Human DNA replication initiation factors, ORC and MCM, associate with *oriP* of Epstein–Barr virus

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The 165-kb chromosome of Epstein–Barr virus (EBV) is replicated by cellular enzymes only once per cell cycle in human cells that are latently infected. Here, we report that the human origin recognition complex, ORC, can be detected in association with an EBV replication origin, *oriP*, in cells by using antibodies against three different subunits of human ORC to precipitate crosslinked chromatin. Mcm2, a subunit of the MCM replication licensing complex, was found to associate with *oriP* during G_1 and to dissociate from it during S phase. The detection of ORC and Mcm2 at *oriP* was shown to require the presence of the 120-bp replicator of *oriP*. Licensing and initiation of replication at *oriP* of EBV thus seem to be mediated by ORC. This is an example of a virus apparently using ORC and associated factors for the propagation of its genome.

Epstein–Barr virus (EBV) is a human herpesvirus that immortalizes human B cells and is involved in several types of cancer of both B cell and non-B cell origin (1, 2). EBV is predisposed to infect cells latently, that is, without producing progeny virus. The 165-kb EBV chromosome is circularized after infection and usually is maintained as an episome that is replicated once per cell cycle by cellular proteins when the infected cells proliferate (3). An understanding of how the EBV chromosome replicates during latent infection would contribute to our understanding of the replication of human chromosomes and could eventually have medical significance.

OriP is a 1.7-kb, bifunctional locus of the EBV chromosome that supports the replication and maintenance of recombinant plasmids in human cells when an EBV-encoded protein, EBNA1, is present (4, 5). A ≈120-bp region called DS, for a dyad symmetry, is the functional replicator of *oriP* (6–8), and it is the approximate site where bidirectional replication initiates (9). Four EBNA1 binding sites within DS are responsible for replicator activity (6, 8). The other functional component of *oriP* is a family of 30-bp repeats (FR), which also binds EBNA1 but functions differently, providing for the mitotic stability of episomes by tethering them via EBNA1 to human chromosomes (10–13). Because EBNA1 lacks the enzymatic activities expected of a DNA helicase, the initial unwinding of DNA for initiation of replication at *oriP* must be performed by cellular proteins (14, 15). Plasmids that depend on *oriP* are limited to one round of replication per cell cycle (16), suggesting that initiation at *oriP* is controlled by replication licensing, the mechanism that is believed to govern the replication of the chromosomes of all eukaryotes (17).

Replication licensing is understood to be the process that leads to the loading of the six-member MCM complex of proteins at origins during the G_1 phase of the cell cycle. The loading of MCM requires the prior loading of two proteins, Cdc6 and Cdt1, which in turn require the origin recognition complex, ORC (18–22). Once MCM has been loaded, ORC and Cdc6 are not needed for replication to initiate normally, at least in *Xenopus* egg extracts (23). MCM is suspected of functioning as a DNA helicase (24, 25), and it seems to move with the replication forks after initiation (20) and to be required continuously for fork progression (26). MCM dissociates from chromatin during S phase (18, 27–29) and is prevented from relicensing by multiple mechanisms until the end of the next mitosis. In light of this, we expected that MCM would be recruited by the replicator of *oriP*, at the minimum, or possibly ORC and all of its associated factors might be involved.

ORC was first discovered in budding yeast as a six-member complex that binds in an ATP-dependent manner to a consensus sequence present at yeast replication origins (30). More recently, *Drosophila* ORC was shown to bind to three control elements within a 440-bp region that drives amplification of the chorion gene (31). ORC is required for DNA replication in *Xenopus* eggs, which at this stage of development occurs without apparent sequence specificity (32). Replication origins of mammalian chromosomes are usually complex and are just beginning to be defined genetically (33). It is widely anticipated that mammalian chromosomal origins will feature sites of ORC binding, but this has not been determined.

Here, we report evidence that MCM and ORC associate with the replicator of *oriP* of EBV. We demonstrate ORC at a replication origin in a vertebrate organism and provide evidence for the involvement of eukaryotic initiation factors in the replication of a viral chromosome.

