental plasmids and final SV40 plasmid construct. **(A)** The annotated p184Mertz SV40 vector with relevant restriction enzyme cut sites labeled (*NgoMIV*/*EcoRV*) obtained from Dr. Janet Mertz. **(B)** The annotated p4241 firefly luciferase plasmid is shown along with the PCR insert that was amplified from the parent plasmid. **(C)** The SVLuc plasmid made by ligating the *XmaI*/*StuI* insert into the *NgoMIV*/*EcoRV* vector is shown.

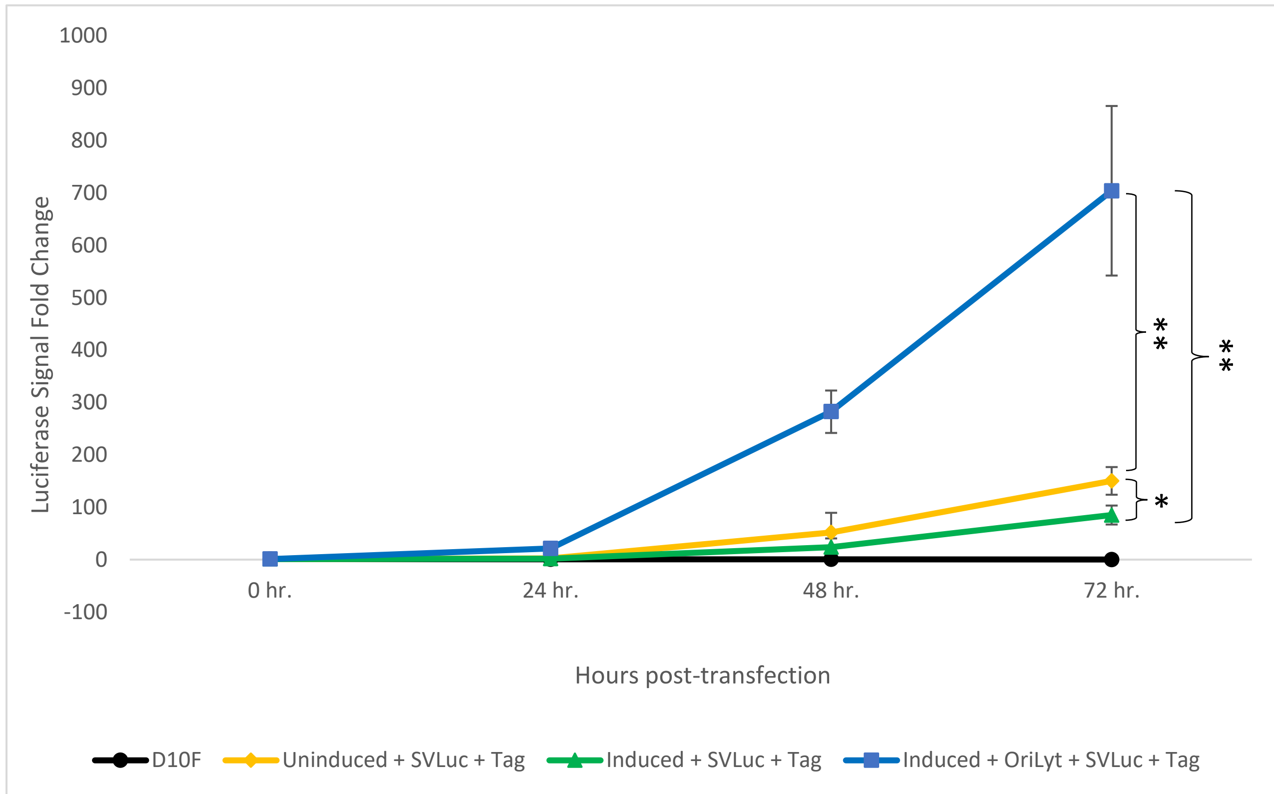


Figure III.2. SVLuc replication in lytically-induced EBV+ cells. 293/2089 EBV Δ*oriLyt* uninduced/induced and transfected with *oriLyt*, SVLuc, and/or SV40 TAg were assayed at 0, 24, 48, and 72 hrs. post-transfection for firefly luciferase expression. Luciferase signal readout was normalized to the average signal per cell assayed (~10^4 cells). D10F is a media-only control. *: p-val < 0.05; **: p-val < 0.01, Student's two-tailed T-test. (n = 3).

