Epstein-Barr Nuclear Antigen 1 (EBNA1)-dependent Recruitment of Origin Recognition Complex (Orc) on oriP of Epstein-Barr Virus with Purified Proteins

STIMULATION BY Cdc6 THROUGH ITS DIRECT INTERACTION WITH EBNA1 *

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Kenji Moriyama‡, Naoko Yoshizawa-Sugata§, Chikashi Obuse§, Toshiki Tsurimoto¶, and Hisao Masai‡1

From the 6 Genome Dynamics Project, Department of Genome Medicine, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan, the 6 Division of Molecular Life Science, Graduate School of Life Science, Hokkaido University, Sapporo 001-0021, Japan, and the 6 Department of Biology, Faculty of Sciences, Kyushu University, Fukuoka 812-8581, Japan

Background: Enzymatic studies on the steps of mammalian DNA replication with purified proteins are essential to elucidate its mechanisms.

Results: Association of Orc with oriP requires EBNA1 and is stimulated by Cdc6 directly interacting with EBNA1.

Conclusion: EBNA1 recruits Cdc6/Orc at oriP, permitting site-specific assembly of pre-RC.

Significance: This study provides novel insight into a mechanism for initiation of mammalian DNA replication with purified factors.

Origin recognition complex (Orc) plays an essential role in directing assembly of prereplicative complex at selective sites on chromosomes. However, Orc from vertebrates is reported to bind to DNA in a sequence-nonspecific manner, and it is still unclear how it selects specific genomic loci and how Cdc6, another conserved AAA+ factor known to interact with Orc, participates in this process. Replication from oriP, the latent origin of Epstein-Barr virus, provides an excellent model system for the study of initiation on the host chromosomes because it is known to depend on prereplicative complex factors, including Orc and Mcm. Here, we show that Orc is recruited selectively at the essential dyad symmetry element in nuclear extracts in a manner dependent on EBNA1, which specifically binds to dyad symmetry. With purified proteins, EBNA1 can recruit both Cdc6 and Orc independently on a DNA containing EBNA1 binding sites, and Cdc6 facilitates the Orc recruitment by EBNA1. Purified Cdc6 directly binds to EBNA1, whereas association of Orc with EBNA1 requires the presence of the oriP DNA. Nuclease protection assays suggest that Orc associates with DNA segments on both sides adjacent to the EBNA1 binding sites and that this process is stimulated by the presence of Cdc6. Thus, EBNA1 can direct localized assembly of Orc in a process that is facilitated by Cdc6. The possibility of similar modes of recruitment of Orc/Cdc6 at the human chromosomal origins will be discussed.

Chromosomal DNA must be replicated once and only once per cell cycle to transmit a precise, complete copy of the whole genetic information to daughter cells. In eukaryotes, acquisition of replication competence in late M or early G1 phase is essential for initiation of DNA replication in subsequent S phase (reviewed in Refs. 1 and 2). This process, called replication licensing, is accomplished by ordered assembly of prereplicative complex (pre-RC)2 on chromosomes. Essential components of pre-RC include origin recognition complex (Orc), Cdc6, Cdt1, and minichromosome maintenance (Mcm) complex. In most organisms so far examined, Orc first docks on a presumptive replication origin, and Cdc6 is associated with it, followed by association of the Mcm complex, facilitated by Cdt1. In budding yeast Saccharomyces cerevisiae, autonomously replicating sequences (ARS) were identified as bona fide origins, to which Orc binds by recognizing a specific DNA sequence termed the ACS (ARS consensus sequence) (3, 4). In contrast, no specific sequence has been identified for origins or for Orc binding in higher eukaryotes, except for a slight preference for AT-rich sequences in some species (5, 6). Such a lack of sequence specificity of Orc has precluded detailed biochemical and structural studies on the geometry and functions of pre-RC components in higher eukaryotes. Thus, a system for site-specific assembly of Orc and other pre-RC components on DNA bearing a defined origin would be highly desired. This would be achieved, for example, by exploiting the latent replication system of Epstein-Barr virus (EBV) or Kaposi’s sarcoma-associated herpesvirus (KSHV) (reviewed in Refs. 7–9). In the latent state, the genome of EBV or KSHV replicates extrachromo-

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This article contains supplemental Experimental Procedures and Figs. S1–S5.

1 To whom correspondence should be addressed. Tel.: 81-3-5316-3231; Fax: 81-3-5316-3145; E-mail: masai-hs@igakuken.or.jp.

2 The abbreviations used are: pre-RC, prereplicative complex; Orc, origin recognition complex; EBV, Epstein-Barr virus; DS, dyad symmetry; FR, family of repeat; Mcm, minichromosome maintenance; ARS, autonomously replicating sequence(s); KSHV, Kaposi’s sarcoma-associated herpesvirus; nt, nucleotide(s); PC, Protein C; NE, nuclear extract; MBP, maltose-binding protein; ExoIII, exonuclease III.
somally in the nucleus as a circular, chromatinized DNA (epi-
some) and persists in a relatively low copy number. Their rep-
lication occurs once in S phase in synchrony with host chromo-
some replication (10). The episomal replication of the EBV genome initially depends on its origin of plasmid replica-
tion (oriP) and EBNA1 (Epstein-Barr nuclear antigen 1), a viral
trans-acting factor that is believed to trigger assembly of
pre-RC (11–13). Similarly, LANa (latent nuclear antigen) of
KHSV also recruits Orc onto the terminal repeat of the KHSV
genome (14, 15). EBNA1 is a dimer that binds to an 18-bp pal-
indromic sequence with its C-terminal DNA-binding and
dimerization domain (16). oriP is necessary for EBNA1-depen-
dent latent replication and for establishment of latent infection
of EBV, containing 24 EBNA1 cognate sites, of which four sites
exist in a 113-bp dyad symmetry (DS) (17–19). The other 20
EBNA1 sites are present as 30-bp tandem repeats in the family
of repeat (FR) region that is required for mitotic segregation of
the oriP-episome by mediating its anchoring to host chromo-
somes (20, 21). Initiation of replication at oriP is believed to
depend on pre-RC formation within or around DS (11–13).
Fourteen EBNA1 binding sites, DS carries three nonamer
repeats, each resembling the telomere repeat unit, and they also
contribute to replication and mitotic persistence of oriP-epi-
some (19, 22). TRF2 (telomere repeat-binding factor 2) and
several other telomere factors associate with DS in a manner
dependent on the nonamers and modulate the efficiency and
timing of oriP-replication (23–27). Because EBNA1 was
reported to associate with Orc in cell extracts (13, 25, 28), it
would be interesting to examine the possibility of in vitro site-
specific assembly of Orc and other pre-RC components on
DNA bearing EBNA1 binding sites. Toward the long term goal
of in vitro reconstitution of mammalian chromosomal replica-
 tion with purified components, EBNA1-dependent, site-spe-
cific assembly of human pre-RC components on oriP DNA in
vitro will provide an excellent model system.

The present study demonstrated that EBNA1 could recruit
purified Orc onto DNA bearing EBNA1 binding sites and that
this process is stimulated by Cdc6. They cooperate to provoke
localized alteration in nucleosome sensitivity on DS and its flan-
kling regions. On the basis of the results presented, we will dis-
cuss how Orc and Cdc6 assemble onto EBNA1-bound DS.

