

## Identification of New Human Origins of DNA Replication by an Origin-Trapping Assay<sup>∇†</sup>

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**Metazoan genomes contain thousands of replication origins, but only a limited number have been characterized so far. We developed a two-step origin-trapping assay in which human chromatin fragments associated with origin recognition complex (ORC) in vivo were first enriched by chromatin immunoprecipitation. In a second step, these fragments were screened for transient replication competence in a plasmid-based assay utilizing the Epstein-Barr virus latent origin *oriP*. *oriP* contains two elements, an origin (dyad symmetry element [DS]) and the family of repeats, that when associated with the viral protein EBNA1 facilitate extrachromosomal stability. Insertion of the ORC-binding human DNA fragments in *oriP* plasmids in place of DS enabled us to screen functionally for their abilities to restore replication. Using the origin-trapping assay, we isolated and characterized five previously unknown human origins. The assay was validated with nascent strand abundance assays that confirm these origins as active initiation sites in their native chromosomal contexts. Furthermore, ORC and MCM2-7 components localized at these origins during G<sub>1</sub> phase of the cell cycle but were not detected during mitosis. This finding extends the current understanding of origin-ORC dynamics by suggesting that replication origins must be reestablished during the early stages of each cell division cycle and that ORC itself participates in this process.**

Transmission of genetic information from one cell generation to the next requires the accurate duplication of the genome. In bacteria, DNA replication initiates at a defined start site, the replicator, which is recognized and bound by an initiator protein (24). The first eukaryotic origins of replication were identified in *Saccharomyces cerevisiae*. In this unicellular organism, there are well-defined genetic elements that support the extrachromosomal replication of plasmids and were therefore called autonomously replicating sequences (ARS). These replication origins contain an 11-bp consensus sequence that is recognized by the origin recognition complex (ORC) (8, 52, 57).

However, in higher eukaryotes, replication origins are more difficult to define because of the increased complexity of these organisms and their genomes. Indeed, the existence of genetically defined replicator elements in higher eukaryotes is still debatable, since chromatin structure and epigenetic features are of fundamental importance for origin selection and activity (4, 9, 15, 19, 41). Many genomic sites can be used as replication origins when placed under appropriate conditions. In *Xenopus* and *Drosophila* embryos, any DNA sequence will efficiently replicate once per cell cycle up to the blastula stage of development when microinjected into embryos or introduced into egg extracts (20, 23, 67). We are only beginning to understand how, for example, higher-order chromatin organization influences origin organization and activity or how the genetic and

epigenetic environment determines origin usage (37, 58). To elucidate how these processes are linked to and regulate replication initiation, it is necessary to identify and characterize DNA sequences that serve as replication origins in higher eukaryotes.

Results of biochemical fractionation, as well as genetic and physical studies, demonstrate that replication in higher eukaryotes initiates at specified start sites (10, 19). In support of genetically defined replicators in higher eukaryotic genomes, specific DNA fragments that contain the dihydrofolate reductase (DHFR),  $\beta$ -globin, *c-myc*, and lamin B2 start sites function as replicators at ectopic locations (15). As for that in budding yeast, ORC binding has been mapped to specific sites in mammalian chromosomes that colocalize with replication start sites (1, 6, 29, 35, 64). Mammalian genomes are thought to contain several thousand replication start sites (26, 39, 40, 56), some of which are hypothesized to colocalize with replicator elements, but the lack of a generally applicable and functional screening assay has hampered their identification and the definition of their characteristic features (63, 64).

Different types of general strategies to identify mammalian replicators have been developed. Early attempts to identify mammalian origins via their presumed ARS activity met with limited success due to poor autonomous replication activity and loss of small plasmids from the cells (19). To alleviate the problem of plasmid loss, Krysan and colleagues screened random mammalian DNA fragments for origin activity by cloning them into a replication-defective Epstein-Barr virus (EBV) *oriP* plasmid (33). Replication of wild-type *oriP* plasmids occurs once per cell cycle in human cells under the control of cellular replication-licensing factors and depends on two *cis*-acting components (11, 61, 68). One, the family of repeats (FR), is responsible for plasmid maintenance during cell divi-

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sion. The other, the dyad symmetry element (DS), serves as a replicator that through its interaction with the *oriP*-binding factor EBNA1 recruits ORC to DS (16, 61). Additional regulation of *oriP* replication occurs through nucleosome remodeling and covalent histone modifications (70, 71). Thus, random human fragments inserted into an *oriP* plasmid lacking DS were expected to replicate in human cells if the fragment served as a replicator. Indeed, Krysan and colleagues demonstrated autonomous replication of some plasmids in mammalian cells over a period of several months under selection (32, 34). Only human fragments of 6 kb or greater displayed replication activity in this assay, and activity-conferring features of the sequences other than their lengths were not identified (21). In recent years it has become clear that epigenetic processes, which are independent of replication competence, are involved in establishing long-term maintenance of plasmids (36). Currently, it is unknown to what extent DNA sequence contributes to the efficiency of establishment.

In a third general approach, a library of potential human DNA origins was prepared by cloning ORC-binding DNA fragments enriched by chromatin immunoprecipitation (ChIP). Origins located upstream of the *Mcm4* and *Top1* genes were identified using this approach (29). However, the effectiveness of this approach is currently uncertain because the efficiency and enrichment of the coprecipitated DNA fragments were not quantified (29). Moreover, ORC subunits may bind *in vivo* to regions of chromatin that serve other functions. For example, the Orc2 protein (Orc2p) has been shown to associate with nonorigin elements, such as heterochromatin, centrosomes, and centromeres (50).

To isolate new origins, we developed a two-step origin-trapping assay suitable to screen for potential replicators in human chromatin. We first used ChIP to enrich for DNA sequences bound *in vivo* by a subunit of ORC. In the second step, we selected for their ability to rescue transient replication of a replication-defective EBV *oriP* plasmid. This two-step origin-trapping assay differs from that used in the early *oriP*-based studies (32, 34) in that the human DNA fragments cloned into *oriP* plasmids are not random but rather are enriched for their ability to bind ORC *in vivo*. To minimize the contributions of epigenetic processes, the period of selection for *oriP* plasmid maintenance was comparatively brief. This new origin-trapping strategy also enhances the effectiveness of the ORC-binding library approach (29) by following it with a plasmid-based replication screen.

In this initial report we describe five new human origins of replication that were identified by the origin-trapping assay. We present evidence that the assay is a stringent screen that allows the unbiased identification of sequences that serve as replication start sites in their native chromosomal context. Finally, we show that the association of replication-licensing proteins with these sites is cell cycle regulated and that Orc subunits are largely absent from origins during mitosis.

#### MATERIALS AND METHODS

**Plasmids.** p2832 is a 12.1-kb *oriP* plasmid containing *oriP*, enhanced green fluorescent protein (GFP), and the puromycin selection marker. p2932 ( $\Delta$ DS) was obtained by deleting the DS element between BsaBI and HpaI sites and replacing it with an AscI site containing a linker element. In addition, a 2.4-kb BamHI fragment containing a 3' long terminal repeat was removed, resulting in

a 9.4-kb plasmid. DNA fragments isolated by ChIP were ligated to the AscI linker, amplified, and cloned into plasmid p2932. The lamin B2 fragment was cloned by PCR from plasmid IRALp962B012Q2 (RZPD) with an AscI-linked primer and cloned into p2932. All plasmid DNA sequences are available upon request.

**Cell culture.** The Burkitts lymphoma cell line A39 (61) and the semiaherent embryonic kidney cell line HEK293/EBNA1 (36) were cultured in RPMI medium (Invitrogen) supplemented with 10% fetal calf serum and 220  $\mu$ g/ml G418 (HEK293/EBNA1). HEK293 cells were drug selected with 80  $\mu$ g/ml hygromycin or 200 ng/ml puromycin. The 143/EBNA1 line was generated from the fibroblast cell line 143 (ATCC CRL-7092) (69) and maintained in Dulbecco's modified Eagle's minimal medium (Invitrogen) supplemented with 10% fetal calf serum and 600  $\mu$ g/ml G418. Adherent HeLa cells (ATCC CCL116) were grown in Dulbecco's modified Eagle's minimal medium (Invitrogen) containing 10% fetal calf serum. For G<sub>1</sub>/S synchronization, the cells were subjected to a double thymidine block (54). They were first arrested by a single thymidine block for 15.5 h and then released for 9 h and blocked again with 2.2 mM thymidine for 15 h. For synchronization in mitosis, cells were treated with 40 ng/ml nocodazole for 9 h and isolated by mitotic shake off. Synchronization was controlled by flow cytometry using standard procedures.

**ChIP cloning.** For cloning of Orc2p-coprecipitated DNA fragments, 10 immunoprecipitations with  $1 \times 10^8$  cells were performed in parallel from A39 cells and isolated as described below. To generate blunt ends, the purified ChIP sequences were pooled, dissolved in 100  $\mu$ l of buffer (50 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 10 mM Tris-HCl [pH 7.9]), and treated with 2 U Klenow enzyme for 1 h. After enzyme inactivation and ethanol precipitation, the DNA fragments were ligated to 10 pmol of annealed AscI linker with 5 U T4 DNA ligase (50- $\mu$ l volume, overnight at 14°C). For PCR amplification, the ligated DNA was ethanol precipitated and dissolved in 20  $\mu$ l water. PCR was performed with 10 pmol AscI PCR oligonucleotides by use of 1 U TaqPlus precision polymerase (Stratagene) in 100  $\mu$ l by adding 2% dimethyl sulfoxide (25 cycles of 1 min at 94°C, 1 min at 64°C, and 2 min at 72°C). PCR products of between 200 and 1,000 bp were isolated from a 0.8% Tris-acetate-EDTA agarose gel and purified (gel extraction kit; QIAGEN). One microgram of amplified DNA fragments was digested with 2 U AscI (100  $\mu$ l) and separated from the nucleotides (PCR purification kit; QIAGEN). These inserts were ligated with 1 U T4 DNA ligase in five parallel samples of 20  $\mu$ l overnight into AscI-digested p2932.

**Chromatin immunoprecipitation.** Chromatin was prepared for immunoprecipitation exactly as described previously (55). For cloning of Orc2p-coprecipitated DNA fragments, 10 immunoprecipitations with  $1 \times 10^8$  cells were performed in parallel from A39 cells. The enrichment of specifically Orc2p-bound chromatin was determined on the mini-EBV episome by use of the PCR fragment p4.2 (sc6) and the reference I3 (55).