A

Whole Field | **Single Cell**

mCherry-H2B GFP Merge mCherry-H2B GFP Merge

Uninduced iD98HR1 + TAg

Induced iD98HR1 + TAg

B

12
10
8
6
4
2
0

% ROCC

N.S.

Induced	-	+	+
SV40 TAg	+	-	+

Figure III.3. SV40 TAg alone does induce or inhibit ROCC in iD98HR1-ZER cells. iD98HR1-ZER cells uninduced or induced with 200 nM 4-OHT and transfected with SV40 large TAg. **A)** Live-cell imaging was performed 48 hours post-transfection with cellular chromatin labeled by mCherry-H2B and SV40 TAg labeled by GFP. Whole-field images (left) and single-cell magnifications (right). Induced cells without SV40 TAg were also imaged (not shown). Scale bar = 10 µm. **B)** Quantification of % ROCC+ cells in the conditions in A) plus induced cells without SV40 TAg. Error bars represent standard deviation of two biological replicates. N > 300 viable cells for each condition. N.S. = Not Significant, Student's two-tailed T-test. (n = 2).

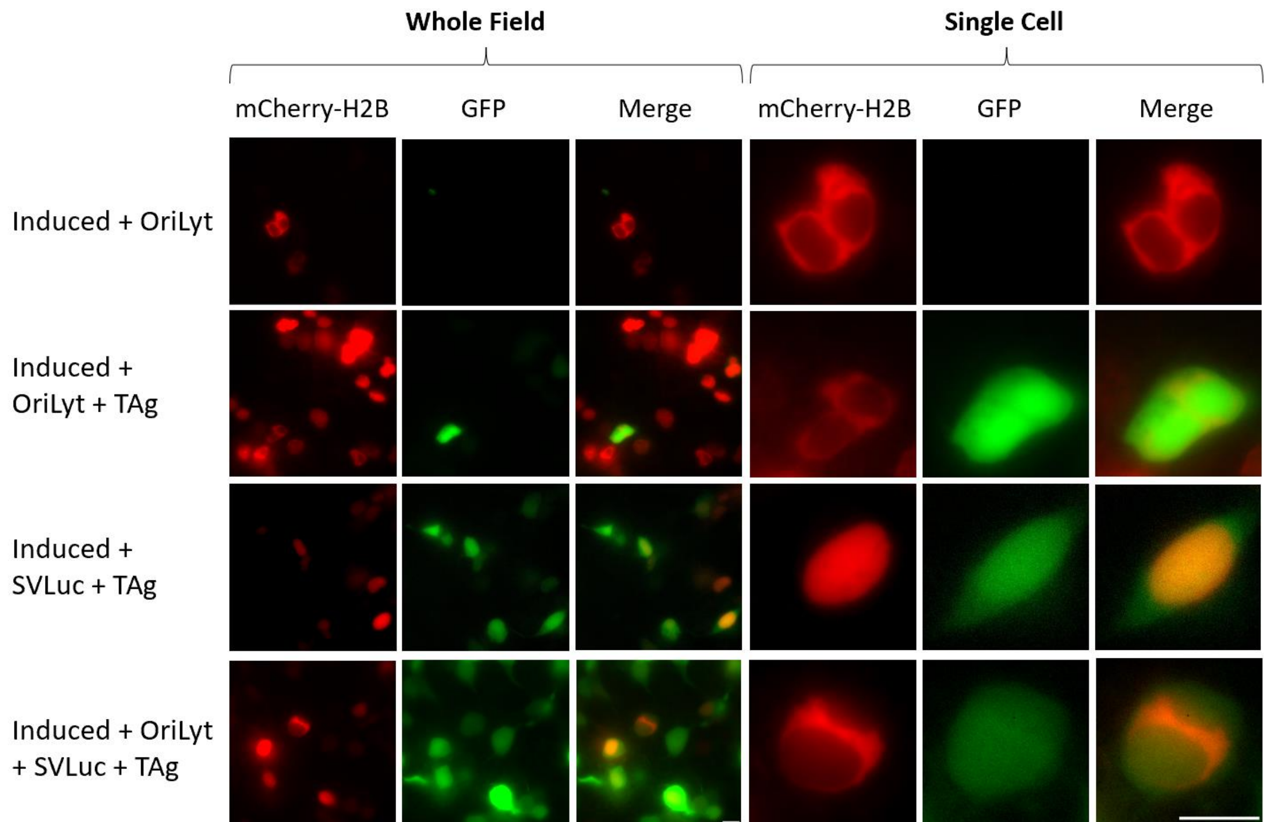


Figure III.4. ROCC+ cells support high levels of SV40 TAg expression. 293/2089 EBV Δ*oriLyt* induced by a plasmid expressing the EBV immediate early transactivator protein BZLF1 and transfected with EBV *oriLyt*, SVLuc, and/or SV40 TAg. Live-cell imaging was performed 48 hours post-transfection with cellular chromatin labeled by mCherry-H2B and SV40 TAg labeled by GFP. Whole-field images (left) and single-cell magnifications (right) are shown. SV40 TAg is expressed in all conditions where SVLuc (containing SV40 TAg) or the SV40 TAg expression plasmid is transfected. Scale bar = 10 µm. (n = 3).

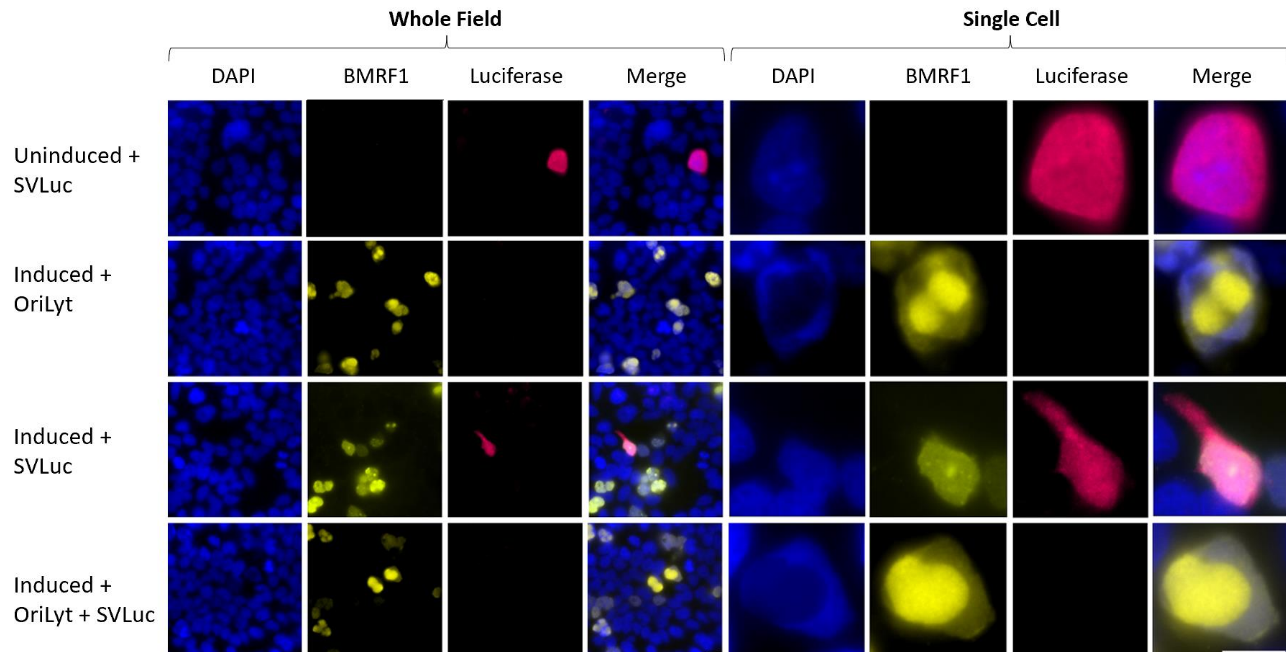
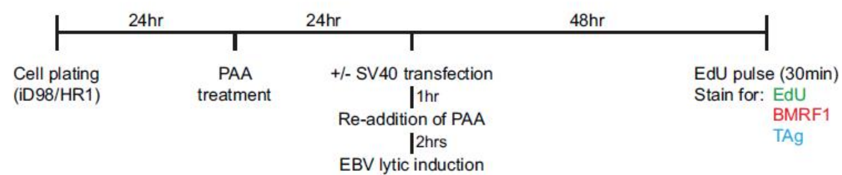
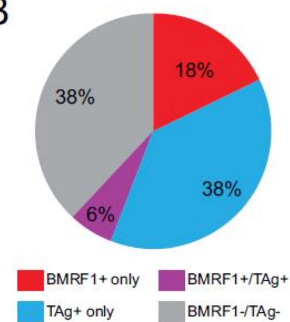
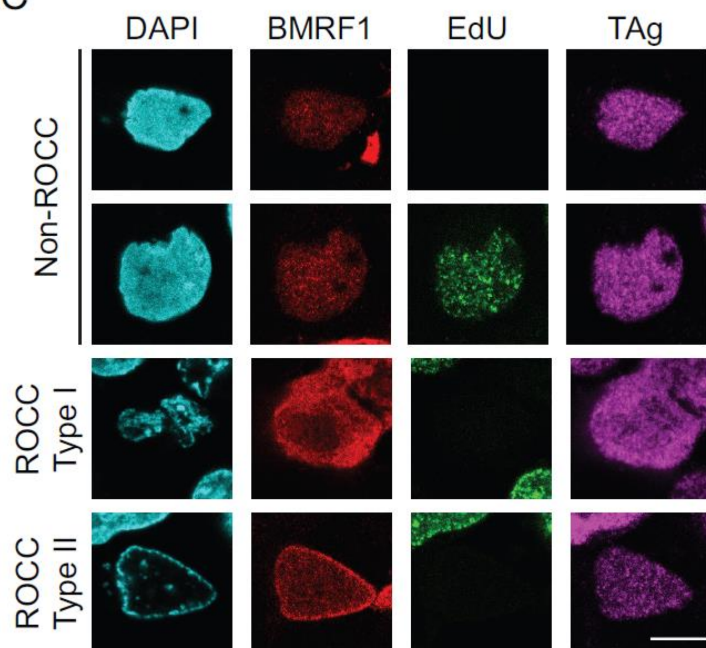
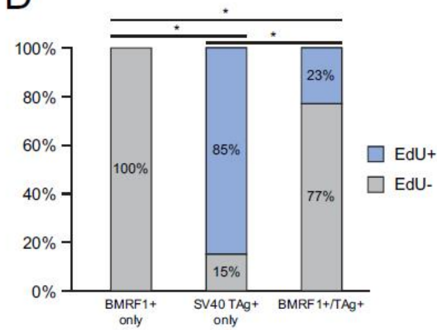