**EXPERIMENTAL PROCEDURES**

Reagents, Antibodies, and Cells—Anti-EBNA1 antibody (rabbit
polyclonal) was kindly provided by Dr. Shirakata. Anti-Orc2
antibody (3B7) was obtained from MBL Co. Anti-TRF2
(4A794.15) was purchased from Imgenex. Anti-GST (B-14),
anti-Cdc6 (sc-9964), and anti-Orc6 (3A4) were from Santa
Cruz Biotechnology, Inc. (Santa Cruz, CA). Other antibodies
were made in our laboratory. Protease inhibitors (Sigma
P-8849) and 1 mM PMSF were included in solutions for cell
extraction and for protein purification unless otherwise indi-
cated. Glutathione-Sepharose 4B, streptavidin-Sepharose HP,
ovalbumin, and poly(dI-dC) duplex were purchased from GE
Healthcare. Anti-protein C affinity matrix and biotin-dUTP
were obtained from Roche Applied Science. Anti-FLAG
M2-agarose beads and 3× FLAG peptides were from Sigma.
TALON metal-chelating resin was from Clontech. Dynabeads-

streptavidin M280 was obtained from Invitrogen. Oligonucleo-
tides were synthesized commercially by Hokkaido System Sci-
ence. Nucleases and other molecular biology enzymes were
purchased from Takara-Bio, TOYOBO, Roche Applied Sci-
ence, Sigma, or New England Biolabs. HEK293 and 293T cells
were cultured in DMEM (Nissui Co.) plus 10% FBS supple-
mented with penicillin and streptomycin (Invitrogen). Sf9 and
Sf21 cells were cultured in SF900II SFM (Invitrogen) plus 5%
FBS. HiFive cells were cultured in serum-free Excell405
medium (Invitrogen). For bacterial expression, Escherichia coli
BL21 harboring pRep4 (Qiagen) was used as host strain and
cultured in 2× YT plus 0.2% glucose. An ATP-regenerating
system consisted of 20 mm phosphocreatin, 40 units/ml creatin
kinase (Sigma), and 3 mM ATP, pH 7.5.

Recombinant Plasmids and Viruses—The glycine-alanine
repeat (amino acids 91–322) of EBNA1 is dispensable for its
function in oriP-dependent replication. This region was
replaced with maltose-binding protein (MBP) encoded on
pMAL-c2 (New England BioLabs) and inserted into pUSR-BS,
an SRα promoter-based mammalian expression plasmid (29).
This construct was designated as pUSR-EB900mbp323. An oriP
plasmid, pKS-EX, and its deletion derivatives, pKS-EXΔDS and
pKS-DS, as well as EBNA1-encoding DNA (derived from the
EBV strain B95-8) were kindly provided by Dr. Shirakata (18).

pKS-ARV, a DS-containing plasmid, was created by inserting
an EcoRV-Alul fragment (8995–9195 nt of EBV B95-8) into a
blunted Sall site of pBluescript II KS(−) vector (Stratagene).
pKS-AHF, another DS-containing plasmid, was created by
inserting a blunted HindI-Alul fragment (8945–9195 nt of EBV
B95-8) between Smal and blunted Sall sites of pBluescript II
KS(−). pSOP-T_{48} was made by replacing the bla-LacZ (2126–
2657 nt) portion of pBluescript II SK(−) from the SV-neo
(G418\textsuperscript{R}) unit from pMAMneo (Stratagene), followed by
inserting 48x
tetO (30) between Sall and Xhol sites and full-length
oriP (from pKS-EX) between EcoRI and XbaI sites of its multi-

cloning site. Recombinant baculoviruses for expression of
human Orc1, FLAG-Orc1, Protein C (PC)-Orc4, Orc2, and
Orc5 were constructed using pFastBac DUAL (Invitrogen).
Baculovirus for MBP-Orc5 was constructed using pVL1392
(Invitrogen), and those for HA-Orc3 and GST-Orc3 were made
using pVL1393. For the above three constructs, cDNAs encod-
ing human Orc3 and Orc5 were supplied by Dr. Yasuyuki
Watanabe (our laboratory). Human Orc6 cDNA was provided
by Dr. Ryo Kitamura (our laboratory) and used for bacterial
expression of His\textsubscript{6}-Orc6 in pQE60-based plasmid (Qiagen).

Human Cdc6 cDNA was obtained from Dr. Hiroko Fujii-
Yamamoto (our laboratory) and used for bacterial expression
of Histag, Orc6 in pQE60-based plasmid (Qiagen). Human
Cdc6 cDNA was constructed using pFastBac DUAL (Invitrogen).

Transient Replication Assays—Subconfluent HEK293 cells
were co-transfected with 2.5 \mu g of pSOP-T_{48} (oriP-plasmid) and
1.5 \mu g of pUSR-EB900mbp323 using TransIT293 reagent
(Mirus) in a 60-mm dish. After 24 h, cells were split into two
100-mm dishes and cultured for 3 days. Cells were harvested in
0.5 ml/dish of chilled PBS plus 5 mM EDTA and collected by

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EBNA1- and Cdc6-mediated Association of Orc at oriP

Preparation of 293T Nuclear Extract and Recombinant Proteins—Mammalian expression plasmids were transfected into 293T cells using TransIT293 reagent. After 48–50 h, nuclear extracts were prepared essentially as described by Dignam et al. (31) with the following modifications. The collected cells were suspended in 0.5 ml of hypotonic buffer A (10 mM Hepes-KOH (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM ATP, and 0.5 mM DTT) for 10 min on ice. The swelled cells were recollected, suspended in 0.2 ml of the same buffer, and homogenized with 10 strokes in a micropestle. The lysed cells were centrifuged for 5 min (2000 rpm, 2 °C). The supernatant was centrifuged (12,000 rpm, 20 min, 2 °C), and the supernatant was stored as low salt extract. The pellet was suspended in 0.2 ml of hypertonic buffer C (20 mM Hepes-KOH (pH 7.9), 5 mM MgCl₂, 450 mM NaCl, 0.2 mM ATP, 0.2 mM EDTA, 0.2 mM EGTA, 0.5 mM DTT, and 25% glycerol). After the addition of 4 µl of 10% Triton X-100, it was rocked for 30 min at 4 °C and centrifuged at 3,000 rpm for 5 min and then at 12,000 rpm for 20 min at 2 °C. The supernatant (high salt extract) was dialyzed in Buffer D (20 mM Hepes-KOH (pH 7.8), 5 mM Mg(OAc)₂, 100 mM NaCl, 0.5 mM potassium glutamate, 0.2 mM EDTA, 0.2 mM EGTA, and 15% glycerol) for 5 h at 4 °C, and it was designated as nuclear extract (NE). Both dialysis buffers contained 0.2 mM ATP and 0.5 mM DTT unless otherwise indicated.

Protein Expression and Purification—MBP-EBNA1 fusion protein was purified using amylase resin (New England BioLabs) according to the manufacturer’s instructions, from nuclear extract prepared from 12 150-mm dishes of human 293T cells after transient expression of EB90mbp323. MBP-EBΔ451 was purified similarly from 20 dishes of transfected 293T cells. The human Orc1–5 complex was extracted from HiFive cells infected with five recombinant baculoviruses (Orc1, Orc2, HA-Orc3, PC-Orc4, and Orc5) for 48 h. Cytoplasmic extract, Triton-soluble nuclear extract, and high salt NE were prepared according to Vashee et al. (6). The NE was applied to an anti-Protein C affinity matrix column. After extensive washing, the Orc1–5 complex was eluted with a solution containing 10 mM EDTA, 2 mM EGTA, 150 mM KCl, 1 mM ATP, 10% glycerol, 20 mM and Tris-HCl (pH 7.5). The Orc2–5 complex (Orc2, GST-Orc3, PC-Orc4, and MBP-Orc5) was extracted similarly, and the NE fraction was applied to glutathione-4B. Then the glutathione-eluate was further purified using an anti-Protein C affinity matrix column as described above. For FLAG-Orc1, 10 150-mm dishes of SF9 cells or a liquid culture (~0.5 liter) of SF21 cells were infected with a recombinant baculovirus. The NE fraction was prepared as above and was applied to anti-FLAG M2-agarse beads. After high salt washing, FLAG-Orc1 was eluted with 3× FLAG peptide. For His-Orc6, GST-Cdc6, and HCBD-TRF2, E. coli BL21 (pRep4) cells carrying each plasmid were collected by centrifugation. The low molecular weight DNA fraction was treated with lysozyme for 30 min at 4 °C, the cell suspension was frozen in liquid nitrogen, thawed, and mixed with the same volume of a chilled lysis solution B (20 mM Tris-HCl (pH 8.0), 0.2 mM DTT, 0.6 mM KCl, and 0.4% Triton X-100). 0.5 mM ATP was included when GST-Cdc6 was extracted. After mild mixing for 20 min at 0–4 °C, lysates were clarified by centrifugation for 30 min at 30,000 rpm (Beckman L90K). His-Orc6 and HCBD-TRF2 were then purified through TALON resin with 30–180 mM imidazole gradient elution. GST-Cdc6 was purified through a glutathione-Sepharose 4B column. When required, Cdc6 was excised from the GST portion with PreScission protease (GE Healthcare). The PreScission protease, uncleaved GST-Cdc6, and cleaved GST were then removed by adsorption to glutathione beads. Purified recombinant proteins were dialyzed in chilled Buffer GD. ATP was omitted from the buffer when EB90mbp323, His-Orc6, and HCBD-TRF2 were purified.