ChIP experiments with HeLa cells were performed essentially as described previously (60). Briefly,  $1 \times 10^8$  HeLa cells were washed with phosphate-buffered saline (PBS) and treated with 1% formaldehyde in prewarmed medium for 4 min at 37°C. To prepare nuclei, cells were resuspended in hypotonic RSB buffer (10 mM Tris-HCl [pH 8.0], 3 mM MgCl<sub>2</sub>), incubated for 10 min on ice, and disrupted by Dounce homogenization. Nucleoprotein was washed and resuspended in NSB buffer (10 mM NaCl, 10 mM Tris-HCl [pH 8.0], 0.1% NP-40, 1 mM EDTA). After centrifugation through a sucrose cushion, immunoprecipitation was performed as described previously (55). Ten micrograms of affinity-purified polyclonal Orc2 antibody or an equivalent amount of preimmune serum was added to 500  $\mu$ g chromatin-DNA. Coprecipitated DNA was analyzed by real-time PCR (LightCycler) according to the manufacturer's instructions by use of the parameters and primer pairs described previously (55, 61).

**Replication assay.** Plasmids (2  $\mu$ g) were transfected into human embryonic kidney cell line HEK293/EBNA1 or human osteosarcoma cell line 143 cells stably expressing EBNA1, by using Polyfect (QIAGEN) according to the manufacturer's instructions. One day posttransfection, the cells were transferred to 15-cm plates. The transfection efficiency was determined by counting GFP-positive cells. Only transfections with comparable efficiencies were taken in account. For long-term replication assays, cells were subjected to puromycin selection (180 ng/ml) for 2 to 3 weeks. The plasmids were extracted by the method of Hirt (22) as follows. Cells were washed on the plate with TEN (10 mM Tris [pH 8.0], 2 mM EDTA, 100 mM NaCl), covered with 1.5 ml TEN, and lysed with an equal volume of 200 mM NaOH, 1% sodium dodecyl sulfate. After neutralization with 750  $\mu$ l 5 M NaCl, the lysate was kept on ice for 4 to 5 h or overnight and centrifuged at 15,300 rpm for 30 min at 4°C. The supernatant was extracted twice with phenol-chloroform, and the DNA was precipitated with isopropanol. After dissolving in TE, 500 ng of each DNA sample extracted from a single transfection was digested for plasmid rescue with DpnI and electropo-

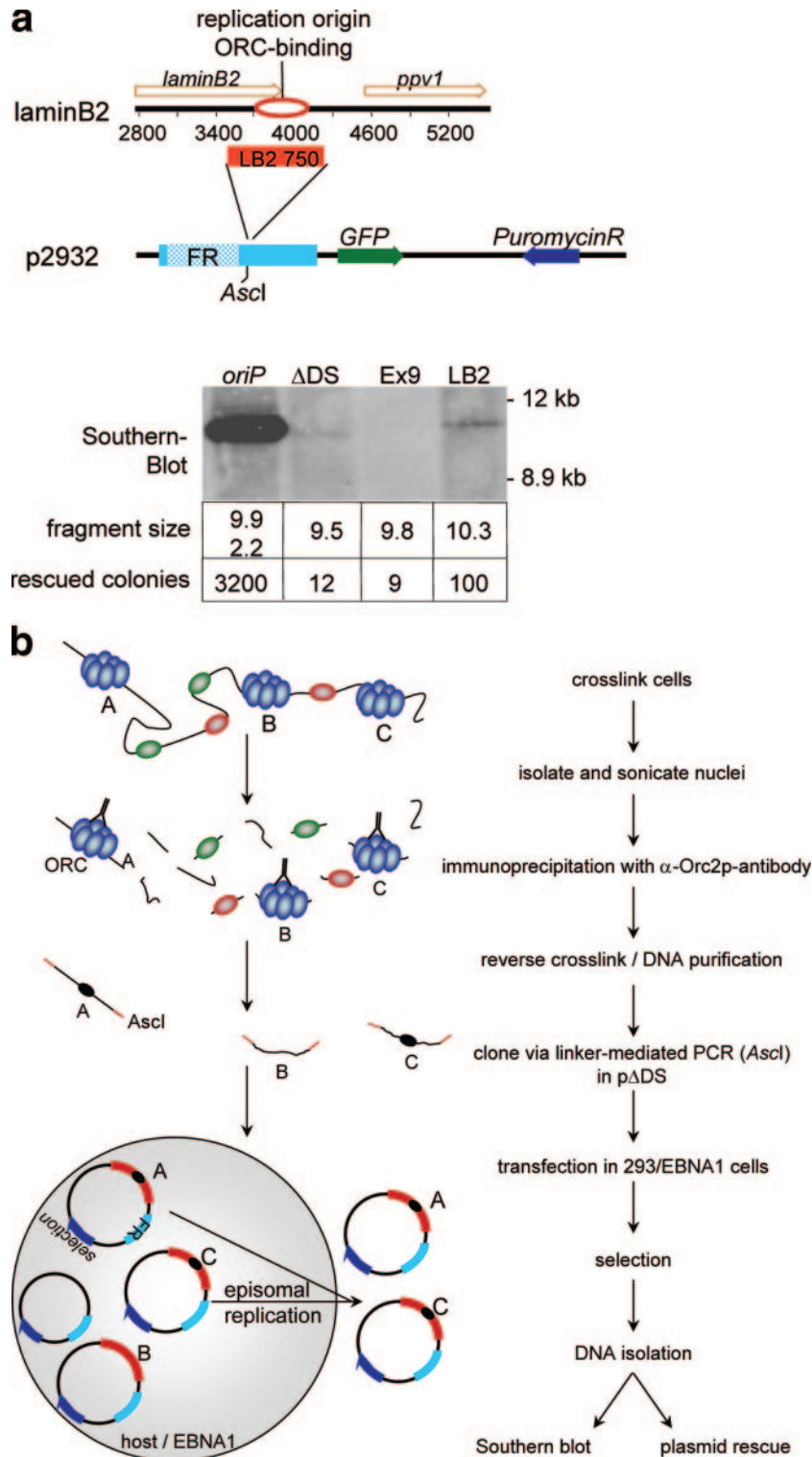


FIG. 1. Establishment of the origin-trapping assay. (a) Schematic representation of test replicators and organization of the lamin B2 locus. The lamin B2 origin is located in the 3' region of the lamin B2 gene (top). The basic vector  $\Delta$ DS contains the maintenance element FR of EBV, the GFP and puromycin genes, and an Ascl site used for cloning of Orc2p-binding fragments. The lamin B2 origin (*ori*LB2; positions 3495 to 4267 of the gene under accession no. M94363) was cloned in place of the DS element by use of the Ascl site. Similarly, the exon9 fragment (Ex9) located near the Mcm4 origin (positions 6342 to 6729, accession no. U63630) was inserted. These plasmids were transfected into HEK293/EBNA1 cells and analyzed for transient replication competence by Southern blotting and plasmid rescuing. For Southern blotting, plasmids were digested with BamHI and DpnI. The sizes of the BamHI fragments are indicated below each lane of the blot. The number of *E. coli* colonies obtained after transfection of 500 ng Hirt-extracted DNA from an exemplary experiment with each plasmid is also indicated. At least three experiments were performed for each analyzed origin, and results are summarized in Table 1. (b) Schematic diagram of the origin-trapping assay (see Results for details).