Figure III.5. SV40 DNA amplification is not detectable in ROCC+ cells. 293/2089 EBV Δ*oriLyt* uninduced or induced by a plasmid expressing the EBV immediate early transactivator protein BZLF1 and transfected with EBV *oriLyt*, SVLuc, and/or SV40 TAg. Live-cell imaging was performed 48 hours post-transfection with the cellular chromatin labeled by DAPI (blue), BMRF1 – lytic marker (yellow), firefly luciferase – proxy for SV40 DNA amplification (magenta). Whole-field images (left) and single-cell magnifications (right) are shown. SV40 DNA amplification is absent from cells in the EBV lytic phase (including ROCC+). Scale bar = 10 µm. (n = 3).


A
24hr
24hr
48hr
Cell plating
(iD98/HR1)
PAA
treatment
+/- SV40 transfection
1hr
Re-addition of PAA
2hrs
EBV lytic induction
EdU pulse (30min)
Stain for: EdU
BMRF1
TAg


B
18%
38%
38%
6%
BMRF1+ only
BMRF1+/TAg+
TAg+ only
BMRF1-/TAg-


C
DAPI
BMRF1
EdU
TAg
Non-ROCC
ROCC Type I
ROCC Type II


D
100%
80%
60%
40%
20%
0%
100%
85%
15%
23%
77%
EdU+
EdU-
BMRF1+ only
SV40 TAg+ only
BMRF1+/TAg+