Pull-down Assay with Amylose Resin—DS of oriP was amplified by PCR using M13 forward and reverse primers from pKS-DS. Control tetO₆ DNA was prepared similarly. One hundred µl of nuclear extract (293T NE) was mixed with DS or tetO₆ DNA for 20 min at 25 °C. Then MBP-EBNA1 in the extract was adsorbed onto amylase resin (New England BioLab) for 60 min. The beads were washed three times with Buffer D containing 0.15 M NaCl and 0.1% Triton X-100 and then eluted with 0.3 µl of the same buffer containing 0.6 M NaCl. The eluates were subjected to SDS-PAGE.

Pull-down Assay with Streptavidin-Sepharose—DS of oriP was amplified by PCR using an M13 primer and a biotinylated reverse primer. FRF, a DNA fragment containing four EBNA1 binding sites of the FR region, was prepared as a biotinylated form as well. Seventy µl of nuclear extract was mixed with 1.5 µg of biotinylated DNA (DS or FRF fragment) for 20 min at 25 °C. The biotinylated DNA and associated proteins were adsorbed onto streptavidin-Sepharose for 20 min. The beads were washed three times with Buffer D containing 0.15 M NaCl and 0.1% Triton X-100, and residual materials were liberated by boiling in SDS sample buffer. In the experiment for Fig. 2, D and E, poly(dl-dc) duplex and an ATP-regenerating system were included in the binding reaction. When purified proteins were used (Fig. 3C), the binding reaction contained 10 µg/ml ovalbumin to suppress nonspecific protein interactions.

Pull-down Assay with DNA-Magnetic Beads—To obtain oriP- or oriPΔDS-magnetic beads, pKS-EX or pKS-EXΔDS was
digested with Xhol and XbaI and then filled in with biotin-dUTP, dATP, dCTP, and dGTP by the Klenow fragment. The biotinylated DNA was further cleaved with Asp718 and SacII. After short Asp718-Xhol and XbaI-SacII fragments were excluded through a Microspin S-400 column, longer DNA fragments were mixed with streptavidin-Dynabeads using the Dynabeads Kilobase-binder kit (Invitrogen). Unlinked vector backbone (SacII-Asp718 ~2.9 kbp) was removed by extensive washing. Sixty-four µl of nuclear extract was mixed with oriP- or oriPΔDS-Dynabeads (3 pmol of DNA) in the presence of 0.1 mg/ml poly(dI-dC) duplex and an ATP-regenerating system. The binding reaction proceeded for 30 min at 25 °C. The magnetic beads were collected by a magnetic separator and washed three times with Buffer GD containing 0.1 M KCl, 0.1% Triton X-100, and 0.5 mM ATP. Then binding materials were eluted by boiling in SDS sample buffer. The oriP-binding assays with purified proteins were similarly performed with magnetic beads to which 1.5 µg of oriP was linked at a single end (Xhol site) in the presence of 10 µg/ml ovalbumin (Fig. 3B).

Release of DS-bound Proteins by DNase I Treatment—Linear ARV DNA (428 bp) was PCR-amplified using biotinylated primers and immobilized at both ends on streptavidin-Dynabeads through the biotin-avidin linkage. Purified recombinant proteins were mixed with the DNA-Dynabeads (corresponding to ~2 pmol DS) in the presence of 0.1 mg/ml poly(dI-dC) duplex and an ATP-regenerating system. After mild mixing for 20 min at 37 °C, the beads were collected and washed three times with Buffer GD containing 0.3 ml of 0.1 M NaCl, 0.05% Triton X-100, and 0.5 mM ATP. Then the beads were treated with 7 units (2 μg) of DNase I (Takara-Bio) in a 45-μl reaction for 30 min at 37 °C. The DNase-released proteins were analyzed by SDS-PAGE followed by silver staining or Western blotting. The detected bands were quantified using densitometric software (MultiGauge, Fujifilm).

Immunoprecipitation of Protein-DNA Complexes with Anti-EBNA1 Antibody—Anti-EBNA1 antibody was kindly provided by Dr. Shirakata and used to immunoprecipitate EBNA1 and associated proteins in the presence or absence of ARV (a DNA fragment bearing DS of oriP). Two μg of anti-EBNA1 antibody was conjugated to Dynabeads-Protein G according to the manufacturer’s instructions (Invitrogen). Recombinant EBNA1, Orc1–5 subcomplex, His-Orc6, and GST-Cdc6 were mixed on ice, and 1 pmol of biotinylated ARV was added, where indicated, in Buffer GD containing 5 mM MgCl2, 40 mM potassium glutamate, 0.01% Triton X-100, 60 ng/µl ovalbumin, and an ATP-regenerating system. After 15 min at 37 °C, the anti-EBNA1 Dynabeads were added, followed by incubation for 40 min at room temperature. The beads were washed three times with Buffer GD containing 0.1 M NaCl, 0.5 mM ATP, and 0.05% Triton X-100 and were boiled in SDS sample buffer for subsequent electrophoresis. The eluted proteins were detected by Western analyses. The eluted DS fragment (biotinylated) was dot-blotted on a Hybond N+ membrane (GE Healthcare) and detected by peroxidase-conjugated streptavidin (Sigma). The bands and dots were quantified using densitometric software (MultiGauge, Fujifilm). The values were converted to the amount of respective proteins used in the entire reaction by using KalediGraph software.

Exonuclease III Protection Assay—The exonuclease III (ExoIII) protection assay was performed as reported (32, 33) with the following modifications. pKS-ARV was cleaved with HindIII (or Xhol) and labeled at the 5’-ends with 32P by a standard method. The end-labeled DNA was cleaved with Xhol (or HindIII), and a 228-bp fragment was isolated as a DS substrate. The substrate (75 fmol) was mixed with purified proteins in 20 mM Tris-HCl, 12.8 mM Hepes-KOH (pH 7.6), 6 mM MgCl2, 3.2 mM Mg(OAc)2, 64 mM potassium glutamate, 1.3 mM DTT, 20 μg/ml BSA, 12% glycerol, and an ATP-regenerating system. The binding reaction was done in 25 μl for 10 min at room temperature and then for 5 min at 37 °C. One μl of ExoIII (100 units) was added and incubated for 6 min at 37 °C. Digestion was terminated by adding SDS and phenol/chloroform. After purification by ethanol precipitation, the digested DNA was dissolved in 80% formamide, denatured, and run on a 7.5% polyacrylamide sequencing gel.

DNase I Protection Assay—DNase I protection assays were carried out as described (34). pKS-AHF contains a 251-bp sequence (Hinfl site at 8945 nt, AluI site at 9195 nt of the EBV B95-8 genome) carrying DS on the pBluescript vector. A 0.44-kbp DNA of this plasmid was PCR-amplified with M13 primers and cleaved by Xhol. A 354-bp fragment was gel-purified and radiolabeled at the 5’-end with 32P by a standard method. Seventy-five fmol of the DS substrate was mixed with EB90mbp323 (3 pmol), TRF2 (3 pmol), Orc1–5 subcomplex (0.15, 0.3, or 0.6 pmol), and/or Cdc6 (0.3, 0.6, or 1.2 pmol) in 25-μl reaction mixtures containing 32 mM Hepes-KOH (pH 7.8), 12 mM Mg(OAc)2, 150 mM potassium glutamate, 1.3 mM DTT, 20 μg/ml BSA, 12% glycerol, and an ATP-regenerating system. The binding reaction was for 12 min at room temperature and then continued for 6 min at 37 °C. One μl of DNase I (0.01 units) was added and incubated for 60 s at 37 °C. Digested DNA was purified and electrophoresed as described for the ExoIII protection assay.