Materials and Methods

Cell Lines and Plasmids. Raji (American Type Culture Collection) is a Burkitt's lymphoma cell line that carries about 50 copies of the EBV chromosome per cell. The subclone of BL30 (Burkitt's lymphoma) cells carrying the EBV mutant P3-ΔDS-33 at about 30 copies per cell has been described (34). The 293 cells, derived from human kidney epithelium (American Type Culture Collection), carrying p818 at about 50 copies per cell were grown in Iscove's modified Dulbecco's medium containing 250 μg/ml hygromycin B (Calbiochem). A clone of the EBV-negative Burkitt's lymphoma cell line, DG75, that is close to tetraploid and carries p818 at about 50 copies per cell was obtained and maintained in RPMI medium supplemented with 9% FBS, penicillin and streptomycin, and hygromycin B at 300 μg/ml. p818 (Fig. 1*A*) is a 12.6-kb plasmid similar to p201 (5) but contains 4 kb of additional EBV DNA extending rightward from *oriP* to the *Bam*HI site at 13215.

Chromatin Immunoprecipitation (ChIP). Cells (2×10^8) were collected, washed in PBS, and then fixed with 1% paraformaldehyde in PBS for 20 min at 37°C. The fixed cells were washed in PBS, and chromatin was solubilized in urea as described (35). Chromatin was sonicated in a volume of 2 ml to an average DNA length of 600–1,300 bp by using a Heat Systems Ultrasonics W220 at setting 6 with the small probe and 10–15 pulses of 15 s separated by cooling on ice. Crosslinked chromatin with a

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Abbreviations: EBV, Epstein–Barr virus; ORC, origin recognition complex; DS, dyad symmetry region; ChIP, chromatin immunoprecipitation; FR, family of 30-bp repeats.

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buoyant density of ≈1.4 gm/ml was purified by equilibrium centrifugation in CsCl as described (36).

Aliquots of chromatin containing about 30 μg of DNA were adjusted to volumes of 50 μl in RIPA buffer (1% Triton X-100/0.1% sodium deoxycholate/0.1% SDS/150 mM NaCl/5 mM EDTA/50 mM Tris·HCl, pH 8.0/1 mM PMSF) and incubated with 1.5 μg of rabbit IgG; 1.5 μg of anti-EBNA1 mAb, EBNA1.OT1x (37), obtained from Jaap Middledorp (Free University Medical Center, Amsterdam, The Netherlands); 1.5 μg of rabbit antibody against human Mcm2, affinity-purified by using BM28 antigen (38); or 1 μl of rabbit antiserum specific to Orc2 (39), Orc3 (40), or Orc4 (39) and kept at 4°C for 4 h. Antibody complexes were recovered by adding 10 μl of 50% (vol/vol) protein A-Sepharose (Sigma), rocking for 1 h at 4°C, and pelleting. Pellets were washed five times with RIPA buffer adjusted to 750 mM NaCl, twice in LiCl buffer (0.25 M LiCl/0.5% Triton/1% SDS/1% sodium deoxycholate/1 mM EDTA/10 mM Tris·HCl, pH 8.0), twice in TE (10 mM Tris·HCl, pH 8.0/1 mM EDTA), and finally resuspended in 100 μl of TE. Recovered chromatin was de-crosslinked by adding SDS to 0.5% and proteinase K to 1 mg/ml and incubating at 37°C overnight followed by 8 h at 65°C. After extracting with 1:1 phenol/chloroform, DNA was recovered by ethanol precipitation in the presence of 20 μg of glycogen as carrier. PCR was performed by using 1/10th to 1/1,250th of the ChIP DNA in 50 μl by using 2.5 units of *Taq* polymerase (Roche Biochemicals) for 35 cycles of 1 min at 95°C, 1 min at 60°C, and 40 s at 72°C. The PCR products were separated by electrophoresis in 2% agarose gels (NuSieve 3:1, BioWhittaker), which were stained with SYBR Green or SYBR Gold (Molecular Probes) and digitally photographed.