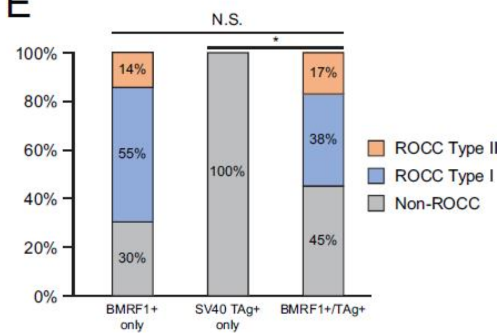
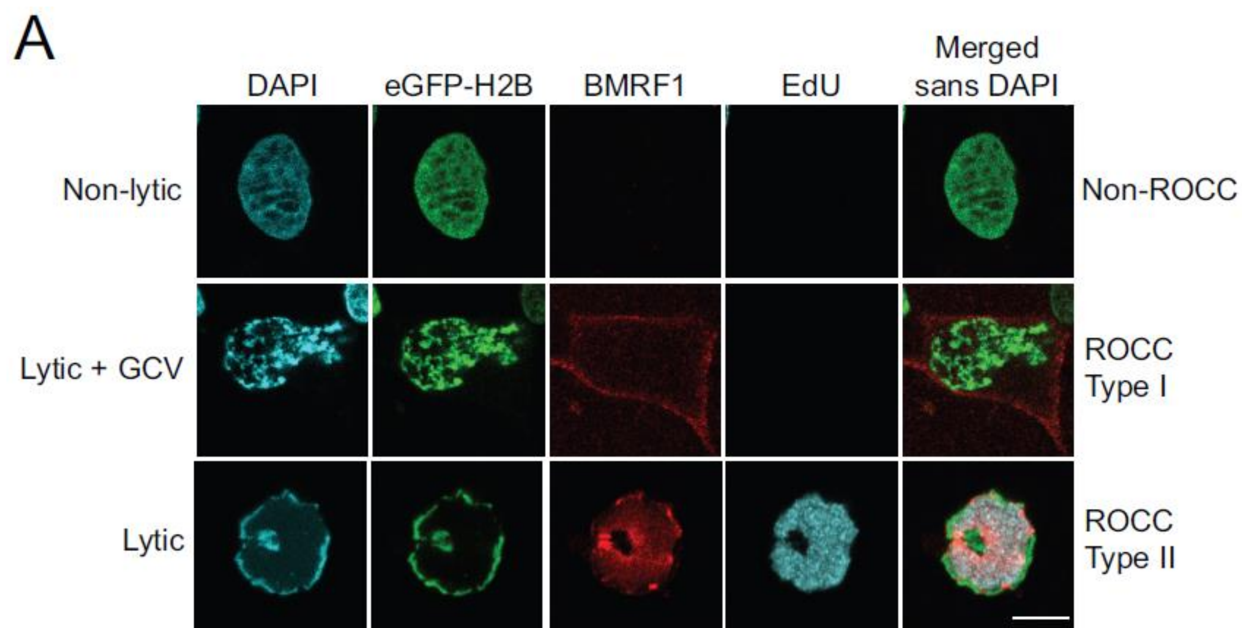

E
N.S.
14%
55%
30%
100%
17%
38%
45%
ROCC Type II
ROCC Type I
Non-ROCC
BMRF1+ only
SV40 TAg+ only
BMRF1+/TAg+