KmnO4 Oxidation Assay—The KMnO4 oxidation assay was carried out according to Hearing et al. (35). EBNA1 (12 pmol), TRF2 (10 pmol), Orc (0.8 pmol of Orc1–5 + 1.2 pmol of His-Orc6), and/or Cdc6 (0.4 pmol) were mixed with 0.5 μg (0.23 pmol) of pKS-AHF (DS-containing plasmid) in 40-μl reaction mixtures containing 33 mM Hepes-KOH (pH 7.8), 154 mM potassium glutamate, 11 mM Mg(OAc)2, 20 μg/ml BSA, 12.8% glycerol, and an ATP-regenerating system. After 15 min at 37 °C, 4 μl of 100 mM KMnO4 was added, and incubation was continued for further 4 min at 37 °C. Oxidation was terminated by adding 4 μl of 14.4 M β-mercaptoethanol and 44 μl of 2% SDS, 40 mM EDTA (pH 8). Oxidized plasmid (0.18 μg) was subjected to primer extension using 32P-labeled M13 primers according to the method of Parsons et al. (36). The extended DNA was dissolved in 80% formamide, denatured, and run on a sequencing gel.

RESULTS

DS-dependent Interaction between Human Orc and EBNA1 in Nuclear Extracts—In order to dissect the molecular interactions involved in initiation of DNA replication at oriP, we first attempted to verify the reported interaction between EBNA1 and human Orc using nuclear extract of 293T cells. Because the
glycine-alanine repeat region of EBNA1 is dispensable for latent replication from oriP (37), we replaced this region with MBP to create a novel recombinant EBNA1 (Fig. 1A). It was previously known that fusion of a large tag at the N terminus or C terminus of EBNA1 results in some difficulty in large scale purification. The Gly-Ala repeat region spanning amino acids 91–322 of EBNA1 was replaced with MBP in EB90mbp323, and the amino acids 85–322 were replaced in EB84mbp323 (Fig. 1A). These two MBP-fused EBNA1 recombinants supported transient replication of oriP-containing plasmid as efficiently as the full-length counterpart in HEK293 cells (Fig. 1B, lanes 2–4). In contrast, EB84mbp356 (lacking LR2; the second linking region, amino acids 323–355) did not support efficient replication (Fig. 1B, lane 5) because LR2 was known to be important for replication of oriP-plasmid (38, 39). Thus, we were able to obtain a new recombinant EBNA1 (EB90mbp323) that permitted efficient replication of oriP-plasmid and that allowed one-step, large scale purification (see below).

Amylose resin efficiently pulled down EB90mbp323 from the nuclear extract of transfected 293T cells (Fig. 2A). However, neither Orc nor other pre-RC proteins were significantly co-precipitated in the presence of a control tetO fragment (Fig. 2B, lane T, and data not shown). When the DS fragment was present during incubation of the nuclear extract with amylose resin, substantial amounts of TRF2 and Orc2 were co-precipitated with MBP-fused EBNA1 and could be eluted with 0.6 M NaCl treatment (Fig. 2B, lane D). Besides four EBNA1 binding sites, DS carries three nonamer repeats, each resembling the telomere repeat unit, and they also contribute to replication and mitotic persistence of oriP-episome (19, 22). One of the major telomere factors, TRF2 was reported to associate with DS in a manner dependent on the nonamers, and it modulates efficiency and timing of oriP-replication (23–27). TRF2 was actually present in an EB90mbp323-bound fraction only in the presence of DS (Fig. 2B, lane D). A small amount of Cdc6 was detected, although it was pulled down to some extent even without DS (Fig. 2B, lane T; see below).

These results suggest that the presence of DS is required for interaction of Orc with EBNA1 in nuclear extracts. DS has four EBNA1 binding sites, but oriP carries 24 EBNA1 binding sites, of which 20 sites exist in the FR region (Fig. 2C). To examine whether a FR-derived DNA fragment could allow EBNA1 to recruit Orc, we compared the abilities of DS and a partial fragment of FR bearing only four EBNA1 binding sites (designated as FRf; Fig. 2C) to recruit Orc. The DS and FRf fragments were biotinylated at one end and incubated with the nuclear extract.
of 293T cells. Then the biotinylated DNA and the associated molecules were adsorbed onto streptavidin-beads. Cdc6 was pulled down with both DS and FRf due to the direct interaction between EBNA1 and Cdc6, as described below. In contrast, the DS, but not the FRf fragment, could recruit Orc2 from nuclear extract in the presence of EBNA1 (Fig. 2, D and E). The addition of increasing amounts of poly(dI-dC) duplex in the nuclear extract augmented the recruitment of Orc2 to DS but not to FRf DNA, whereas the amount of recruited EBNA1 did not change (Fig. 2, D and E). Slightly more EBNA1 is eluted from DS than from FRf, probably reflecting the affinity of EBNA1. However, this will not affect the interpretation of the results because the level of Cdc6, which depends on the level of EBNA1, does not differ significantly between DS and FRf. Similar results were
obtained when purified calf thymus DNA was used in place of poly(dI-dC) duplex (data not shown). These nonspecific competitor DNAs may sequester the contaminating DNA-binding molecules that might otherwise interfere with Orc2 binding to DS. Alternatively, the DNA may act as a mediator that facilitates binding of Orc2 to EBNA1-bound DS. These results indicate that Orc2 is specifically recruited to DS but not to FR. As expected, immobilized full-length oriP also recruited Orc2 in the presence of MBP-EBNA1 (EB90mpb323), but it was abolished when DS was deleted from oriP (supplemental Fig. S1). These results demonstrate the essential role of the DS sequence in EBNA1-dependent recruitment of Orc in nuclear extracts.

**Purified EBNA1 Recruits Purified Human Orc and Cdc6 onto oriP**—Interaction in the nuclear extracts could be indirect and may be mediated by other proteins. Therefore, we next examined the interactions using purified proteins. MBP-EBNA1 (EB90mpb323) was purified from transfected 293T cells (Fig. 3A). Orc is known to be present in different subassemblies. Differential cell cycle regulation of Orc1 and functions of Orc6 independent from the complex have also been reported (40–43). Therefore, we prepared various forms of Orc. FLAG-Orc1 was purified from Sf9 or Sf21 insect cells after infection with the recombinant baculoviruses. The human Orc1–5 and Orc2–5 complexes were purified from HiFive insect cells co-infected with multiple baculoviruses. HCBD-tagged TRF2, GST-Cdc6, and His6-tagged Orc6 were purified from E. coli. The purified EBNA1 bound efficiently to immobilized full-length oriP (Fig. 3B). In the presence of nonspecific competitor DNA (poly(dI-dC) duplex), TRF2, Orc, or Cdc6 did not associate with oriP when EBNA1 was absent (Fig. 3B, lane 3). The purified EBNA1 recruited purified TRF2, the Orc2–5 subcomplex, and Cdc6 onto oriP (Fig. 3B, lane 5). TRF2 was not required for EBNA1 to recruit Orc and Cdc6 (Fig. 3B, lane 2). The presence of Cdc6 appears to stimulate the binding of Orc to oriP (Fig. 3B, compare lanes 1 and 5). However, this conclusion needs to be carefully evaluated because more EBNA1 is bound to oriP DNA (Fig. 3B, lane 5) (see below). It is interesting to note that Cdc6 was recruited to oriP in the absence of Orc (Fig. 3B, lanes 4–6). Unexpectedly, Orc1 only slightly stimulated the binding of the purified Orc2–5 subcomplex to oriP (Fig. 3B, lanes 4 and 5). Thus, EBNA1 is able to recruit Orc to oriP on its own, and this process occurs in the absence of Orc1 and is facilitated by Cdc6.