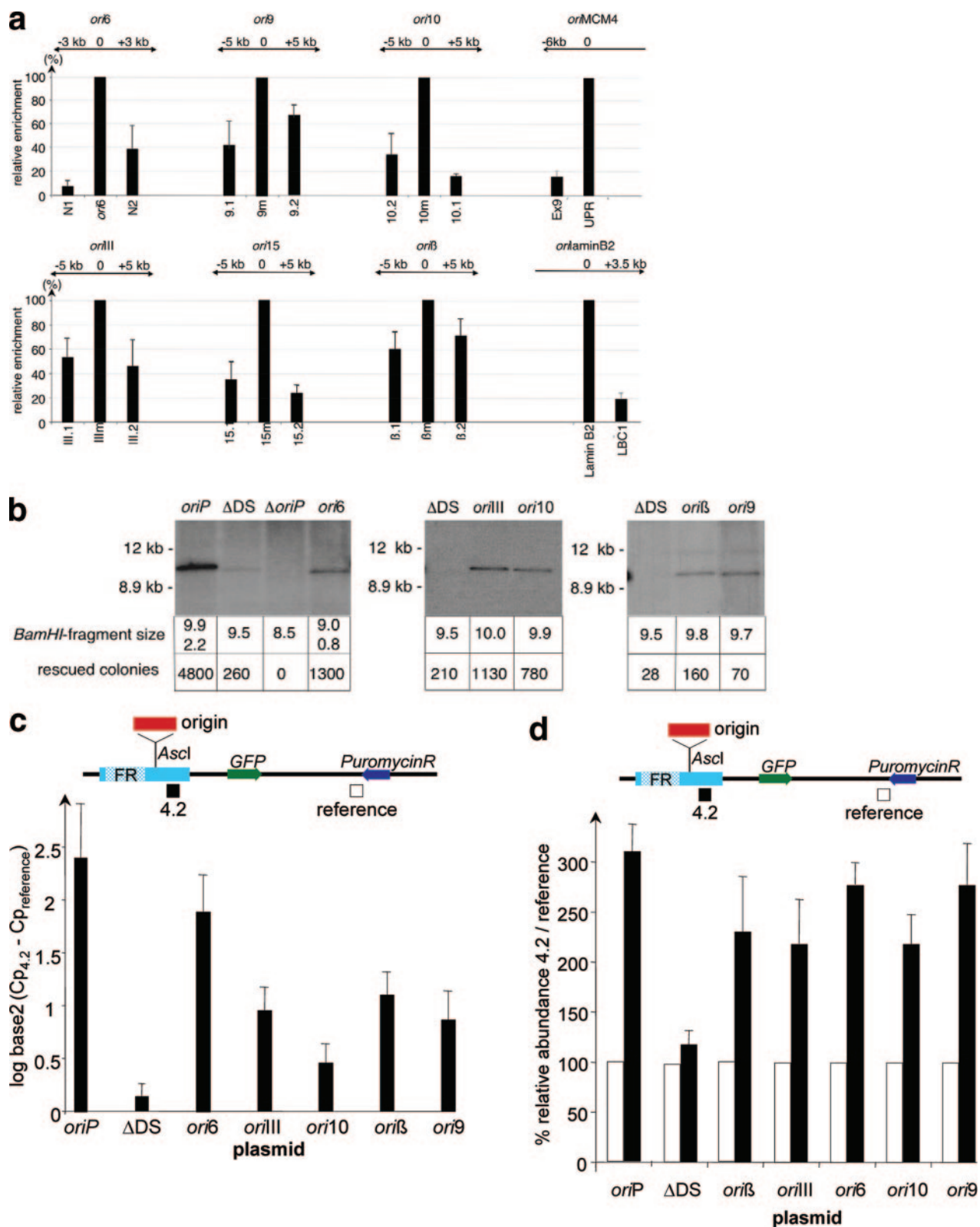


FIG. 2. Confirmation of potential human replicators. (a) The abundance of sequences corresponding to the potential human origins was quantified for nascent genomic DNA (800 to 1,300 nucleotide fragments) prepared from  $1 \times 10^7$  A39 cells. For each potential replicator, we designed primer pairs located 5 kb and 3 kb down- and upstream, respectively, of the origin region. The putative initiation site is located in the middle primer set (m), and this center bar was defined as 100%. The abundance at the flanking regions was calculated relative to that at the potential origin site. The MCM4, lamin B2, and hypoxanthine phosphoribosyltransferase origins (data not shown) served as internal positive controls for each preparation with primer pairs previously described (13, 18, 35). The name of each element is indicated at the top of each

rated into DH10B bacteria (Invitrogen). To linearize plasmids for Southern blot analysis, 2  $\mu$ g of Hirt DNA was incubated with DpnI and BamHI. DNA was subjected to electrophoresis on 0.8% Tris-acetate-EDTA gels and transferred to a nitrocellulose membrane. The membrane was hybridized to  $^{32}$ P-labeled p2832. For short-term replication experiments, transfected cells were grown for 4 days without selection. Plasmid DNA was enriched as described above, and 500 ng of each transfected sample was digested with DpnI. To monitor the DpnI digestion, one nontransfected sample was spiked with 500 ng pUC. The samples were transfected into DH10B bacteria by electroporation. To measure the total DNA recovered as reference, 5 ng DNA not digested with DpnI was electroporated in parallel.

**Nascent strand analysis.** Nascent strand analysis was performed as described previously (28). HEK293/EBNA1 or A39 cells ( $2 \times 10^7$ ) were washed with PBS and resuspended in PBS with 10% glycerol. Cells were lysed for 10 min in slots of a 1.2% alkaline agarose gel (50 mM NaOH, 1 mM EDTA). Afterwards, DNA was separated by electrophoresis overnight (low-melting-temperature agarose; Biozym). Fragments of from 0.8 to 1.3 kb were extracted from the gel by use of a QIAquick gel extraction kit (QIAGEN). Nascent strand abundance was determined by quantitative real-time PCR.

**Separation of BrdU-substituted DNA.** HEK293/EBNA1 cells transfected with the different replicator plasmids were drug selected for 2 weeks and then transferred for 18 h to a cell culture medium containing 5-bromodeoxyuridine (BrdU; 10  $\mu$ M) to increase the buoyant density of replicated DNA for CsCl equilibrium gradient centrifugation. Total DNA was isolated from  $1 \times 10^7$  cells and digested with the restriction enzymes BamHI and DpnI. CsCl gradients were adjusted to a refractive index of 1.403 and spun in a Beckman SW41 rotor at 40,000 rpm for 48 h. Fractions of 250  $\mu$ l were collected, and their refractive indexes were determined. The content of bulk genomic DNA was determined with a fluorometer (Beckmann). Samples were diluted, precipitated, and further analyzed by quantitative PCR.

**Primers.** All primers used in this study are summarized in Table S3 in the supplemental material and are also available on <http://haema143.gsf.de/>.

## RESULTS

**Establishment of a plasmid replication assay.** The goal of the origin-trapping assay was to design a functional tool capable of effectively identifying active origins. We made use of the latent origin of replication of Epstein-Barr virus (*oriP*), a replicator that supports extrachromosomal replication of plasmids once per cell cycle in an ORC-dependent manner. We reasoned that since ORC-binding sites colocalize with chromosomal replication start sites, human ORC-binding sequences might confer plasmid DNA replication activity in human cell lines on *oriP* plasmids lacking the viral replicator element DS. If so, a plasmid replication assay might be suitable as a functional screen for origin trapping.

To assess the feasibility of this idea, a 750-bp fragment of the human lamin B2 origin (*oriLB2*) that binds to ORC in vivo (18) and the control fragment (Ex9) located in exon 9 of the *Mcm4* gene, 6.5 kb upstream of the *Mcm4* origin (*oriMcm4*) (35). The exon9 fragment does not bind ORC and was cloned into an *oriP* plasmid lacking the DS origin but retaining the viral maintenance element FR (Fig. 1a). These plasmids were separately transfected into HEK293 cells expressing the viral protein EBNA1, which binds to FR and DS (36, 69). After 2 weeks of antibiotic selection, the amount of plasmid maintained in each transfected culture was determined by two methods, Southern blot hybridization and plasmid rescue (Fig. 1a). The high signal intensity in Southern blots and the large number of colonies obtained by plasmid rescue indicated efficient replication of the positive control *oriP* plasmid, while the negative control  $\Delta$ DS plasmid yielded only a faint signal in Southern blots and few colonies by plasmid rescue. The plasmid bearing the 750-bp *oriLB2* fragment reproducibly yielded colony numbers up to eightfold greater than the  $\Delta$ DS plasmid did. In contrast, only residual replication activity similar to that of  $\Delta$ DS was observed with the plasmid containing the nonorigin fragment exon9 (Fig. 1a). These results indicate that an ORC-binding human DNA fragment conferred transient replication activity on the  $\Delta$ DS plasmid, albeit with an activity lower than that of the wild-type *oriP*.

**Orc2p-binding elements support replication of  $\Delta$ DS plasmid DNA.** The origin-trapping assay combines a ChIP protocol and a plasmid replication assay (Fig. 1b). To prepare a library of potential human replicators, we enriched Orc2p-binding fragments from the established human mini-EBV cell line A39 by ChIP cloning (61). Cross-linked chromatin fragments containing DNA of 1 kb or less were generated by sonication and partial digestion with micrococcal nuclease. Chromatin fragments were immunoprecipitated with antibodies directed against the human Orc2p, and the precipitates were pooled after the precipitation step. To assess the specificity of this approach, we determined the *oriP*-specific enrichment of Orc2p as an internal control. Using primer set 4.2, which binds near the *oriP* origin, DS, we measured at least a 10-fold enrichment of Orc2-bound DNA relative to what was seen for a distal reference fragment (data not shown) (55). Coprecipi-

histogram, which depicts the average and standard deviation of at least three independent experiments. The middle primer set (m) defines the isolated origins (*ori6*, *ori9*, *ori10*, *oriIII*, *ori15*, and *ori $\beta$* ). The primer sets on the left and right define reference fragments. UPR, PCR fragment spanning *oriMCM4*; N1 and N2, reference fragments of *ori6*; LBC1, control fragment of *oriLB2*. (b) Each plasmid bearing a human Orc2-binding sequence was transfected individually into HEK293/EBNA1 cells, and transient replication was evaluated after 2 weeks by plasmid rescue analysis and Southern blotting. The size of the DNA fragment obtained after BamHI digestion is indicated below each lane of the blot. The number of *E. coli* colonies rescued in the same experiment is indicated below. The result of one exemplary experiment is shown. At least three experiments were performed for each analyzed origin, and results are summarized in Table 1. Similar results were obtained for the human osteosarcoma cell line 143 stably expressing EBNA1 (data not shown). (c) ChIP experiments with HEK293/EBNA1 cells were performed to measure Orc2p association at the potential human replicators inserted at the *AscI* site relative to that at a distal sequence in the plasmid. Relevant features of the replicator plasmids are diagrammed at the top. The primer set 4.2, near the *AscI* site, and the reference primer set, located at the puromycin gene, are depicted below. The site-specific enrichment ( $\Delta C_p = C_p$  at 4.2 -  $C_p$  at reference) of Orc2p was calculated on a logarithmic scale (log base 2) as the difference between the primer locus 4.2 and the reference. The averages and standard deviations of triplicates are shown. (d) Nascent strand abundance at primer set 4.2 and at the reference primer set was analyzed 4 days after plasmid transfection into HEK293/EBNA1 cells. The histogram shows the abundance of fragment 4.2 relative to that of the reference fragment. The number of nascent strand molecules was first determined by a standard curve for the amplified regions. All plasmids except the negative control,  $\Delta$ DS, had similar amplification rates. The number of molecules determined for the reference was set at 100% (white bars). The black bars show the ratios between the abundance of the reference and those of the 4.2 sequences in nascent DNA. The graph shows the mean values and standard deviations, indicated by error bars, from three independent experiments.

TABLE 1. Summary of results for human replicators in plasmid context<sup>a</sup>

Origin plasmid	Value after 2-wk selection		Avg test plasmid/ $\Delta$ DS plasmid ratio after 4 days (no selection)	Extrachromosomal maintenance for 6 wk (under selection)	Relative site-specific enrichment ratio for <sup>c</sup> :	
	Avg no. of colonies rescued (no. of expts <sup>b</sup> )	Avg test plasmid/ $\Delta$ DS plasmid ratio			Orc2 binding	Nascent strand
<i>oriP</i>	3,828 $\pm$ 445 (7)	21.6	20.4	Yes	5.28	3.08 $\pm$ 0.3
$\Delta$ DS	177 $\pm$ 68 (7)	1	1	No	1.14	1.18 $\pm$ 0.15
<i>oriLB2</i>	712 $\pm$ 246 (3)	4.0	ND	No	ND	ND
<i>ori6</i>	577 $\pm$ 20 (3)	3.3	6	No	3.81	2.27 $\pm$ 0.21
<i>ori10</i>	600 $\pm$ 224 (3)	3.4	2.2	No	1.38	2.11 $\pm$ 0.31
<i>ori<math>\beta</math></i>	457 $\pm$ 161 (3)	2.6	4.5	No	2.2	2.25 $\pm$ 0.5
<i>oriIII</i>	773 $\pm$ 248 (3)	4.4	5.4	No	1.91	2.11 $\pm$ 0.47
<i>ori9</i>	289 $\pm$ 75 (3)	2.3	2.4	No	1.81	2.27 $\pm$ 0.4

<sup>a</sup> The replication and maintenance competences of the individual plasmids were tested in transient replication assays. Exemplary results thereof are shown in Fig. 1a and 2b. Colony numbers were normalized to the absolute amount of extracted DNA by calculating the ratio between 500 ng of DpnI-treated and 5 ng of untreated DNA sample. ND, not determined.