Figure III.6. EBV’s ROCC is incompatible with histone-bound DNA replication in lytic cells. A) Experimental workflow for the EBV and SV40 co-transfection ROCC assay. iD98/HR1 cells were pretreated with 100 mg/mL PAA 24 h prior to transfection. The cells were then mock transfected or transfected with SV40 viral DNA, retreated with PAA, and induced for EBV’s lytic phase by treatment with 200 nM 4-OHT. Forty-eight hours later, cells were pulsed with EdU for 1 h, after which samples were stained for EdU, EBV’s BMRF1, and SV40’s large T antigen (TAg). **B)** Proportion of BMRF1+, TAg+, and BMRF1+/TAg+ cells in the sample population. n = 306 cells. **C)** Representative images of samples that were PAA-treated, induced for EBV’s lytic phase, and transfected with SV40 viral DNA are shown. All images have the same scale; bar, 10 mm. EdU signals were found only in BMRF1+/TAg+ cells displaying non-ROCC. **D and E)** EdU status **D)** and ROCC types **E)** were classified for BMRF1+, TAg+, or BMRF1+/TAg+ cells in samples that were PAA-treated, induced for EBV’s lytic phase, and transfected with SV40 viral DNA. BMRF1+/TAg+ double-positive cells have a reduced proportion of EdU+ cells compared to TAg+ -only cells but a similar distribution of ROCC types compared to BMRF1+ -only cells. This finding indicates that EBV’s ROCC and SV40’s histone-associated DNA amplification are incompatible, and co-transfections favor EBV’s ROCC. n > 50 cells per group. For EdU classification **D)**, *, P value <0.01, Fisher’s exact test. For ROCC classification **E)**, *, P value <0.01; N.S., P value <0.05, 2 x 3 Fisher’s exact test.


A
DAPI
eGFP-H2B
BMRF1
EdU
Merged sans DAPI
Non-lytic
Lytic + GCV
Lytic
Non-ROCC
ROCC Type I
ROCC Type II

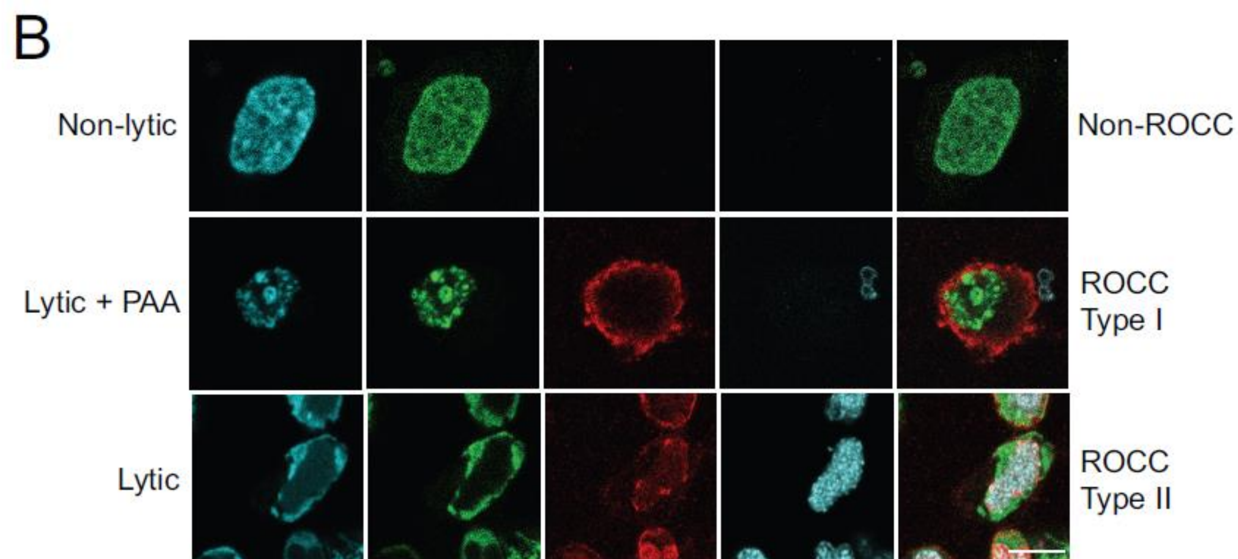

B
Non-lytic
Lytic + PAA
Lytic
Non-ROCC
ROCC Type I
ROCC Type II

Figure SIII.1. Detectable lytic DNA synthesis is dispensable for chromatin condensation but required for its margination. iD98/HR1 cells were treated with GCV or PAA and induced to enter the lytic phase. **A and B)** Representative cells from GCV **A)** and PAA **B)** experiments are shown. Uninduced (nonlytic) cells do not express BMRF1 and do not display ROCC. Induced, lytic cells robustly express BMRF1, have EdU signals localizing to viral replication compartment(s), and support ROCC type II, in which cellular chromatin is both condensed and marginated. Lytic cells treated with GCV or PAA robustly express BMRF1, and >90% lack EdU signals, confirming the inhibition of viral DNA synthesis in them. The majority of these cells display ROCC type I, in which cellular chromatin is condensed but not marginated. All images have the same scale; scale bar = 10 µm.