The regions amplified and the sequences (B95–8 strain) of upper and lower strand primers (5′ to 3′) were as follows: DS or ΔDS (for Figs. 1 and 2), 435 bp or 315 bp, EBV 8758–9192, ODJ3 gggggcgtcacctgaaaccttgtttt and ODJ11 gccctcttgagggtccgcttatcggt; DS (for Fig. 3), 254 bp, EBV 8762–9115, OJY216 gcgtcacctgaaaccttgttttcg and OHX-8987 tgggagatatcgctgttccttaggaccct; E1 5′, 334 bp, EBV 107656–107989, OJY129 aatccccatccctaccgtcca and OJY130 ggccatttccaggtcctgtacc; E1 3′, 248 bp, EBV 109726–109973, OJY127 atcagggtgactgtgtgcagcttt and OJY128 caccaacagcacgcatgatg; Qp, 155 bp, EBV 62398–62552, OJY205 cgctttgcgaaaacgaaagtg and OJY206 gggtccccaaacatacaccg; and hph, 182 bp, 275–456 of GenBank K01193, OJY132 tctccgacctgatgcagctc and OJY131 ttccccaatgtcaagcacttcc. To obtain even, simultaneous amplification by PCR, the primer pairs had to be used at different concentrations, which required adjustment for different chromatin preparations or for use with different thermal cyclers. For the experiments shown, the concentrations were as follows. Fig. 1: DS, 400 nM; E1 5′, 46 nM; E1 3′, 32 nM; hph, 36 nM. Fig. 2: Raji, DS, 430 nM; E1 3′, 32 nM; Qp, 260 nM; P3-ΔDS-33:ΔDS, 120 nM; E1 3′, 41 nM; Qp, 600 nM. Fig. 3: DS, 100 nM; E1 5′, 47 nM.

Centrifugal Elutriation. DG75/p818 cells (2×10^8) were collected and resuspended in 10 ml of ice-cold culture medium. A Beckman model JE 5.0 elutriator with a Sanderson chamber was used at 4°C. Conditions were, in sequence: 1,500 rpm for 20 min at 5 ml/min to remove debris followed by 13.32 ml/min at increasing rotor speeds to collect cells, 1,500 rpm for 15 min for G_1, 1,250 rpm for 20 min for early S, 1,100 rpm for 20 min for late S, and finally 900 rpm for 15 min for G_2. Because the yield of G_1 cells was only about 4%, the procedure was done four times, and the cells were pooled. A portion of the cells was stained with propidium iodide for cytometric analysis of DNA content (41) on a Becton Dickinson FACScan analyzer.

Results

Detection of ORC and MCM At or Near the Replicator of *oriP*. ChIP assays were used to investigate whether MCM associates with *oriP*. A 12.6-kb *oriP*-based plasmid (p818, Fig. 1*A*) was introduced into the human epithelial cell line 293, and cells were treated with formaldehyde and sonicated sufficiently to reduce the average size of DNA to ≈700 bp. Crosslinked chromatin was purified and tested by using rabbit antibodies against human Mcm2 or a mouse mAb specific to EBNA1. DNA was recovered from antibody–chromatin complexes and tested by PCR by using four sets of primers simultaneously to detect four different regions of the plasmid (Fig. 1*A*). The four regions were amplified fairly evenly and uniformly when PCR was performed on DNA from chromatin that had not been immunoprecipitated (Fig. 1*B*, lanes 6–8). As expected, the DS region was selectively enriched in chromatin that was precipitated by the EBNA1-specific antibody (Fig. 1*B*, lanes 3 and 4). Antibodies against Mcm2 also selectively precipitated DS (lanes 1 and 2). With rabbit IgG used as a control, DS was not detected (lane 5). In some experiments, all regions of the plasmid were detected at a low level with this control but without significant enrichment of the DS region. Very similar results were obtained with p818 carried by a different cell line, DG75, derived from a Burkitt's lymphoma (data not shown).

The EBNA1-specific antibody also reproducibly enriched the region of the plasmid that is 3′ of the EBNA1 gene (E1 3′ in Fig. 1*B*) but with a lower efficiency. This region lies within 600 bp of FR of *oriP*, to which EBNA1 also binds, and would not have been efficiently separated from it by sonication. The antibodies specific to Mcm2 never caused any enrichment of this region. This