<sup>b</sup> At least three independent experiments were performed with each plasmid.

<sup>c</sup> The Orc2 binding and the start site mapping data indicate the enrichment (*n*-fold) of the origin-proximal PCR fragment 4.2 versus the reference. Each experiment was performed in triplicate.

tated Orc2p-binding fragments of 200 to 1,000 bp were size selected, amplified by AscI linker-mediated PCR, purified, and cloned into the  $\Delta$ DS plasmid by use of the AscI site present in the inserted linker. This library was then screened to identify  $\Delta$ DS plasmids bearing human DNA elements able to support extrachromosomal replication.

Plasmids that preferentially contained 200- to 500-bp inserts were tested in pools of four to six origin candidates for transient replication activity in HEK293 cells stably expressing EBNA1. This cell line was chosen because it is easily transfected with high efficiency and is established as a cell line that supports *oriP*-dependent replication (36). After 2 weeks of selection, plasmids that had been extrachromosomally maintained were rescued back into *Escherichia coli* and analyzed by restriction enzyme digestion (see Table S1 in the supplemental material). Two readouts reflect the stringency of this replication assay: (i) the number of colonies obtained after transformation into *E. coli* compared to that obtained with  $\Delta$ DS and (ii) the enrichment of a dominant candidate fragment in each pool. In 10 out of 20 pools, up to fourfold more colonies were obtained with plasmids bearing a human DNA fragment than with the negative control  $\Delta$ DS. Most replication-competent pools contained one predominant clone (see Table S1 in the supplemental material).

**Potential origins of replication serve as chromosomal initiation sites.** To test whether the isolated sequences were indeed active origins of replication, we measured in the mini-EBV cell line A39 and in HEK293/EBNA1 cells their initiation activities in their native chromosomal context with the nascent strand abundance assay (5, 13, 35). Nascent strand abundance at the hypoxanthine phosphoribosyltransferase, MCM4, and lamin B2 origins was measured as a positive control (Fig. 2a and data not shown) (13, 18, 35). A panel of primer pairs was generated for each potential replicator, encompassing up- and downstream elements of the corresponding chromosomal regions (for chromosomal locations and primers, see Tables S1 and S2 in the supplemental material). The relative amount of nascent strand DNA in the neighboring regions was determined and compared with that at the potential origin, set as 100%. Nascent DNA enrichment ranging from 10 to 75% at the distal regions was determined for the six potential origins compared

to the reference locus (Fig. 2a). Measurements of nascent strand abundance at these loci in the HEK293/EBNA1 cell line confirmed that all six trapped sequences reside at active chromosomal replication origins (data not shown).

To assess the effectiveness of the origin-trapping assay in enriching for putative origin fragments, we compared the chromosomal replication initiation activity of the six replicator candidates shown in Fig. 2a with that of one of the DNA sequences in groups 3 and 7, which contained no obvious origin candidates in the transient replication screen (see Table S1 in the supplemental material). None of these sequences showed any chromosomal initiation activity (data not shown). These findings indicate that the origin-trapping assay selected quite effectively for active chromosomal replication origins.

**Replicators support extrachromosomal replication but not long-term establishment.** To further characterize the novel origins as replicators, we reexamined them individually in plasmid replication assays after 2 weeks of drug selection. Five of the six plasmids with potential origin sequences generated colony numbers similar to those obtained with a plasmid containing *oriLB2*; these colony numbers were 2.3- to 4.4-fold higher than those obtained with  $\Delta$ DS (Fig. 2b; Table 1). The remaining candidate, *ori15*, did not support significant plasmid replication activity and was therefore not further analyzed (data not shown). We confirmed the results of the replication assay for these replicators with another EBNA1-expressing cell line, 143/EBNA1 (69), clearly verifying these replicators as active replication origins in two different cell lines.

To assess whether the origin-containing plasmids could also be maintained in human cells without antibiotic selection, equal numbers of origin and negative control ( $\Delta$ DS) plasmid molecules were separately transfected into HEK293/EBNA1 cells, cultivated for 4 days, and recovered from the cells. If the recovered plasmid DNA had undergone replication in human cells, it should be resistant to DpnI digestion and would generate colonies after electroporation into *E. coli*. Thus, the replication competence of each plasmid was calculated as the ratio of the colony number generated by DpnI-treated relative to untreated DNA. The replication competences of the five origin plasmids in human cells without drug selection were two- to sixfold greater than that of the negative control  $\Delta$ DS

(Table 1). These results confirm that the trapped genomic fragments support plasmid replication in human cells.

Next, we investigated plasmid maintenance in HEK293/EBNA1 human cells after 1 month. The cells were transfected with wild-type *oriP*, the negative control plasmid  $\Delta$ DS, and  $\Delta$ DS derivatives containing the isolated replicator *ori6* or *oriLB2*. The cells were placed under selection 2 days after transfection, and puromycin-resistant populations were grown for 2 weeks. At this time point, cultures were split in half and cultured in the presence or absence of selection. Plasmid loss in the absence of puromycin was monitored every third day. At different times following transfection, plasmid DNA was isolated by Hirt extraction (22) and digested with DpnI, and 500 ng of DNA was transfected into *E. coli*. We found that  $\Delta$ DS was lost from HEK293/EBNA1 cells 6 days after withdrawal of selection, whereas the *ori6*- and *oriLB2*-containing plasmids were maintained for 9 days under nonselective conditions. HEK293/EBNA1 cells transfected with the origin plasmids (*ori6*, *ori9*, and *oriLB2*) and control plasmids (*oriP* and  $\Delta$ DS) were also kept under selection and analyzed 2, 4, and 6 weeks posttransfection for the extrachromosomal status of plasmids. Plasmid DNA was isolated from the different cell lines and transfected back into *E. coli*. None of the isolated small DNA fragments maintained long-term episomal replication of their  $\Delta$ DS derivatives for at least 6 weeks (Table 1; also data not shown).

**The trapped human replicators serve as ORC binding and initiation sites in the  $\Delta$ DS plasmid.** The new human replicator elements described here were selected as Orc2p-binding fragments able to confer replication competence to a defective *oriP* plasmid. To assess whether Orc2p is indeed specifically associated with the human replicator elements, ChIP experiments were performed. The replicator plasmids were individually transfected into HEK293/EBNA1 cells and drug selected for 2 weeks prior to the ChIP. As positive and negative controls, we used the *oriP* and  $\Delta$ DS plasmids. The association of Orc2p with the origin sequences was compared to that of Orc2p at a distal reference site (see map in Fig. 2c). As expected, *oriP* showed reproducible enrichment of Orc2p at the wild-type viral origin relative to what was seen for a distal reference fragment, whereas  $\Delta$ DS exhibited no Orc2p enrichment at primer set 4.2 near the *AscI* site (Fig. 2c). The replicator *ori6* demonstrated enrichment of Orc2p at primer set 4.2 that was 3.8-fold greater than that for the reference site (Fig. 2c; Table 1). The sequences of the *oriIII*, *ori $\beta$* , *ori9*, and *ori10* elements were significantly enriched in Orc2p-chromatin immunoprecipitates compared to the corresponding sequences in the negative control  $\Delta$ DS but to a lower extent than the sequences for *ori6* and *oriP* were.

To investigate whether the newly identified human replicators serve as the preferred initiation sites for plasmid replication, we performed nascent strand abundance analysis. As shown in Fig. 2d, the 4.2 sequence was greatly enriched relative to the reference in nascent DNA from cells transfected with each of the human replicator plasmids and the *oriP* plasmid. As expected, no preferred initiation site was observed in the  $\Delta$ DS plasmid. In addition, the copy number of nascent strand DNA obtained from  $\Delta$ DS-transfected cells was several hundredfold lower with that obtained from the same number of transfected cells containing the human origin plasmids, indicating a severe reduction in replication competence. From these results, we

conclude that the human replicator elements isolated by the origin-trapping assay function in their plasmid context as site-specific origins of DNA replication.

**Once-per-cell-cycle plasmid replication is EBNA1 independent.** The results shown in Fig. 2, which are summarized in Table 1, indicate that the newly identified human DNA origins have several properties predicted for a human chromosomal replicator. Another property predicted for a human replicator is that it should direct initiation of DNA replication only once per cell cycle. The ability of the newly identified origins to initiate replication once per cell cycle can be measured in their plasmid context with the classic Meselson-Stahl density approach (2, 45). To test this prediction, we analyzed *ori6* and *oriLB2*, as well as wild-type *oriP* and  $\Delta$ DS control plasmids, with this approach. HEK293/EBNA1 cells transfected with the different plasmids were drug selected for 2 weeks and then transferred to medium containing 10  $\mu$ M BrdU for 18 h. Total DNA was isolated, digested with BamHI, and fractionated by equilibrium density gradient centrifugation. The amount of genomic DNA in each fraction was determined by measuring the absorbance at 260 nm and confirmed by quantitative PCR using a primer pair located at *oriMCM4* (35). The distribution of the plasmid sequences within the fractions was determined by real-time PCR, using the plasmid-specific primer set 4.2. We found that  $\Delta$ DS, which lacks an efficient replication origin, incorporated BrdU only very inefficiently (Fig. 3, upper panel), consistent with a previous report stating that an EBV plasmid containing only the FR element of *oriP* supported replication poorly (36). In contrast, the *oriP* control plasmid and the *oriLB2* and *ori6* plasmids shifted from a being light fraction to having a density near that of a heavy-light hybrid (Fig. 3, upper panel). These data clearly demonstrate that plasmids containing these two human chromosomal origins replicated efficiently once in the HEK293/EBNA1 cell cycle.

