Complex protein-DNA dynamics at the latent origin of DNA replication of Epstein-Barr virus

Marion Ritzi[1], Kristina Tillack[1], Jeannine Gerhardt[1], Elisabeth Ott[1], Sibille Humme[1], Elisabeth Kremmer[2], Wolfgang Hammerschmidt[1] and Aloys Schepers[1,*]

[1]Department of Gene Vectors, GSF-National Research Center for Environment and Health, Marchioninistrasse 25, 81377 München, Germany
[2]Institute for Immunology, GSF-National Research Center for Environment and Health, Marchioninistrasse 25, 81377 München, Germany
*Author for correspondence (e-mail: schepers@gsf.de)

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Summary

The sequential binding of the origin recognition complex (ORC), Cdc6p and the minichromosome maintenance proteins (MCM2-7) mediates replication competence at eukaryotic origins of DNA replication. The latent origin of Epstein-Barr virus, *oriP*, is a viral origin known to recruit ORC. *OriP* also binds EBNA1, a virally encoded protein that lacks any activity predicted to be required for replication initiation. Here, we used chromatin immunoprecipitation and chromatin binding to compare the cell-cycle-dependent binding of pre-RC components and EBNA1 to *oriP* and to global cellular chromatin. Pre-replicative-complex components such as the Mcm2p-Mcm7p proteins and HsOrc1p are regulated in a cell-cycle-dependent fashion, whereas other HsOrc subunits and EBNA1 remain constantly bound. In addition, HsOrc1p becomes sensitive to the 26S proteasome after release from DNA during S phase. These results show that the complex protein-DNA dynamics at the viral *oriP* are synchronized with the cell division cycle. Chromatin-binding and chromatin-immunoprecipitation experiments on G0 arrested cells indicated that the ORC core complex (ORC2-5) and EBNA1 remain bound to chromatin and *oriP*. HsOrc6p and the MCM2-7 complex are released in resting cells. HsOrc1p is partly liberated from chromatin. Our data suggest that origins remain marked in resting cells by the ORC core complex to ensure a rapid and regulated re-entry into the cell cycle. These findings indicate that HsOrc is a dynamic complex and that its DNA binding activity is regulated differently in the various stages of the cell cycle.

Key words: *oriP*, Replication, Cell cycle, EBNA1, ORC

Introduction

In recent years, considerable progress has been made in understanding the molecular mechanisms that control eukaryotic DNA replication initiation. Replication initiates from specialized sequences, replication origins that are recognized in eukaryotes by the origin recognition complex (ORC). This complex, like most replication initiation proteins, is conserved throughout evolution. It is generally accepted that the initiation mechanism follows the same principles in all eukaryotes. Nevertheless, there are some significant differences between simple systems like yeasts and more complex multicellular organisms (for reviews, see Bell and Dutta, 2002; Blow, 2001; Bogan et al., 2000; Fujita, 1999; Kelly and Brown, 2000).

Following the separation of the replicated chromatids at mitosis, the genome has to acquire replication competence during the subsequent G1 phase. This is achieved by the sequential formation of pre-replicative complexes (pre-RC), a process also called licensing. ORC is chromatin bound during the entire cell cycle, whereas the Cdc6 and Cdt1 proteins, the first factors of the pre-RC assembly, are recruited in early G1 or late mitosis of the proceeding cell cycle. Both Cdc6p and Cdt1p are required for loading the minichromosome maintenance complex (MCM2-MCM7) onto chromatin (Cocker et al., 1996; Coleman et al., 1996; Maiorano et al., 2000; Nishitani et al., 2000; Tanaka and Diffley, 2002). Mcm2p-Mcm7p are not only essential for the initiation process but are also required for the progression of replication forks during S phase (Labib et al., 2000). Once Mcm2p-Mcm7p are assembled on chromatin, the functions of Cdc6p and Cdt1p are no longer necessary (Cook et al., 2002; Jares and Blow, 2000). In the next step, the pre-RCs are reorganized to establish the pre-initiation complexes (Diffley and Labib, 2002; Takisawa et al., 2000). This process depends on the presence of the MCM2-MCM7 complex. The subsequent activation of origins during initiation requires the activity of two cell-cycle-regulated kinases: cyclin-dependent kinases and Cdc7 (Bousset and Diffley, 1998; Donaldson et al., 1998a,b).

Eukaryotes possess multiple safeguard mechanisms that prevent re-replication within a single cell cycle. These include mechanisms that negatively regulate ORC subunits, Cdc6p, Cdt1p and Mcm2p-Mcm7p (Bell and Dutta, 2002; Kelly and Brown, 2000; Nguyen et al., 2001). One such mechanism in metazoan eukaryotes appears to involve the release of Orc1p from chromatin during S phase (Asano and Wharton, 1999; Keller et al., 2002; Kreitz et al., 2001; Ladenburger et al., 2002; Li and DePamphilis, 2002; Mendez and Stillman, 2000; Mendez et al., 2002; Natale et al., 2000). However, it is currently controversial whether this ORC subunit is completely released from chromatin during S phase and degraded, or whether Orc1p is only selectively released from DNA with ongoing replication (Kreitz et al., 2001; Ladenburger et al., 2002; Li and DePamphilis, 2002; Mendez et al., 2002; Natale

et al., 2000). By contrast, other laboratories report that Orc1p remains chromatin associated throughout the cell cycle (Okuno et al., 2001; Tatsumi et al., 2000). The smallest ORC subunit, Orc6p, although essential, seems to be dispensable for origin recognition. It was shown that budding yeast Orc6p is not necessary for autonomous replicating sequence (ARS) binding and complex building (Lee and Bell, 1997). The human homologue is bound to chromatin throughout the cell cycle (Mendez et al., 2002). Co-immunoprecipitation experiments with other ORC subunits indicate that HsOrc6p is the most weakly bound ORC constituent (Dhar and Dutta, 2000; Vashee et al., 2001).