When nuclear extract was used as a source of Orc, EBNA1 (EB90mpb323) recruited Orc onto DS but not onto FR (Fig. 2 and supplemental Fig. S1). In contrast to the nuclear Orc, purified Orc could bind to both FRf and DS fragments in the presence of EBNA1 (Fig. 3C, lanes 1, 2, 5, and 6). Purified Cdc6 also bound to both FRf and DS only in the presence of EBNA1 (Fig. 3C). These results indicate that EBNA1 has an intrinsic ability to recruit Orc onto both DS and FR as long as they carry EBNA1 binding sites. The results also suggest that nuclear extracts contain some factor(s) that prevent Orc from associating with FR (see “Discussion”).

**Purified Cdc6 Enhances EBNA1-dependent Recruitment of Orc1–5 Complex to DS**—The results above indicate that EBNA1-dependent loading of Cdc6 and Orc onto DS can be reconstituted with purified proteins. Furthermore, Cdc6 appears to stimulate the loading of Orc onto DS. Therefore, we next performed more quantitative analyses on the interaction of Orc and Cdc6 with DS using modified pull-down methods. In this assay, we used the Orc1–5 complex (Fig. 3A), which gave basically the same results as Orc2–5 supplemented with FLAG-Orc1. In the pull-down experiments, we sometimes observed background binding of Cdc6 to affinity resins or beads in the absence of DNA and/or EBNA1 (e.g., see supplemental Fig. S1). Yeast Cdc6 has been reported to have a similar background binding (44, 45). To minimize the background binding, DS-bound proteins were released from DS-conjugated magnetic beads by DNase I treatment instead of boiling the beads in an SDS solution. The DNase digestion successfully eluted the DNA-bound Cdc6 but did not release that adhered to beads. The DNase-released proteins were electrophoresed in parallel with the defined amount of proteins (0.1–10% of input) to quantify the fractions of the bound proteins (Fig. 4 and supplemental Fig. S2). Purified Orc1–5 was recruited to DS in an EBNA1-dependent manner, and Cdc6 enhanced this recruitment by 1.8–3.3-fold (Fig. 4, B and C, compare lanes 4 and 6 or lanes 5 and 7; supplemental Fig. S2, lanes 3 and 5). Purified Orc6 also bound to DS, but this binding did not require EBNA1 (Fig. 4, B and C, lanes 9 and 10), probably reflecting its intrinsic DNA binding activity with low sequence selectivity (46). However, such EBNA1-independent binding of Orc6 to DS did not lead to recruitment of Orc1–5 to DS (Fig. 4B, lanes 9 and 10). The addition of Orc6 did not significantly affect the amount of Orc1–5 or Cdc6 recruited to DS by EBNA1 (Fig. 4, B and C, compare lanes 2–8). Cdc6 was also recruited to DS by EBNA1 alone (Fig. 4B, lane 2). Orc1–5 slightly stimulated this recruitment (supplemental Fig. S2). These quantitative analyses establish that Cdc6 facilitates recruitment of Orc to EBNA1-bound DS.

**Purified Orc Is Recruited to DS in a Manner Dependent on the EBNA1-binding Sequences in the Presence of EBNA1**—We next examined the sequence specificity of Orc recruitment to DS in the purified system. To evaluate the requirement of EBNA1 binding sites, we prepared a mutant DS (DS-4AT) that carried a TA-to-AT substitution at the center of every four EBNA1-binding sites, we prepared a mutant DS (DS-4AT) that carried a TA-to-AT substitution at the center of every four EBNA1-binding sites in the presence of EBNA1 (Fig. 5A). The same mutation was reported to cause ~20-fold decrease in the binding affinity of EBNA1 to a single, 18-bp binding sequence (47). In our gel shift assay, however, the 4AT mutation resulted in only ~4-fold decrease in the binding affinity to DS (Fig. 5B), probably due to the presence of four EBNA1 binding sites on DS. In pull-down assays as well, less EBNA1 was pulled down with 4AT compared with the wild-type DS (Fig. 5C, compare lanes 4 and 5 with lanes 7 and 8). In accordance with the reduced EBNA1 binding, the amount of Orc pulled down decreased with the 4AT mutant (Fig. 5, D and E; compare lanes 3–5 with lanes 8–10). Orc was not pulled down on a negative control tetO9 DNA (Fig. 5, D and E, lanes 12–14). These results indicate that the interaction of Orc with DS depends on the EBNA1-binding sequences on DS.

**Cdc6 Directly Interacts with EBNA1 and Facilitates Orc Assembly on DS**—We next examined the protein interactions on DS by immunoprecipitation with anti-EBNA1 antibody. Of Orc1–5, Cdc6, and Orc6, none was pulled-down in the absence of EBNA1 (Fig. 6A, lane 14). In the presence of DS, Cdc6,
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A

B Pull-down with oriP (2.2 kb)-conjugated dynabeads (single-biotinylated)

C Pull-down with streptavidin-Sepharose beads
EBNA1- and Cdc6-mediated Association of Orc at oriP

EBNA1- and Cdc6-mediated Association of Orc at oriP

Orc1–5, and Orc6 on DS in an EBNA1-dependent manner. Orc6 depended more on Cdc6 than on Orc1–5 for its association with the EBNA1-DS complex, and this could be due to possible Cdc6-Orc6 interaction. Quantitative analyses revealed almost equimolar interaction between Cdc6 and an EBNA1 dimer, whereas up to one Orc1–5 complex could be recruited per two dimers of EBNA1 in the presence of DS (Fig. 6C).

The N-terminal Region of EBNA1 Is Necessary for Recruitment of Cdc6 and Orc—RNA has been implicated in recruitment of the pre-RC components to EBNA1/TRF2. Therefore, we next examined whether the observed interactions among DNA, EBNA1, Cdc6, and Orc may be mediated by RNA. We pretreated the purified proteins with RNase or DNase and conducted immunoprecipitation with anti-EBNA1 antibody (Fig. 7). GST-Cdc6 and EB90mbp323 were separately incubated with RNase A (Fig. 7, lanes 3 and 7), RNase T1, (Fig. 7, lanes 4 and 8), or DNase I (Fig. 7, lane 5) for 30 min at 37 °C before the binding assay. The amounts of the nucleases added were 50-fold more than that required to degrade 10 μg of RNA (for RNases) or 2 μg of DNA (for DNase I in the presence of 0.5 mM CaCl2) under the same condition (data not shown). The nuclease treatment only slightly diminished the interaction between Cdc6 and EB90mbp323 (Fig. 7, compare lane 2 with lanes 3–5). Moreover, pretreatment with RNases did not decrease the co-precipitated Cdc6 at all when a DS fragment was added in the binding reaction (Fig. 7, compare lane 6 with lanes 7 and 8). Thus, we concluded that the interaction between Cdc6 and EB90mbp323 is most likely direct, not mediated by RNA or DNA. When the DS fragment was present, the pretreatment with RNases did not affect Orc recruitment to EB90mbp323 either (Fig. 7, compare lanes 12 and 13), precluding the role of RNA in binding of Orc to EBNA1-DS in our system.

In order to obtain insight into the domain of EBNA1 required for recruitment of Cdc6 and Orc, we generated MBP-EB451, a variant of MBP-EBNA1 that bears the C-terminal DNA-binding/dimerization domain and an acidic tail region fused to the MBP tag at the N terminus (Fig. 1A). The MBP-EB451 was

FIGURE 4. Quantitative analyses of DS-bound proteins after release with DNase I digestion. A 428-bp DNA fragment (ARV) bearing DS was biotinylated at both ends and immobilized on streptavidin-Dynabeads. Then EB90mbp323 (11 pmol), Orc1–5 complex (0.6 pmol), His-Orc6 (2 pmol), and Cdc6 (1.8 pmol) were mixed with DNA-Dynabeads (2 pmol, DS) in the presence of an ATP-regenerating system plus 0.1 mg/ml poly(dI-dC) duplex. The beads were washed three times with 0.3 ml of Buffer GD containing 0.1 M NaCl, 0.05% Triton X-100, and 0.5 mM ATP. Then the Dynabeads were digested with DNase I. The DNase eluates were analyzed on SDS-PAGE followed by silver staining (A) or Western blotting (B). C, the detected bands were quantified using densitometric software (MultiGauge). The values were converted to the amount of respective proteins used in the entire reaction, and expressed in pmol on the bar graph.