We have previously shown that EBNA1 is in a complex with ORC, thus recruiting ORC to the latent EBV origin *oriP* (61). Therefore, it is formally possible that EBNA1, by binding to the FR, recruits ORC to the different plasmids. To test this possibility, we asked whether the plasmids containing human replicators are duplicated independent of EBNA1. Some residual EBNA1-independent replication of *oriP* plasmids might occur in the first 48 h after transfection, but continued replication at 96 h after transfection is dependent on EBNA1 (3). The density transfer experiments were repeated in HEK293 cells transiently transfected for 96 h with the same plasmids as before. After being labeled with BrdU for 18 h, total DNA was isolated, digested with BamHI and DpnI, and fractionated by CsCl gradient centrifugation as before. The fractions were collected and analyzed as before. At 18 h after the addition of BrdU, when most cells had progressed through S phase, the  $\Delta$ DS plasmid and the *oriP* plasmid remained near the light density, indicating that they did not replicate in the absence of EBNA1 (Fig. 3, lower panel). In contrast, the *ori6*- and *oriLB2*-containing plasmids had shifted to the heavy-light hybrid fraction (Fig. 3, lower panel). At 96 h after transfection, the transfected cells still contain a large number of nonreplicated input plasmids. To test the completeness of the DpnI digestion, we used a plasmid-specific primer pair that contains two DpnI recognition sites and amplifies only DNA fragments generated by cellular replication. These control reactions gave an ampli-

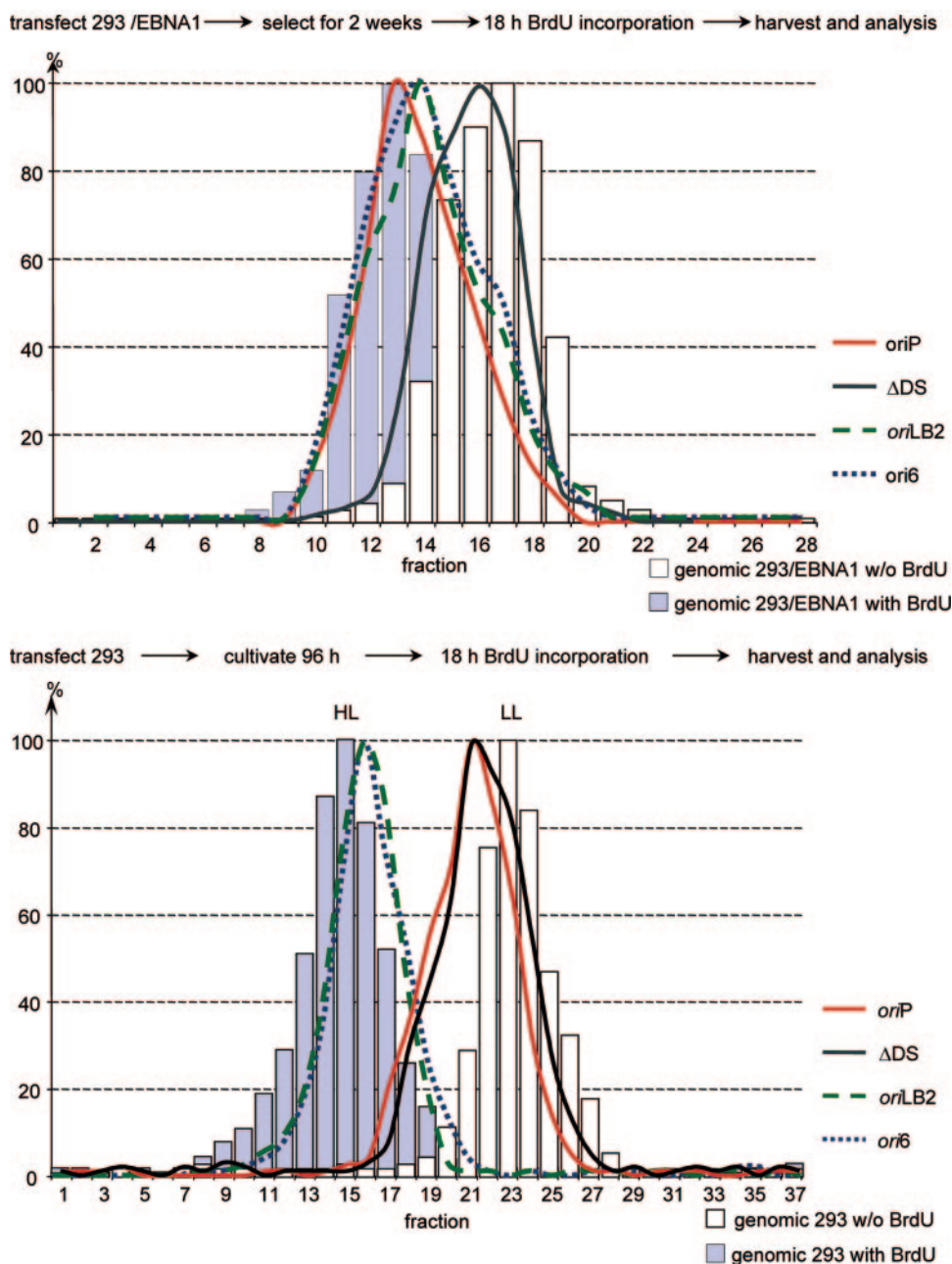


FIG. 3. Human replicator plasmids are duplicated once per cell cycle. Equimolar amounts of wild-type *oriP*,  $\Delta$ DS, *ori6*, and lamin B2 plasmids were introduced independently into HEK293/EBNA1 (upper panel) and HEK293 (lower panel) cells. Ninety-six h after transfection, the cells were labeled with bromodeoxyuridine for 18 h. Total DNA was isolated from  $1 \times 10^7$  cells, digested with BamHI, and separated by equilibrium centrifugation in a CsCl density gradient. The refractive index and the amount of total genomic DNA (absorbance at 260 nm) were determined for every second fraction and confirmed by PCR amplification at the lamin B2 origin (primer pair lamin B2 [Fig. 2]). Plasmid DNA in the light-light (LL) and heavy-light (HL) hybrid fractions was digested with DpnI and quantified using real-time PCR and a plasmid-specific primer pair that amplifies a sequence containing DpnI sites. For each of four independent experiments, the level of replicated test plasmid was set to 100%, and the relative DNA concentration was plotted versus fraction number. No DNA was detected at the density at which heavy-heavy hybrid fraction DNA should migrate.

fication level the same as that with primer pair 4.2. These data indicate that the plasmids containing human chromosomal origins replicated independently of EBNA1.

**Characterization of the replication origin *ori6*.** The replication efficiency of the isolated replicator elements is moderate in comparison to that of *oriP*. To analyze whether the efficiency can be improved, we multimerized *ori6* (Fig. 4a) and tested the

different plasmids in transient replication assays (Fig. 4b). As a positive control we used *oriP* (data not shown), and as a negative control we used  $\Delta$ DS (Fig. 2b). The multimerization of the *ori6* sequence resulted in a linearly increased replication efficiency, indicating that additional elements might be required for an improved origin activity.