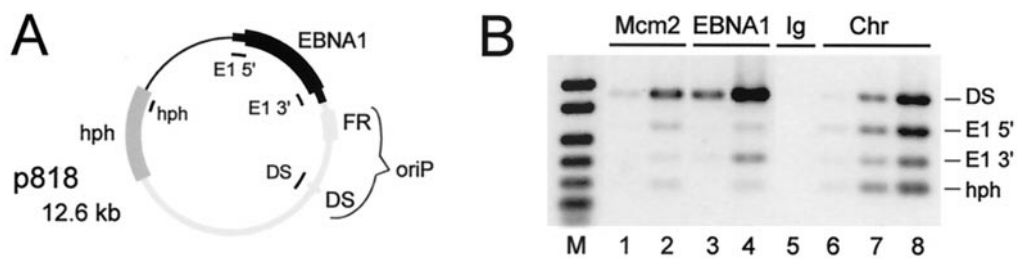

Fig. 1. Detection of Mcm2 at DS of *oriP* by ChIP. (*A*) Diagram of p818, a 12.6-kb plasmid carrying *oriP* and flanking sequences (light gray), the EBNA1 gene and flanking sequences (black), and hygromycin B-resistance cassette (hph, dark gray). The regions that were amplified by PCR for ChIP are indicated inside the circle. The distal end of the control region, E1 3′, is 560 bp from the edge of FR and 2.1 kb from DS, whereas E1 5′ is 2.1 kb farther away from *oriP*. hph is about 6 kb from *oriP*. (*B*) ChIP of chromatin from 293 cells carrying p818. Chromatin (30 μg) was precipitated by using antibodies to Orc2, Mcm2, or EBNA1 or by using nonimmune rabbit IgG, as indicated. Either 1/10th (lanes 2, 4, and 6) or 1/50th (lanes 1, 3, and 5) of the recovered DNA was amplified by PCR to detect the four indicated regions of the plasmid. DNA from the chromatin preparation was tested in lanes 6–8, by using 6.4, 32, and 160 ng, respectively. PCR products were separated by agarose-gel electrophoresis and detected by staining with SYBR Green. A reverse image is shown. M, marker. The specific PCR products ranged from 184 bp (hph) to 435 bp (DS).

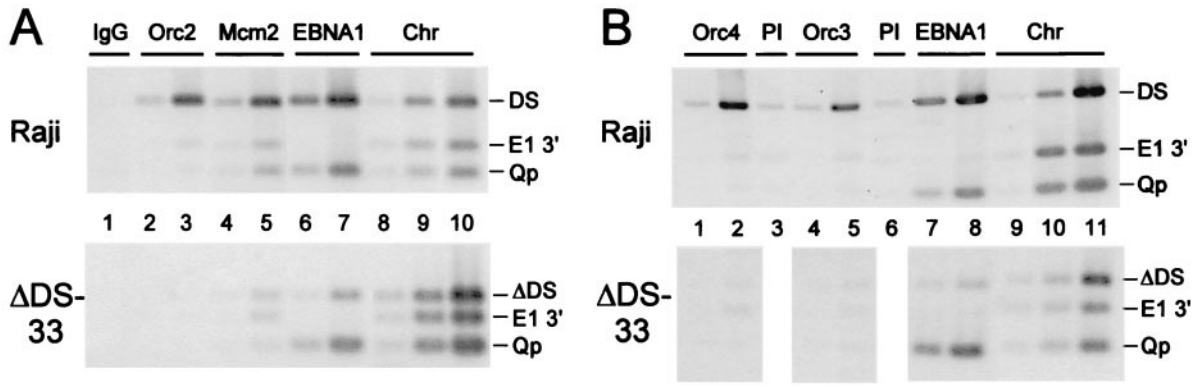

Fig. 2. The association of ORC and MCM with *oriP* requires the replicator, DS. ChIP analysis of 30 μg of chromatin from Raji cells or BL30 cells carrying the EBV mutant P3-ΔDS-33. Preimmune sera (PI) corresponding to the antisera against Orc4 and Orc3 were used for *B*, lanes 3 and 6, respectively. The amounts of ChIP DNA used for PCR were 1/250th (lanes 1, 3, 5, and 7) and 1/1,250th (lanes 2, 4, and 6) (*A*) and 1/250th (lanes 2, 3, 5, 6, and 8) and 1/1,250th (lanes 1, 4, and 7) (*B*). The amounts of DNA tested from input chromatin were for Raji, 0.06, 0.3, and 1.5 ng (lanes 8–10 in *A*, lanes 9–11 in *B*) and for P3-ΔDS-33, 0.1, 0.5, and 2.4 ng (*A*) and 0.05, 0.25, and 1.25 ng (*B*).

finding is consistent with MCM complexes associating specifically with DS of *oriP* and not with FR.