Orc1–5, and Orc6 were efficiently co-immunoprecipitated with EBNA1 (Fig. 6A, lanes 8–13). As expected, the DS fragment was also precipitated with EBNA1 (Fig. 6A, lanes 8–13, biotin-DS). The addition of Cdc6 increased the amount of Orc1–5 coprecipitated with EBNA1 (Fig. 6, A and B, lanes 9 and 11), consistent with the results with immobilized DS (Fig. 5). Cdc6 is coimmunoprecipitated with EBNA1 in the absence of DNA or Orc, suggesting direct interaction between EBNA1 and Cdc6 (Fig. 6A, lane 1). Orc6 was precipitated with EBNA1 in the presence of a DS fragment, and Cdc6 significantly enhanced this interaction (Fig. 6, A and B, lanes 10–13). This stimulation was seen also in the absence of DNA, suggesting that Cdc6 may interact with Orc6. Thus, Cdc6 directly binds to EBNA1 and facilitates assembly of Orc1–5 and Orc6 on DS in an EBNA1-dependent manner. Orc6 depended more on Cdc6 than on Orc1–5 for its association with the EBNA1-DS complex, and this could be due to possible Cdc6-Orc6 interaction. Quantitative analyses revealed almost equimolar interaction between Cdc6 and an EBNA1 dimer, whereas up to one Orc1–5 complex could be recruited per two dimers of EBNA1 in the presence of DS (Fig. 6C).

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puriﬁed using amylose resin (Fig. 3A, second panel), and it could associate with a DS fragment as efﬁciently as EB90mbp323 (Fig. 5C, lanes 2 and 3). When MBP-EBΔ451 was used in place of EB90mbp323 for anti-EBNA1 immunoprecipitation, neither Cdc6 nor the Orc1–5 complex was co-precipitated even in the presence of the DS fragment (Fig. 7, lanes 9, 10, and 14). Therefore, recruitment of Cdc6 and Orc depends on the N-terminal 451 amino acids of EBNA1. The role of the N-terminal segment of EBNA1 in association of Orc with oriP is consistent with a previous report (28). This result also precludes the possibility that the MBP moiety attached to EBNA1 adventitiously facilitates the binding of Cdc6 and Orc.

Cooperative Action of Orc and Cdc6 Revealed by Exonuclease III Protection Assays—The above results indicate that Cdc6 and Orc associate with EBNA1-bound DS. In order to clarify the modes of interactions of these proteins with the DS fragment, we employed ExoIII protection assays. ExoIII digests one strand of duplex DNA in a 3’ to 5’ direction and has been used for detecting the outer limits of a protein binding site on dsDNA substrate (32, 33). When the 5’-end-labeled DS substrate (Fig. 8A) was challenged with ExoIII, EBNA1 (EB90mbp323) prevented ExoIII action at both outer edges of DS, generating intense “Exo-stop” bands (Fig. 8B, lane 3, arrows). In the absence of EBNA1, Orc and Cdc6 generated smeared ladders of faint stop bands on upper regions (Fig. 8B, lanes 2 and 7), suggesting weak nonspeciﬁc binding of Orc and Cdc6 to substrate DNA in the absence of competitor DNA. The addition of either pure Orc or Cdc6 with EBNA1 did not signiﬁcantly change the DNA ladder pattern generated by EBNA1 (Fig. 8B, lanes 4 and 6). In contrast, when both Orc and Cdc6 were simultaneously added with EBNA1, the intense Exo-stop band almost completely disappeared on both strands (Fig. 8B, lane 5). This suggests that Orc and Cdc6 provoked a characteristic inﬂuence on EBNA1-bound DS in a highly cooperative manner. Lack of Exo-stop indicates displacement of EBNA1 or blocks of ExoIII action near the end of DNA. The ﬁrst possibility is unlikely because the DNase I footprinting data indicate that the EBNA1 stays bound to DS regardless of the presence or absence of other proteins (see below). Thus, the results instead suggest gradual blocks of ExoIII action near the end of DNA. The absence of thick, discrete stop bands would be due to a fast off-rate or low sequence speciﬁcity of human Orc, consistent with previous reports (6). EBNA1 recruited the Orc2–5 core complex to oriP in vitro in the absence of either Orc1 or Orc6 (Fig. 3B). Therefore, we further examined whether Orc1 and Orc6 subunits are required for Orc to exhibit this cooperative effect in ExoIII assays (Fig. 8, C and D). The addition of Cdc6 diminished the Exo-stop signal in the presence of Orc1–5 alone (Fig. 8C, compare lanes 3 and 4). Cdc6-induced reduction of the Exo-stop signal was observed also with the combination of the Orc2–5 subcomplex and FLAG-Orc1 (Fig. 8D, compare lanes 3 and 5). Under this condition as well, Orc6 was dispensable for this effect of Cdc6 to be observed (Fig. 8D, compare lanes 5 and 6). In contrast, the EBNA1-protected Exo-stop band did not decrease in the presence of Cdc6 when only FLAG-Orc1 or Orc2–5 was present (Fig. 8D, lanes 7 and 8). Thus, both Orc1 and Orc2–5 are required for cooperative action with Cdc6 to generate speciﬁc protein-DNA structures on EBNA1-bound DS, whereas Orc6 is dispensable. The intensity of the Exo-stop band correlated with the amounts of both Orc1–5 and Cdc6 (supplemental Fig. S3, B and C, lanes 5–7 and lanes 7–9). We speculate that Cdc6 stabilizes the Orc-DNA complex on DS, which leads to distributed termination of ExoIII digestion within the regions ﬂanking the EBNA1 binding sites. Regardless of its true molecular mechanism, these experiments revealed that Orc and Cdc6 cooperatively acts on DS in a manner dependent on both Orc1 subunit and Orc2–5 subcomplex.

Puriﬁed Orc and Cdc6 Generate Localized Footprints on DS—In order to more precisely localize the binding sites of Orc and Cdc6 on DS, we employed DNase I protection assays. In a DNase I footprinting assay with an end-labeled DS fragment (AHF; Fig. 9A), EBNA1 (EB90mbp323) protected its four cognate sequences with some DNase I-sensitive sites at the boundary of the four motifs (Fig. 9B, lane 2). The addition of TRF2 protected the nonamer-a, -b, and -c sites (Fig. 9B, lanes 15 and 16), and the addition of EBNA1 and TRF2 generated some sensitive sites outside of DS as well as in the EBNA1 binding sites (E1 and E2) (Fig. 9B, lane 3). The simultaneous addition of Orc generated weak footprints on the regions ﬂanking both the nonamer-a and nonamer-c (Fig. 9B, compare lanes 4–6 with lane 3). This effect of Orc was enhanced and extended when Cdc6 was added (Fig. 9B, lanes 7–9, marked with green bars and solid and broken blue bars), although Cdc6 alone did not generate any protection in the ﬂanking segments even in the presence of EBNA1 (Fig. 9B, lanes 12–14). Generally, the effect of Orc plus Cdc6 was stronger near DS and became weaker at sites farther from DS (Fig. 9B, lanes 7–11). These features are also detected when pKS-ARV, a DS-containing circular plasmid, was used as the substrate (supplemental Fig. S4B), although the Orc-mediated protection was less apparent than on the linear substrate. In summary, these results suggest that Orc is loaded at the vicinity of DS in the presence of EBNA1. Cdc6 does not show any interaction with speciﬁc DS sequences on its own but facilitates the binding of Orc. The relatively weak protection from DNase I may indicate the highly mobile DNA binding, low sequence speciﬁcity, or quick off-rate. These results are consistent with the ExoIII protection data that show cooperative blocks of ExoIII action on the outside segment by Orc plus Cdc6 (Fig. 8).