We also performed a deletion analysis of *ori6* in order to



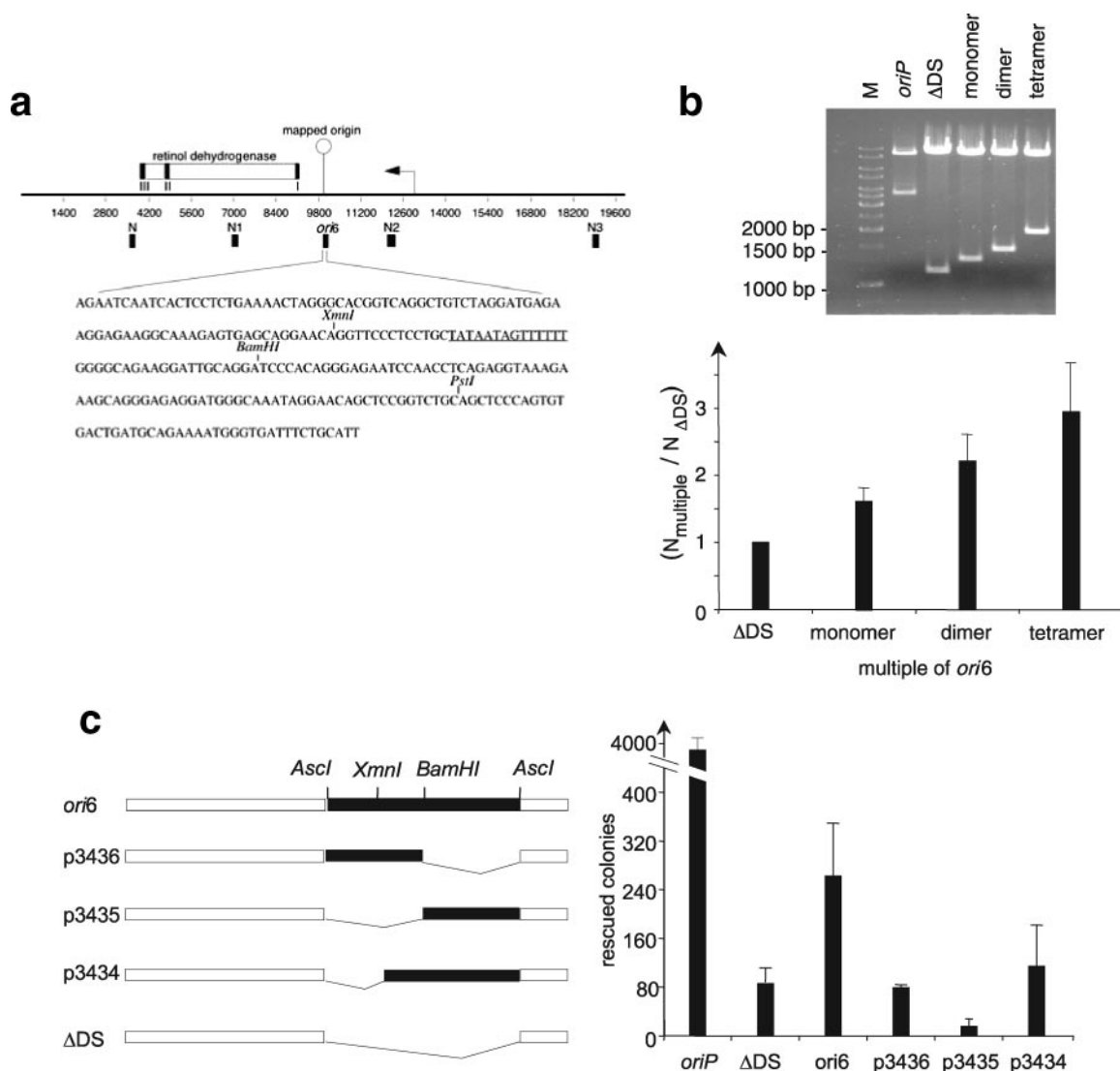


FIG. 4. Genetic analysis of *ori6*. (a) The Orc2-precipitated *ori6* sequence is located in the 5' untranslated region of the retinol dehydrogenase gene. The genomic organization of this region is shown on top. The arrowhead depicts the transcription start site, and the putative exons are shown as black boxes (I to III). The location of the cloned Orc2-binding fragment is shown below the line (*ori6*), as are the locations of the control regions amplified by quantitative PCR (N to N3). The 249-bp-long fragment, including the restriction enzymes used for the deletion mutants as well as an AT-rich region (underlined), is depicted below. (b) *ori6* fragments were multimerized in a head-to-tail fashion. The *ori6* sequence was PCR amplified from the *ori6* plasmid with one primer containing an *Ascl* restriction site and one containing an *AflIII* restriction site. After the PCR, *ori6* fragments were ligated to each other by a head-to-tail cloning strategy and ligated into plasmid p2932 ( $\Delta$ DS). Replication competence was determined as described above in three independent transient replication assays. (c) Deletion analysis of *ori6* located at the retinol dehydrogenase gene. Different deletion mutants of *ori6* are depicted on the left. Using the indicated internal restriction sites, *XmnI* and *BamHI*, generated the deletion mutants. The replication competences of the different mutants were determined in transient replication assays with HEK293/EBNA1 cells and quantified by counting the rescued colonies. The histogram shows the average values and standard deviations for triplicates.

determine whether or not one particular DNA motif of *ori6* was required for ORC binding and replication efficiency. The different *ori6* mutants (Fig. 4c, left) were tested in a transient replication assay. For comparison, we used *oriP*, *ori6*, and  $\Delta$ DS and observed that not one particular element is crucial for extrachromosomal replication but that the deletion of any element results in a loss of replication function (Fig. 4c right). These results indicated that the whole *ori6* sequence is needed for initiation of replication.

**Binding of ORC and MCM subunits at origins in different phases of the cell cycle.** The colocalization of the newly iden-

tified human replicators with chromosomal initiation sites (Fig. 2) and their abilities to bind ORC and direct once-per-cell-cycle plasmid replication in an EBNA1-independent fashion (Fig. 2 and 3) suggested that in their native chromosomal context, these replicators are likely licensed during G<sub>1</sub> phase to initiate replication. This prediction was tested by measuring Orc2p enrichment at the potential origins *ori6* and *ori9* in their native chromosomal contexts in ChIP assays relative to preimmune control precipitates and the input DNA (35, 60). In initial experiments with asynchronously proliferating HeLa cells, we found that the origin sequence (UPR) (Fig. 5a) lo-

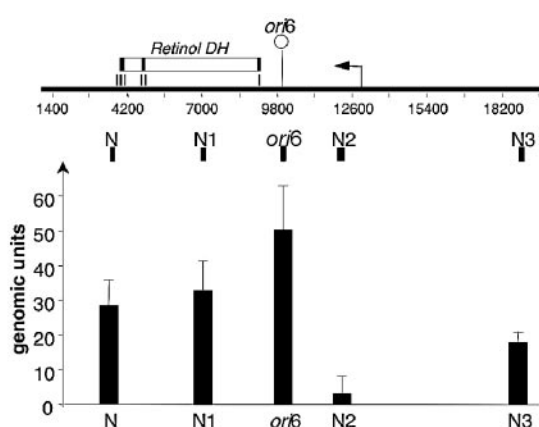
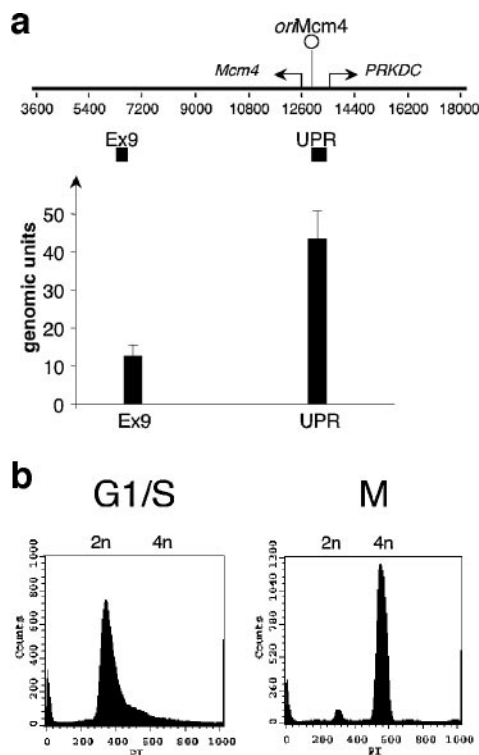


FIG. 5. Orc2p localizes near newly identified origins in chromatin from human cells. (a) Cross-linked chromatin fragments prepared from asynchronously growing HeLa cells were enriched by ChIP using antibodies against Orc2p. DNA sequences that coprecipitated with Orc2p were quantified using primer sets specific for the *Mcm4* origin (left) and *ori6* (right). The genomic units were calculated relative to values for serially diluted genomic DNA by quantitative real-time PCR as described previously (60). Diagrams of the genomic regions flanking the *Mcm4* and *ori6* origins show the locations of primer sets (small black boxes) and the start sites and direction of gene transcription (arrow). UPR is the PCR fragment spanning *oriMCM4*, whereas exon9 (Ex9) is a reference site located 6.5 kb apart from *oriMCM4* (60). DH, dehydrogenase. (b) Flow cytometry of HeLa cells synchronized at G<sub>1</sub>/S with thymidine and at G<sub>2</sub>/M with nocodazole. PI, propidium iodide. (c and d) ChIP analysis with antibodies directed against Orc2, Orc3, Mcm3, and Mcm7 was performed using HeLa cells synchronized in G<sub>1</sub>/S and G<sub>2</sub>/M. Coprecipitated genomic sequences surrounding the *MCM4* (c) and *ori6* (d) replicators were quantified by real-time PCR using the primer sets used for the experiment shown in panel a (27). The results of at least three independent experiments are shown.

cated between the *Mcm4* and *PRKDC* genes was enriched threefold in Orc2p immunoprecipitates relative to the origin-distal exon9 control sequence (Fig. 5a). Chromatin from the same ChIP experiments was moderately enriched in sequences at *ori6* relative to the reference sequences in the flanking retinol dehydrogenase gene (Fig. 5a, N and N1) and more highly enriched relative to reference sites N2 and N3. Similarly, the *ori9* replicator sequence displayed moderate enrichment in the Orc2p precipitate relative to reference sequence 9.2 but significant enrichment relative to control sequence 9.1 (data not shown).

To monitor the cell cycle dependence of ORC localization, ChIP assays were performed using chromatin isolated from HeLa cells synchronized at G<sub>1</sub>/S with a double thymidine block and in mitosis with nocodazole (Fig. 5b). The specific enrichment of Orc2p, Orc3p, and Orc5p in G<sub>1</sub>/S at the *ori6* and *ori9* origins was similar to that at *oriMcm4* (Fig. 5c) (see Fig. S1 in the supplemental material) (60). This enrichment was much greater than that observed with chromatin from asynchronous cultures (Fig. 5a). Mcm3p and Mcm7p immunoprecipitates of chromatin fragments from G<sub>1</sub>/S cells were also efficiently enriched for the origin-specific sequences relative to the more distal sequences (Fig. 5d). As expected, the licensing factors Mcm3p and Mcm7p were released from the origins in mitosis, and the amplification at the sites was as low as that at the neighboring regions (Fig. 5d). Similarly, no enrichment of *Mcm4*, *ori6*, or *ori9* origin sequences was detected in all ORC immunoprecipitates prepared from mitotic chromatin (Fig. 5c) (see Fig. S1 in the supplemental material). These observations

confirm and extend a previous report of cell cycle-regulated association of Orc2p at the *oriLB2* origin (1).

To rule out the possibility that excess nocodazole caused the loss of ORC-binding at origins in mitotic chromatin, we repeated the ChIP experiments using cells that had been synchronized at G<sub>1</sub>/S as described above and then released into medium without any inhibitor. The site-specific association of Orc2p and Mcm7p was measured at *ori6*, *ori9*, and *oriMcm4* in chromatin immunoprecipitated from blocked cells and from cells harvested at 5 h (S phase), 7 h (G<sub>2</sub>/M), and 17 h (G<sub>1</sub>) after release from the G<sub>1</sub>/S block (Fig. 6). Both Orc2p and Mcm7p were specifically enriched at origins during early cell cycle phases (G<sub>1</sub> and G<sub>1</sub>/S), while little Orc2p or Mcm7p was detected at origins in later stages of the cell cycle. These results are in accord with those shown in Fig. 5c and d and demonstrate that ORC and MCM associate with these human origins in G<sub>1</sub> HeLa cells and are largely absent from these sites in mitotic chromatin.

## DISCUSSION

We have established a novel two-step origin-trapping assay and validated it as a useful tool to identify active origins of replication in human cells. The trapped origins are recognized by ORC *in vivo* and serve as replication start sites in a plasmid and in their native chromosomal contexts.