In this and similar experiments, serial 5-fold dilutions of the recovered DNA were tested by PCR as a way to titrate the different amplified regions of the plasmid against the input chromatin. In this way, it could be estimated that the EBNA1-specific antibody enriched DS chromatin by more than 25-fold relative to two of the control regions of the plasmid (E1 5′ and hph). The antibody against Mcm2 gave less enrichment of DS, 5-fold in this experiment and ranging from 2.5- to 5-fold, in part because this reagent usually gave a higher level of nonspecific recovery of all regions of chromatin. The efficiency of recovery of DS chromatin in these experiments, determined by comparing PCR yields to those of input chromatin, was 1–2% with the Mcm2 antibody, which is more efficient than reported for the recovery of yeast replication origins by ChIP with antibodies to ORC and other factors (19, 20). The EBNA1-specific antibody allowed the recovery of as much as 7% of the DS DNA from the input chromatin, but there are eight EBNA1 molecules bound to DS throughout the cell cycle (as four dimers) to contribute to this unusually high efficiency.

In similar experiments, antibodies specific to three different subunits of human ORC also were found to precipitate DS chromatin specifically from cells carrying p818 (Fig. 3, and data not shown). Antibodies against human Orc2 precipitated up to 3% of DS chromatin, giving a 5- to 10-fold enrichment of the region. To test whether the apparent association of ORC and MCM with *oriP* in the vicinity of DS depends on DS itself, we investigated an EBV mutant that lacks DS.

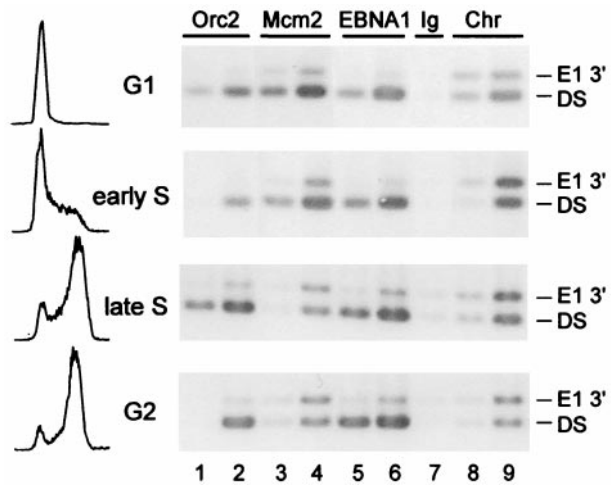

Fig. 3. Cell cycle dependence of the association of Mcm2 with DS of *oriP*. DG75 cells carrying p818 were separated by centrifugal elutriation into four populations enriched for cells in G_1, early S, late S, and G_2; 30 μg of chromatin from each was analyzed by ChIP with antibodies against Orc2, Mcm2, or EBNA1, or with nonimmune rabbit IgG, as indicated. Flow cytometric analysis of the cells for DNA content is shown (*Left*) in the conventional manner. For this experiment, a 254-bp region bordering DS was amplified to detect the presence of DS, rather than the 435 bp spanning DS as done for Figs. 1 and 2. 1/50th (lanes 2, 4, and 6) or 1/250th (lanes 1, 3, and 5) of the recovered DNA were tested. For lanes 8 and 9, 2.4 ng and 12 ng of DNA from input chromatin were tested.

The Association of ORC and MCM with *oriP* Requires the Replicator DS. A 120-bp deletion removing most of DS from the EBV chromosome was shown to eliminate detectable origin activity at *oriP*. During latent infection, the circular chromosome of such ΔDS EBV mutants is replicated exclusively by forks that initiate away from *oriP* (34). ChIP was performed on crosslinked chromatin from BL30 cells carrying the EBV mutant P3-ΔDS-33 and, for comparison, on chromatin from the EBV-positive cell line Raji, in which *oriP* is functional (42). For this experiment, the recovery of DS DNA was compared with two regions of the EBV chromosome that are far from *oriP*, the region 3′ to the EBNA1 gene and the promoter, Qp. EBNA1 binds to two regulatory sites at Qp, so this region was also precipitated by the EBNA1 antibody, although less efficiently than was DS (Fig. 2*A*, lanes 6 and 7). The ΔDS deletion removed all four EBNA1 binding sites at DS, but chromatin containing the DS deletion was still precipitated by the EBNA1 antibody, with a lower efficiency, because the amplified region is only 1 kb away from FR, to which EBNA1 also binds; for this experiment, the average DNA length after sonication was close to 1 kb.