FIGURE 5. Recruitment of the puriﬁed Orc1–5 complex depends on the EBNA1 binding sequences on DS. A, locations of base substitutions of the 4AT-mutant on DS. B, electrophoretic mobility shift assay on agarose gel. Fifty fmol of DS-containing DNA fragment (ARV) or its 4AT mutant (ARV-4AT) was challenged with ExoIII, EBNA1 (EB90mbp323) prevented ExoIII action at both outer edges of DS, generating intense “Exo-stop” bands (Fig. 8B, lane 3, arrows). In the absence of EBNA1, Orc and Cdc6 generated smeared ladders of faint stop bands on upper regions (Fig. 8B, lanes 2 and 7), suggesting weak nonspeciﬁc binding of Orc and Cdc6 to substrate DNA in the absence of competitor DNA. The addition of either purified Orc or Cdc6 with EBNA1 did not signiﬁcantly change the DNA ladder pattern generated by EBNA1 (Fig. 8B, lanes 4 and 6). In contrast, when both Orc and Cdc6 were simultaneously added with EBNA1, the intense Exo-stop band almost completely disappeared on both strands (Fig. 8B, lane 5). This suggests that Orc and Cdc6 provoked a characteristic inﬂuence on EBNA1-bound DS in a highly cooperative manner. Lack of Exo-stop indicates displacement of EBNA1 or blocks of ExoIII action near the end of DNA. The ﬁrst possibility is unlikely because the DNase I footprinting data indicate that the EBNA1 stays bound to DS regardless of the presence or absence of other proteins (see below). Thus, the results instead suggest gradual blocks of ExoIII action near the end of DNA. The absence of thick, discrete stop bands would be due to a fast off-rate or low sequence speciﬁcity of human Orc, consistent with previous reports (6). EBNA1 recruited the Orc2–5 core complex to oriP in vitro in the absence of either Orc1 or Orc6 (Fig. 3B). Therefore, we further examined whether Orc1 and Orc6 subunits are required for Orc to exhibit this cooperative effect in ExoIII assays (Fig. 8, C and D). The addition of Cdc6 diminished the Exo-stop signal in the presence of Orc1–5 alone (Fig. 8C, compare lanes 3 and 4). Cdc6-induced reduction of the Exo-stop signal was observed also with the combination of the Orc2–5 subcomplex and FLAG-Orc1 (Fig. 8D, compare lanes 3 and 5). Under this condition as well, Orc6 was dispensable for this effect of Cdc6 to be observed (Fig. 8D, compare lanes 5 and 6). In contrast, the EBNA1-protected Exo-stop band did not decrease in the presence of Cdc6 when only FLAG-Orc1 or Orc2–5 was present (Fig. 8D, lanes 7 and 8). Thus, both Orc1 and Orc2–5 are required for cooperative action with Cdc6 to generate speciﬁc protein-DNA structures on EBNA1-bound DS, whereas Orc6 is dispensable. The intensity of the Exo-stop band correlated with the amounts of both Orc1–5 and Cdc6 (supplemental Fig. S3, B and C, lanes 5–7 and lanes 7–9). We speculate that Cdc6 stabilizes the Orc-DNA complex on DS, which leads to distributed termination of ExoIII digestion within the regions ﬂanking the EBNA1 binding sites. Regardless of its true molecular mechanism, these experiments revealed that Orc and Cdc6 cooperatively acts on DS in a manner dependent on both Orc1 subunit and Orc2–5 subcomplex.
Orc and Cdc6 Do Not Cause Detectable Melting of Duplex DNA on or around DS but Suppress the EBNA1-induced KMnO4 Sensitivity—Bacterial initiator DnaA and archaeal Orc1/Cdc6 analogues have been reported to exhibit origin-specific DNA unwinding activity (48, 49). However, no unwinding activity has been ascribed to Orc or Cdc6 in eukaryotes. We addressed this possibility with the purified proteins. Duplex opening activity was first assayed by using nuclease P1, a single-
strand-specific endonuclease (50). However, Orc and/or Cdc6 did not significantly sensitize DS-containing plasmid to nuclease P1 even in the presence of EBNA1 (data not shown).

Next, we examined permanganate (KMnO₄) sensitivity (35, 51). Permanganate oxidizes the 5,6 double bond of unpaired thymine and is much more sensitive than nuclease P1 for detection of single-strandedness, although it also detects other structural alteration of DNA, such as distortion or bending of dsDNA (52, 53). The oxidized thymine can be localized by primer extension on the substrate DNA with the Klenow fragment because this DNA polymerase cannot progress over the KMnO₄-oxidized thymine. A control experiment successfully detected a T7 RNA polymerase-induced, melted site within the T7 promoter on a circular plasmid at 10 mM KMnO₄ (supplemental Fig. S5B).

EBNA1 induced remarkable KMnO₄ sensitivity at the 265th (bottom strand, EBNA1 site 1) and at the 201st (top strand, EBNA1 site 4) thymidine (Fig. 10A, lanes 2, arrows I and III), as reported previously (35, 51). We also noticed an additional, weaker sensitive site on the bottom strand of EBNA1 site 2 (Fig. 10A, left, lane 2, filled arrowhead II). However, Orc, Cdc6, or TRF2 failed to induce additional KMnO₄-sensitive sites within or near DS (Fig. 10A, lanes 3–9). Thus, little duplex opening or structural change that can be detected by this reagent was evoked by Orc, Cdc6, or TRF2. On the contrary, Orc decreased the EBNA1-induced KMnO₄ sensitivity at the above three sites (Fig. 10A, lanes 4–6 and 8, arrows I and III and filled arrowhead II). When the density of each band is divided by the average density of the upper ladder region (Fig. 10A, broken lines), the Orc-dependent decrease in signal intensities is more apparent (Fig. 10B, lanes 4–6). This protection suggests a possibility that Orc, in the presence of EBNA1, interacts with the DS segment spanning these nucleotides and protects them from reacting with MnO₄⁻. Alternatively, Orc might affect conformation of EBNA1-bound DS so as to diminish KMnO₄ sensitivity at these sites.

DISCUSSION

The enzymatic analyses of the processes of DNA replication depend on a faithful in vitro system reconstituted with purified proteins. Despite progress in yeast and Xenopus systems (45, 54, 55), the progress in enzymatic reconstitution of mammalian DNA replication has lagged behind. As was shown in the signifi-
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A

| ARV (201 bp) | Bam HI | Hind III | Eco RV |
|-------------|--------|----------|--------|
|             | a      | b        | c      |

B

| DS (113 bp) | Hind III | Eco RV |
|-------------|----------|--------|
|             | a        | b      |

C

| EBNA1 | Orc1-5 | His-Orc6 | Cdc6 |
|-------|--------|----------|------|
|       | +      | +        | +    |

D

| EBNA1 | FLAG-Orc1 | Orc2-5 | His-Orc6 | Cdc6 |
|-------|------------|--------|----------|------|
|       | +          | -      | -        | +    |