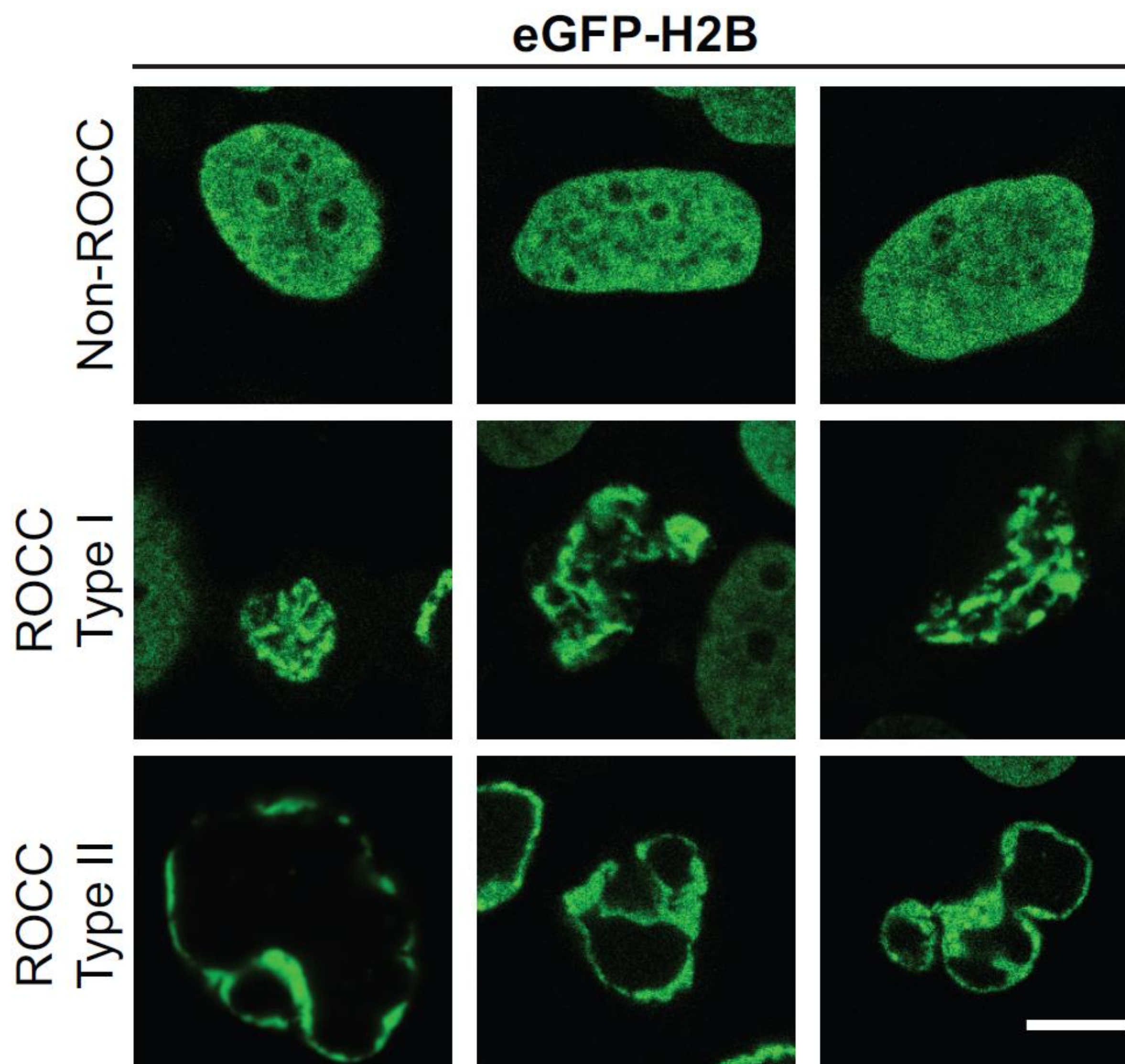


Figure SIII.2. Representative images of cells not displaying ROCC or displaying ROCC Type I or Type II. iD98/HR1 cells stably maintaining an expression vector for eGFP-H2B were imaged using scanning confocal microscopy. Various types of ROCC were captured and representative images are shown. Non-ROCC cells have their cellular chromatin distributed largely evenly across the nucleus. Cells with ROCC Type I have their chromatin condensed but not marginated, whereas those with ROCC Type II have their chromatin condensed and marginated towards the periphery of the nucleus. All images have the same scale; scale bar = 10μm.