As shown in Fig. 2*A*, antibody against Orc2 specifically precipitated DS chromatin from Raji cells but did not precipitate the corresponding region of chromatin of the ΔDS mutant. The antibody against Mcm2 caused an enrichment of DS over the two control regions in chromatin from Raji. In contrast, with ΔDS-33 the Mcm2 antibody did not cause any enrichment of the DS region preferentially compared with the two control regions (Fig. 2*A*, lanes 4 and 5). Qp was recovered as efficiently by the EBNA1 antibody in the case of P3-ΔDS-33 as it was with Raji, so it is unlikely that the failure of the antibodies against Orc2 and Mcm2 to selectively precipitate chromatin containing the DS deletion was because of any fault with the chromatin preparation. The results concerning Orc2 were corroborated by a similar

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experiment by using antibodies against two other ORC subunits, Orc3 and Orc4. As shown in Fig. 2*B*, both antibodies selectively precipitated DS chromatin prepared from Raji cells but not from cells carrying the ΔDS mutant. Again, the recovery of Qp with the EBNA1-specific antibody validated the ChIP assays. From these experiments, we can conclude that ORC and MCM require DS, the *oriP* replicator, to associate specifically with this region of the EBV chromosome.

The Specific Association of Mcm2 with *oriP* Is Cell-Cycle-Dependent. If the observed association of Mcm2 with *oriP* is indicative of origin licensing, then this association should be detected during the G_1 phase of the cell cycle but not during the G_2 phase. DG75 cells carrying p818 were separated by centrifugal elutriation to obtain a nearly pure population of small G_1 cells, a population enriched for late G_1 and early S (early S), a population enriched for late S and G_2 (late S), and a population of large cells that were 85% in G_2 (Fig. 3). With chromatin prepared from the G_1 cells and the early S cells, the Mcm2 antibody precipitated the DS region 3.3-fold and 3.7-fold more efficiently, respectively, than a control region flanking the 5′ end of the EBNA1 gene (Fig. 3, lanes 3 and 4). With chromatin from the late S cells, an enrichment of DS by the Mcm2 antibody was barely discernible (1.4-fold), and with the G_2 chromatin, there was no enrichment (1.0-fold). (The ratios are averages of two independent experiments and take into account any unequal amplification of the DS and control region in the chromatin control, Fig. 3, lanes 8 and 9, in each case.) In contrast, antibodies against EBNA1 and Orc2 precipitated DS specifically from chromatin prepared from all phases of the cell cycle (Fig. 3, lanes 1 and 2, 5 and 6). These results indicate that Mcm2, and presumably the MCM complex, associates with DS during G_1 and disassociates during S phase, consistent with the licensing and delicensing of the *oriP* replicator during the cell cycle.

We investigated whether the nonspecific precipitation of chromatin from cells in late S or G_2 by the Mcm2 antibody might arise from crosslinking to delicensed MCM, which can be removed by washing nuclei with Triton X-100 (28). Cells were washed with Triton X-100 before fixation, which eliminated staining of G_2 nuclei as seen by immunofluorescence, but this did not reduce the precipitation of nonspecific chromatin with the Mcm2-specific antibody (data not shown). This antibody thus appears to interact with chromatin nonspecifically under our conditions of ChIP. Nevertheless, specific enrichment of DS chromatin by the antibody was always observed in several experiments involving three different cell lines. The only experiments in which this antibody ever failed to enrich DS during ChIP were those involving the EBV mutant, ΔDS-33, or cells synchronized to late S or G_2.

Discussion

ORC and Replication Licensing at *oriP* of EBV. We have presented evidence that ORC and MCM associate with a specific replication origin in a vertebrate organism. ChIP assays were particularly powerful when combined with genetic approaches to reveal the presence of replication initiation factors and their dependency relationships at yeast replication origins (19). For studies of mammalian DNA replication, this approach has been slowed by the lack of appropriate antibodies and the lack of genetically defined origins. In the case of *oriP* of EBV, the replicator is confined to a region of less than 120 bp called DS, and DS-deleted EBV mutants are available. This and the availability of antibodies against subunits of human ORC and human MCM allowed us to obtain clear evidence by using ChIP that ORC mediates replication licensing at *oriP* of EBV.