FIGURE 8. Exonuclease III protection analyses of protein-DNA interactions on the DS fragment: co-activity among Cdc6, Orc1, and Orc2–5 subcomplex on the EBNA1-bound DS. A, pKS-ARV carries the 201-bp sequence (from the EcoRV site at 8995 nt to the Alu site at 9195 nt on the EBV B95-8 genome) containing DS on the pBlueScript vector. The EBNA1 binding sites and the nonamer sequences are shown by four ovals (E1–E4) and by three triangles (α–c), respectively. B, a HindIII-XhoI (228-bp) fragment of pKS-ARV was labeled with 32P at one end (HindIII site (left) or XhoI site (right)). The end-labeled DS substrate was mixed with EB900bp323 (1.2 pmol), Orc (0.4 pmol of Orc1–5 + 0.6 pmol of His-Orc6), and Cdc6 (0.4 pmol) in the presence of an ATP-regenerating system. After incubation at 25 °C for 10 min and 37 °C for 5 min, ExoIII was added, and incubation was continued for 6 min at 37 °C. The digested DNA was purified and resolved on sequencing gels. Locations of the EBNA1 binding sites and nonamer sites are indicated by vertical lines (E1–E4) and by triangles (α–c), respectively. The full-length substrates are marked by open arrowheads, and intense Exo-stop bands are indicated by arrows. Orc/Cdc6-induced ladders of faint Exo-stop bands are indicated by a dotted line (left). Note that these experiments were conducted in the absence of competitor DNA because its presence inhibited the ExoIII action. Thus, non-specific binding of Orc to DNA would be expected under this condition. C and D, a 0.44-kbp DNA of pKS-AHF (Fig. 9A or supplemental Fig. S3A) was PCR-amplified with M13 primers and cleaved by XbaI. A 356-bp fragment was gel-purified and radiolabeled at the 5′-end with T4 kinase. The DS substrate was mixed with EB900bp323 (1.4 pmol), FLAG-Orc1 (0.2 pmol), Orc2–5 subcomplex (0.2 pmol), His-Orc6 (1.5 pmol), and/or Cdc6 (1.5 pmol) in the presence of an ATP-regenerating system. ExoIII digestion and electrophoresis were carried out as above. Only the Exo-stop bands are shown. In the lower panels, the intensities of EBNA1-induced Exo-stop bands were quantified and expressed as a ratio to the total substrate intensity.
EBNA1- and Cdc6-mediated Association of Orc at oriP

FIGURE 9. DNase I footprinting analyses of protein-DNA interactions on the DS fragment. A, pKS-AHF contains a 251-bp sequence (HinfI site at 8945 nt; AluI site at 9195 nt of the EBV B95-8 genome) carrying DS on the pBluescript vector. B, a 0.44-kbp DNA of pKS-AHF was PCR-amplified with M13 primers and cleaved by XhoI. A 354-bp fragment was gel-purified and radiolabeled at the 5′-end with T4 kinase. The DS substrate was mixed with indicated amounts of EB90mbp323, TRF2, Orc1–5 subcomplex, and/or Cdc6 in the presence of an ATP-regenerating system. The protein-bound DS substrate was digested by DNase I for 60 s, and the digestion was stopped by the addition of SDS and phenol/chloroform. After purification by ethanol precipitation, the purified DNA chains were denatured and resolved on sequencing gels. The exposed gel image is shown in low contrast (left) and in high contrast (right). Locations of EBNA1 binding sites and nonamer sites are indicated by vertical lines (E1–E4) and by triangles (a–c), respectively. Green and blue lines (solid and broken) show protected regions observed when Orc was added with EBNA1 and TRF2 (lanes 4–11). The addition of Cdc6 augmented the Orc-induced protection (lanes 7–11).

is consistent with the previous report that the tethered Cdc6 can recruit Orc and initiates DNA replication (64).

ExoIII and DNase I protection assays suggested the protein interaction domains on DS and provided further evidence for cooperation of Cdc6 and Orc in assembly at oriP. The EBNA1-induced strong Exo-stop band disappeared or diminished in the presence of both Cdc6 and Orc but not in the presence of either protein alone. Because EBNA1 stays bound to DS regardless of the presence or absence of Cdc6/Orc, it is most likely that binding of Orc at both sides of DS prevented the digestion by ExoIII at short distances from the ends of the DNA fragment. Indeed, a series of weak Exo-stop bands are detected at 20–30 bp from the end of the fragment (Fig. 8B, dotted line). Because both Cdc6 and Orc are required to prevent the ExoIII digestion, we speculate that Cdc6 stabilizes the DS-EBNA1-Orc complex so it can block the movement of ExoIII.

Similarly, DNase I protection assays also suggested the interaction of Cdc6/Orc with the segments outside the DS in the presence of EBNA1 (Fig. 9 and supplemental Fig. S4). In the presence of TRF2, the partially protected segments extended from the DS segment into both sides by more than 100 bp upon the addition of Orc, suggesting that Orc interacts with both sides of DS. The extent and strength of interaction are enhanced in the presence of Cdc6. Because Cdc6 alone cannot make this protection on either side of DS, it is likely that Orc is responsible for the extended protection. The protection is generally stronger near DS and becomes weaker at locations farther from DS, although the footprints are generally weaker compared with a strong protection shown by budding yeast Orc and/or Cdc6 (4, 59, 65). Rather weak protection may reflect the mobile nature of the complex, low sequence specificity, or high off-rate of the Orc binding.
TRF2 was reported to stimulate EBNA1-dependent recruitment of Orc to DS (25), but our pull-down assays indicated that TRF2 did not stimulate Orc association with immobilized oriP (Fig. 3B). However, TRF2 stimulated Orc footprints on DS (supplemental Fig. S4). This may be related to the fact that Orc2–5 (in the absence of Orc1) can be loaded onto DS in pull-down, whereas Orc1 is required for extended binding (as revealed by the ExoIII assay; Fig. 8D). TRF2 may facilitate Orc assembly through interacting with Orc1.

Although EBNA1 can induce localized melting within oriP, Cdc6 and/or Orc did not show any evidence for their ability to melt DNA within oriP. To the contrary, Orc partially suppressed the EBNA1-induced KMnO₄ sensitivity within DS, suggesting that Orc may somehow physically interact with the hypersensitive sites within DS or that Orc binding may induce conformational change of the DS-EBNA1 complex, which may lead to the reduced sensitivity to KMnO₄ (Fig. 10).

**Insight into Orc Assembly at Cellular Replication Origins**

We propose a potential model for Orc assembly at oriP (Fig. 11). Although we do not know the exact numbers of Orc assembled at oriP, our quantitative analyses of co-immunoprecipitation assays suggest a molar ratio of 1:2:2 among Orc, Cdc6, and an EBNA1 dimer assembled on DS (Figs. 6 and 7). This stoichiometry forces us to speculate some structural changes of DS and/or its flanking regions upon recruitment of Cdc6 and Orc (Fig. 11). In this model, a 1:1 complex between Cdc6 and an EBNA1 dimer may play a crucial role in recruiting Orc. This model could be extended to Orc assembly at specific sites on eukaryotic chromosomes. A transcription factor with a site-specific DNA binding activity has been implicated in the site-specific assembly of Orc. This possibility was previously proposed at the rat aldolase promoter carrying AIF-C binding sites and other sites (66, 67). More recently, Cdc6 was reported to bind to the E-cadherin promoter region (E-box sequences) and
Association of Cdc6 with EBNA1 and recruitment of Cdc6 at oriP

FIGURE 11. A model for recruitment of Orc at EBV oriP. EBNA1 directly binds to Cdc6 (with a 1:1 ratio) and brings it to DS. Recruitment of Orc to oriP is facilitated by the presence of Cdc6, although Orc can be recruited to oriP by EBNA1 alone with less efficiency (not shown in the figure). Association of Orc with oriP strictly depends on the presence of DNA bearing EBNA1 binding sites. The DS segment is shown in thicker blue lines, whereas the flanking segments are shown in thinner blue lines. Permanganate sensitivity is detected in EBNA1-bound DS (red arrows), but it is reduced in the presence of Orc (pink arrows), reflecting Orc binding near the sensitive sites or some conformational change caused by Orc binding. Orc probably interacts with the DNA segments flanking DS, and this binding may be more stable in the presence of Cdc6, resulting in protection of the flanking region from DNase attack. This cooperative binding may also cause the ExoIII digestion to halt at the outer edges of the Orc-bound segment. Although only one molecule of Orc is shown to associate with each flanking segment (on the basis of the quantitation from Figs. 6 and 7), more Orc complexes might be recruited. At the cellular replication origins, a transcription factor or other chromatin factor may play a role similar to that played by EBNA1 in recruitment of Cdc6 and Orc.

to suppress transcription and stimulate replication initiation (68). Thus, transcription factors may facilitate Orc recruitment at specific chromosome sites through direct interaction with Cdc6.

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