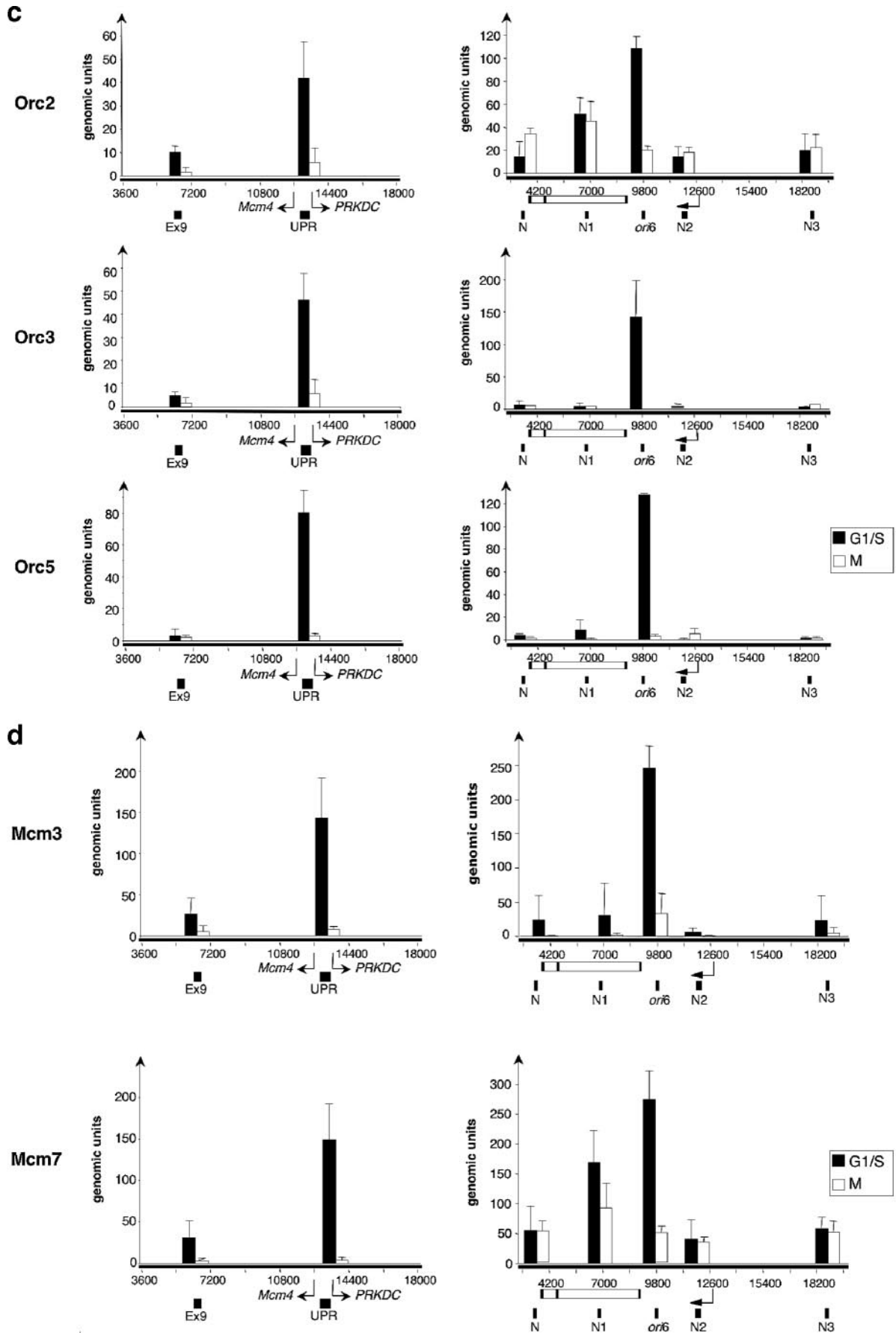


FIG. 5—Continued.

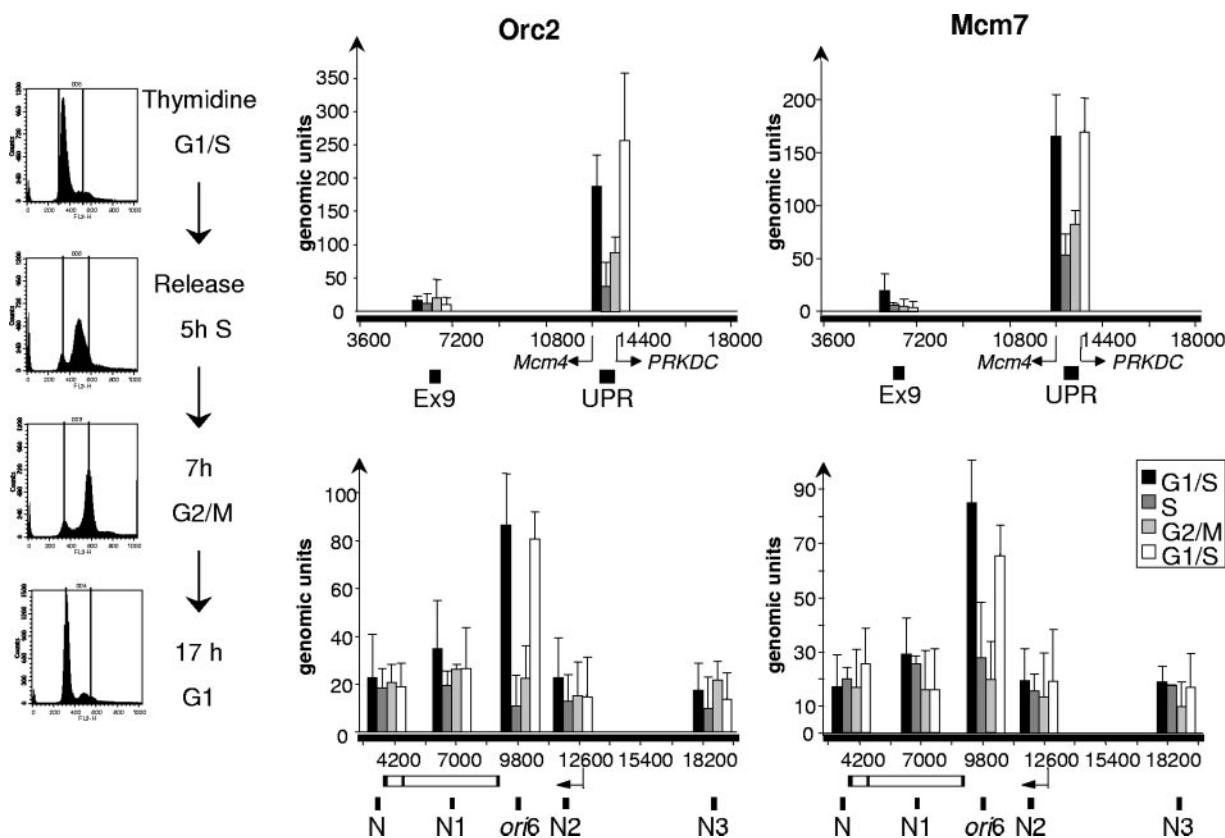


FIG. 6. Cell cycle-dependent localization of Orc2p and Mcm7p at human chromosomal origins. HeLa cells were synchronized at  $G_1/S$  with a double thymidine block and released into fresh medium for 5, 7, or 17 h. The cell cycle distribution of each population was determined by flow cytometry (left). The histograms (right) show ChIP enrichment of Orc2p and Mcm7p at the MCM4 and *ori6* origins during the different stages of the cell cycle. The data shown are the mean values and standard deviations for three independent experiments. Diagrams of the genomic regions flanking the MCM4 and *ori6* origins are shown in Fig. 5.

#### An effective screen for active human replication origins.

This origin-trapping strategy differs from earlier approaches developed in the Calos and Knippers laboratories in several respects. Keller and colleagues at the Knippers laboratory used the ChIP technique to enrich ORC-binding sequences that were then used to identify ORC-binding elements in the corresponding genomic regions, followed by nascent strand analysis to confirm them as active origins (29). Although the efficiency of this approach was not determined quantitatively, 2 out of 20 precipitated and cloned elements were reported to show high ORC enrichment in confirmatory ChIP experiments. These observations highlight the need for a screen, such as that used in our origin-trapping assay, to functionally assess the origin activity of coprecipitated ORC-binding fragments.

Krysan and coworkers at the Calos laboratory used an unbiased cloning approach to identify human DNA sequences that supported autonomous replication of *oriP*-like plasmids in human cells under drug selection for 2 months (33). Larger fragments supported autonomous replication more efficient than that of smaller fragments, but the possibility that the DNA sequence influenced replication competence of these plasmids was not excluded. It is possible that large human DNA fragments are more likely to contain an origin. Alternatively, large fragments may favor establishment of epigenetic features that allowed plasmid maintenance for several months

in human cells. To minimize the contributions of largely uncharacterized epigenetic events, we used a relatively short selection period of 2 weeks. Consistent with this possibility, we found that *oriP*-like plasmids containing trapped small human fragments (less than 1,000 bp) support plasmid replication but were not maintained for longer than a month.

**Newly identified human origins have properties of chromosomal replicators.** The five human replicators identified in our assay replicate once in each cell cycle in the context of a  $\Delta DS$  plasmid, and replication begins near the human origin fragment in these plasmids and in the native chromosomal context in human cells. Although the newly isolated origins share no sequence homology, all of them are located in a distance of 7 to 10 kb from matrix attachment regions (see Table S2 in the supplemental material). All newly identified origins are located in actively or putatively transcribed regions (see Table S2 in the supplemental material). This strongly supports the hypothesis that transcription may be supportive of origin activity or origin selection. ORC associated with each newly identified human origin in context of the  $\Delta DS$  plasmids and was localized at the corresponding sites in human chromosomes during the  $G_1$  phase of the cell cycle. Unexpectedly, Orc2p was not detected at the analyzed origins in mitosis, which appeared to contradict the biochemical analysis of cell cycle-fractionated chromatin showing that Orc2p and other components of ORC might be

stably associated with origins throughout cell division (44, 48, 54). However, Orc2p is known to associate with nonorigin elements *in vivo* (50, 51). It is important to differentiate between the global chromatin binding of Orc proteins as it is studied with chromatin binding experiments (43) and their specific binding to replication origins as it is studied with ChIP experiments. Chromatin binding experiments measure physical properties of complexes bound to chromatin and allow the identification and characterization of subcomplexes with different affinities. ChIP experiments, however, allow for the characterization of complexes at specific sites of the chromatin.