To summarize, antibodies against ORC subunits Orc2, Orc3, and Orc4, and against the MCM subunit, Mcm2, selectively precipitated crosslinked chromatin containing the DS region of the EBV chromosome or of an *oriP*-based plasmid but did not precipitate this region of a DS-deleted EBV strain more than at background levels. The association of ORC and MCM with *oriP* thus requires the replicator and correlates with origin function. The specific association of Mcm2 with DS was detected in cells synchronized to the G_1 phase of the cell cycle and was absent in cells in G_2, which is consistent with a loss of MCM during S phase and thus with its role in licensing *oriP* for replication. ORC appeared to remain associated with DS throughout S and G_2, as it does at undetermined sites on human chromatin (43) and as does yeast ORC at yeast replication origins (44).

Parallel work from two different laboratories supports these conclusions. Schepers and coworkers (45) have found in similar experiments that Orc1, Orc2, and Orc3 associate with *oriP* and that a small amount of these ORC subunits can be coprecipitated with EBNA1 from cell lysates by using specific antibodies. A study by A. Dutta and colleagues (K. Yoshida, S. Dhar, and A.D., unpublished work) has shown that replication of *oriP*-dependent plasmids requires a wild-type *ORC2* gene and, furthermore, that geminin can inhibit replication from *oriP*. This also implicates Cdt1 in licensing at *oriP* because geminin functions to block Cdt1 (46, 47), which is required along with Cdc6 to load MCM at sites of ORC binding (21, 22).

How Might ORC Associate with the Replicator of *oriP*? The key issue is where ORC associates at or near DS. Functionally DS is at most 119 bp long and includes two pairs of EBNA1 binding sites (8), with each pair spanning 39 bp that have been shown by *in vivo* footprinting to be occupied by EBNA1 throughout the cell cycle (ref. 48; H.X. and J.Y., unpublished data). The remaining DS DNA that are not bound by EBNA1, 15 bp between the pairs of EBNA1 sites and at most 13 bp at each end, are too short to accommodate ORC (30), and because these regions include sites that are hypersensitive to cleavage by DNase I, they are unlikely to be bound continuously by proteins *in vivo* (48). The *in vivo* footprinting data did not reveal signs of ORC binding immediately flanking DS. If ORC binds to DNA outside of DS (to nonreplicator DNA), then DS must in some manner facilitate ORC binding. Alternatively, if ORC is present at DS, then it must be bound indirectly. This is of interest in either case because it is not known how sites of ORC binding are determined on human chromosomes (33).

In light of this report, considerable circumstantial evidence suggests that EBNA1 is responsible for recruiting ORC to DS or its vicinity. Not only is EBNA1 the only protein that appears to bind to DS directly (48), the EBNA1 binding sites at DS are the only sequences that are essential for replicator activity (8). Replicator function requires the presence of two EBNA1 binding sites that are spaced exactly 21 bp apart center to center (J. Bashaw and J.Y., unpublished data), implying that a precise structure must be formed by two adjacent EBNA1 dimers. EBNA1 represses transcription by binding to Qp (49) and fosters episome segregation by binding to FR of *oriP* (10–13). However, EBNA1 does not support replication at these sites (refs. 6 and 8 and unpublished data) or appear to recruit ORC (Fig. 2 and unpublished data), which might be because EBNA1 dimers bind at these sites with spacings other than 21 bp. EBNA1 cannot activate replication from *oriP* in rodent cells, implying a failure of EBNA1 to interact with a necessary host factor. This host factor could be either ORC or something that facilitates ORC binding.

Licensed Replication of EBV and Other Viruses. Replication of the EBV chromosome during latent infection initiates not only at *oriP* but elsewhere as well, notably within a long initiation zone spanning 30 kb or more (34, 42). This initiation zone reflects the complexity that is found with most replication origins of mammalian chromosomes (33) and might well function simi-

larly because no viral protein is likely to be involved. Several members of the gamma subfamily of herpesviruses to which EBV belongs, most notably the Kaposi sarcoma-associated virus, HHV8 (50), are also likely to use licensed replication to maintain their chromosomes during latent infection, and discovering their mechanisms could offer insight into the possible different ways that licensed replication origins might be configured. In the cases of medically significant viruses such as EBV and HHV8, for which genome maintenance during latent infection sustains pathology, the information could have additional relevance.

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