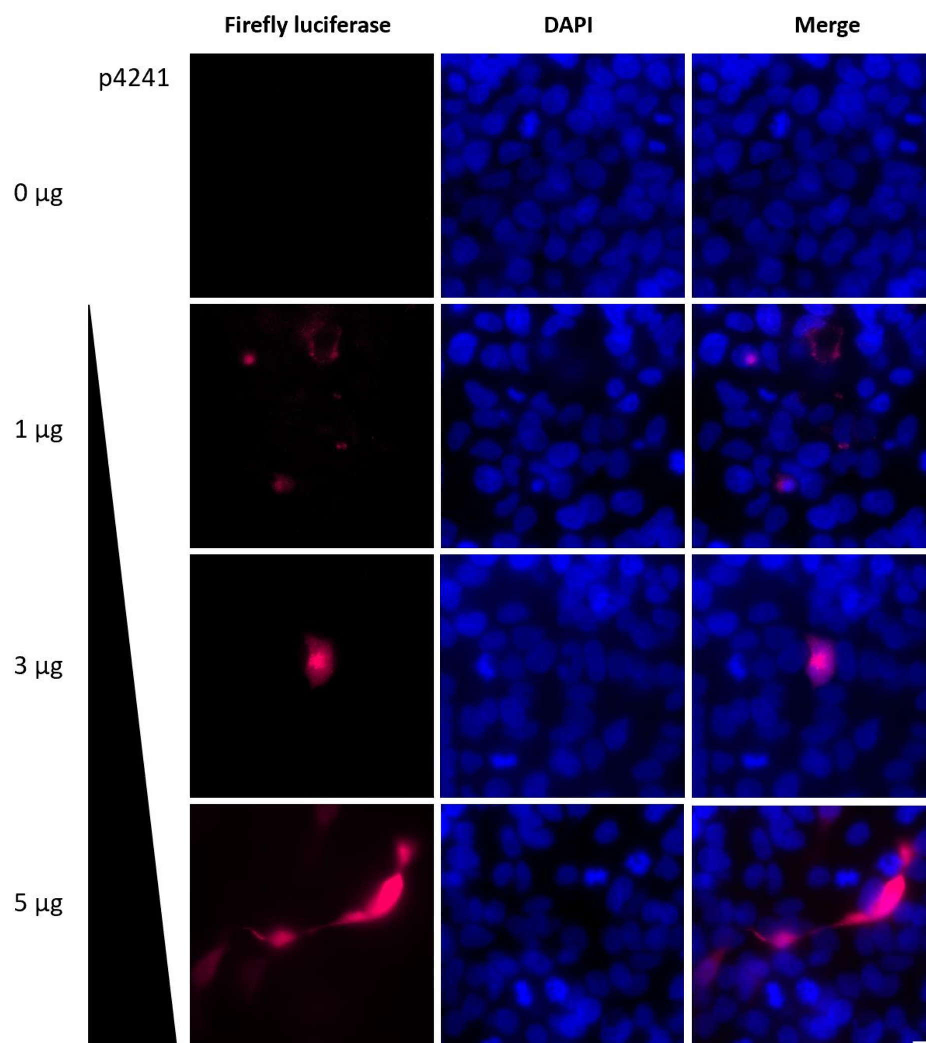


Figure SIII.3. Dose-dependent expression of firefly luciferase detected with a monoclonal luciferase antibody. 293 cells transfected with increasing quantities of p4241 (plasmid that constitutively expresses firefly luciferase under the control of the HSV-Tk promoter). 48 hours post-transfection, the cells were fixed with 4% PFA, permeabilized with 0.2% Triton X-100, stained with primary/secondary antibodies, and treated with 1.5 µg/ml DAPI (nuclei staining). Firefly luciferase staining intensity increased exponentially as the transfected quantity of p4241 increased. Scale bar = 10 µm.

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