From the discussion above, it is quite possible to conclude that only a minority of ORC is actually bound to origins in a cell cycle-dependent manner and that the majority is involved in other chromatin-associated processes that are not cell cycle regulated. We confirmed that three different Orc subunits and, by implication, ORC itself as well as Mcm3p and Mcm7p, localize at the chromosomal *oriMCM4* and at the five new origins in human cells in G<sub>1</sub>, dissociate during S phase and G<sub>2</sub>/M, and relocalize at origins in G<sub>1</sub> (Fig. 5c and d and 6). The behavior of the Mcm proteins at *oriMCM4* and *ori6* is in line with that previously reported (1, 17, 35, 60). The origin association of different Orc subunits along the cell cycle is currently less clear. It is generally accepted that Orc1p is selectively released from origins and, in some cases, degraded (1, 55, 60). The reduced Orc2-binding level at *ori6* and *oriMCM4* in mitosis is in agreement with reports for *oriLB2* (1, 17) but differs from what has been reported for *oriP* of EBV and the *c-myc* origin (11, 17, 55). The exact point in time of Orc2p-Orc5p release during S phase is currently unknown. Orc6p has not been analyzed in this respect on chromosomal origins. The laboratories of Falaschi and Knippers reported that Orc2p is associated with origins during S phase (1, 59), but we did not detect ORC subunits at origins during S phase. One possible explanation for the initially contradictory-seeming results is that the laboratories of Falaschi and Knippers analyzed Orc2p binding at *oriMCM4* and *oriLB2* in early S phase, whereas our samples were examined later in S phase (Fig. 6). Furthermore, it is also possible that Orc2p-Orc6p are not dissociating from origins during S phase but experience a conformational change. Individual experimental setups (cross-linking condition, UV or formaldehyde cross-linking) might lead to differing results. The exact point in time of a conformational change has to be determined in detail. Our findings, which are summarized in a model in Fig. 7, argue against possible epitope masking in mitosis and extend reports of ORC dynamics at human origins. Taken together, the properties of the trapped human origins are those expected for chromosomal replicators.

**Human replicators as extrachromosomal replication origins.** We found that the replication activity of the plasmids selected in the origin-trapping assay is lower than that of wild-type *oriP*. This observation is perhaps not surprising, since the latent viral plasmid has evolved to maintain the EBV genome in a lifelong manner in its human host. Thus, the viral DNA binding protein EBNA1 promotes cell cycle-independent ORC binding to DS of *oriP*, likely rendering the viral replicator resistant to host mechanisms that might modulate origin activity (11, 55). DS interacts via EBNA1 with other elements of *oriP*, such as FR, possibly to improve origin efficiency in a manner that is not mimicked by the human origin fragments.

Another reason could be that additional distal chromosomal elements regulate origin activity. The importance of such distal elements has been demonstrated for origins located near the human  $\beta$ -globin and the hamster DHFR loci (12, 27, 31). In embryonic extracts from frogs, flies, or fish, many genomic sites can be used as replication origins. If these conditions change as cells differentiate, most of these sites are suppressed, but others are enhanced. Furthermore, mammalian ORCs do not bind to specific sequences *in vitro*, and yet ORC appears to be bound preferentially to specific replication origins *in vivo*. It is therefore not surprising that, as mentioned in the introduction, mammalian DNA fragments that support extrachromosomal replication are not as active as origins that are defined mainly by sequence, such as ARS elements in yeast or *oriP* of EBV (14, 19, 23, 66). A deletion analysis of *ori6* supported the idea that not one particular DNA motif is required for ORC binding and replication efficiency. The multimerization of the same sequence resulted in an increased efficiency, further indicating that additional elements might be required for an improved origin activity (Fig. 4).

The new chromosomal replicator plasmids also differ in several important respects from pEPI, another recently described plasmid that supports autonomous replication in human cells (7, 59). ORC binding to pEPI is not localized to a particular site *in vivo* but rather is delocalized with a slight preference for AT-rich regions, much like the weak DNA sequence preference of purified human ORC observed *in vitro* (65). Replication start sites in pEPI were also distributed throughout the plasmid, despite the fact that each plasmid replicated once per cell cycle (59). The differences between pEPI and the newly identified human origin plasmids suggest the existence of at least two different mechanisms to promote plasmid replication in mammalian cells and yet restrict it to once per cell cycle.

The establishment of autonomously replicating plasmids (*oriP* and pEPI) is an infrequent event in mammalian cells (25, 36). Regardless of their replication competence, plasmids are lost from cells at a rate of >25% per cell generation during the first 2 weeks after transfection (36). In contrast, cells selected to maintain established *oriP* plasmids have a 2 to 4% rate of plasmid loss after withdrawal of selection (30, 62). Thus, it seems likely that epigenetic events, in addition to replication competence, contribute to the long-term establishment of *oriP* plasmids. To exclude cell-type-specific features, we used different EBNA1-expressing cell lines (HEK293/EBNA1 and 143/EBNA1). The nature of the epigenetic events that facilitate plasmid maintenance in mammalian cells is currently unclear. It is possible that the DS element of *oriP* provides additional features that support the long-term establishment of wild-type *oriP* plasmids. Such events are probably not supported by the chromosomal replicator in the *oriP* plasmids analyzed in this study.

Our origin-trapping assay is based on the assumption that at least a subset of human origins is also defined by DNA sequence. Some origins, such as the lamin B2 and Mcm4 origins, are localized within a few kilobases and should be identifiable by origin trapping. Other replicators have an initiation zone with dispersed start sites, such as DHFR and *myc* origin. These replication origins are probably defined by a combination of DNA sequence elements, local chromatin structure, and DNA topology (53) and may be difficult to isolate in a trapping assay.

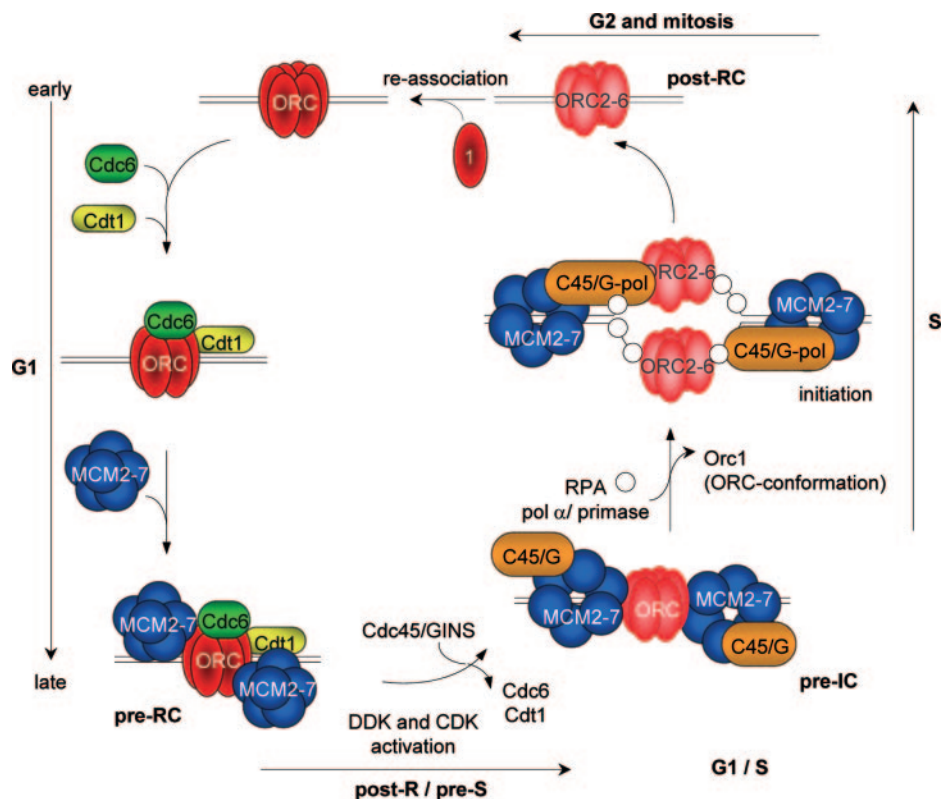


FIG. 7. Model for the origin cycle at a human origin. The origin recognition complex is assembled at origins in the early stages of G<sub>1</sub>. Cdc6 and Cdt1 are required for the association of the Mcm2-7 protein family, resulting in the formation of the prereplicative complex (pre-RC). After activation of the DBF4-dependent kinase (DDK) and cyclin-dependent kinase (CDK) complexes, the transition from the pre-RC to the preinitiation complex (pre-IC) occurs at G<sub>1</sub>/S and is characterized by the formation of the CMG complex (consisting of Cdc45p, Mcm2-7p, and GINS) (47). After origin activation, Orc1p is selectively released from origins. This leads probably to a conformational change of the Orc2p-Orc6p subcomplex, resulting in a decreased sensitivity to formaldehyde and UV cross-linking (this study and reference 1). The exact time point of ORC reassociation has to be determined.

Origins of replication in higher eukaryotes are also influenced by transcriptional activity, as seen for *Drosophila melanogaster* and *Sciara* (38, 40), and by differentiation and development (15, 42, 49). A recent analysis of origins located in the X-inactivation center confirmed an association between replication, transcription, and epigenetic regulation, although there is no clear dependency (58). Whether such origins can be identified by modifications of the trapping assay remains unknown.

The high genome complexity in mammalian cells and the associated decrease in assay sensitivity hampered the identification and characterization of origins. Clearly, there is a need for additional models in studying the features of human replication origins. While this work was in review, two new methods for isolating mammalian replication origins were published (46, 64). The two-dimensional gel-mapping method identified DNA sequences containing active but inefficient origins, such as DHFR origin (46, 64). In one of these reports, Mesner et al. showed that all isolated origins derived from active initiation sites. In a different approach, Todorovic et al. generated a library of nascent strand DNA fragments that identified replication origins with higher resolution, but it was dependent on an additional fine-mapping step by quantification of nascent strand DNA by competitive PCR (46, 64). Thus, both recently published origin-mapping

methods are based on physical features of the replicated DNA rather than on functional properties. The two-step origin-trapping assay reported here is based not only on the physical property of ORC binding but also on a functional selection for origins. Our assay allows for the first time the identification of active, efficient replication origins which also support replication of extrachromosomal DNA. Taken together, these three new origin-mapping methods should enable the identification and characterization of new mammalian origins, their composition and biochemical properties, and their roles in chromosome structure and function in mammalian cells.

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