Viral systems have always played an important role in studying eukaryotic replication. During latency, Epstein-Barr virus (EBV) replicates exactly once per cell cycle by using the cellular replication machinery (Yates, 1996). The latent origin of DNA replication of EBV, *oriP*, was originally discovered as an element that supports the replication and maintenance of extra-chromosomal episomes (Yates et al., 1984; Yates, 1996). The *oriP* is a 1.8 kbp fragment that consists of two essential elements – the family of repeats (FR) and the dyad symmetry (DS) element (Reisman et al., 1985). FR is a cluster of 20 binding sites for the viral transactivator EBNA1. This element mediates the maintenance of *oriP*-dependent episomes and functions as a transcriptional enhancer (Aiyar et al., 1998; Reisman et al., 1985; Wysokenski and Yates, 1989). The DS element contains four EBNA1 binding sites and is the site at or near which initiation occurs. We and others have shown that the presence of this element is crucial for recruiting ORC to the latent origin (Chaudhuri et al., 2001; Schepers et al., 2001). Deleting the DS element not only abolishes ORC binding but also reduces replication initiation at *oriP* to background levels (Norio et al., 2000). These data are complemented by the observation that EBNA1 and ORC interact with each other, supporting the suggestion that EBNA1 functions as a loading factor tethering ORC to *oriP* (Schepers et al., 2001). The assumption that *oriP* is regulated like a chromosomal origin is indirectly supported by the findings that Geminin, a cell-cycle-regulated inhibitor of Cdt1p (Tada et al., 2001; Wohlschlegel et al., 2000), blocks latent viral replication and that HsMcm2p is found at this origin (Chaudhuri et al., 2001; Dhar et al., 2001; Hirai and Shirakata, 2001).

In this study, we compare the cell cycle dynamics of protein complexes site specifically at *oriP* and at cellular chromatin using chromatin-immunoprecipitation (ChIP) and chromatin-binding assays. We show that EBNA1, HsOrc3p and some HsOrc6p are associated with specific sequences throughout the cell cycle, whereas HsOrc1p and Mcm2p-Mcm7p are recruited to *oriP* and to global chromatin in a cell-cycle-dependent manner. In addition, we evaluate the chromatin and *oriP* association of pre-RC components in G0-arrested cells. Components of the core ORC remain associated with DNA, whereas Mcm2p-Mcm7p and HsOrc6 are completely released from global chromatin and *oriP*. The affinity of HsOrc1p changes in G0-arrested cells but this subunit is not completely liberated from DNA.

Materials and Methods

Cell lines

Raji, a Burkitts lymphoma derived cell line, contains 20-50 EBV episomes (Adams, 1987; Nonoyama and Pagano, 1973; Pritchett et al., 1976). A39 is a lymphoblastoid B-cell line (LCL) generated from human primary B cells with EBV virions containing the mini-EBV 1478.A. The viral episome is maintained with a copy number of five to ten per cell (Schepers et al., 2001).

Antibodies and affinity purification

Polyclonal antibodies directed against HsMcm3p and HsMcm7p were raised as described (Burkhart et al., 1995; Schulte et al., 1995). HsOrc1p-, HsOrc3p- and EBNA1-specific antibodies have already been described (Schepers et al., 2001). Polyclonal antibodies were affinity purified against bacterially expressed antigen using the Sulfolink kit (Pierce) according to the manufacturers instructions. Rat monoclonal antibodies directed against HsOrc6p were raised against bacterially expressed full-length HsOrc6p as described (Schepers et al., 2001). *HsOrc6* was obtained from the Resource Centre and Primary Database (clone IMAGp222413).

Commercially available antibodies used in this study are: anti-HsOrc4 (Transduction Laboratories; code #83120), anti-Cyclin B1 (Neomarkers, AB1; clone V152), anti-Cyclin A (Neomarkers, Ab2; clone HE12) and anti-Cyclin E (Neomarkers, Ab6; Clone CyA06).

Centrifugal elutriation and flow cytometry

Centrifugal elutriation (Beckman J6-MC centrifuge) was used to separate the different cell cycle phases. For ChIP experiments, 5×10^9 logarithmically growing A39 cells were washed with PBS and resuspended in 50 ml Hanks' balanced salt solution (HBSS) supplemented with 1% foetal calf serum (FCS), 1 mM EDTA and 0.25 U ml^{-1} DNase I (Roche). Cells were injected in a JE-5.0 rotor with a large separation chamber at 1500 rpm and a flow rate of 30 ml per minute controlled with a Cole-Palmer Masterflex pump. The rotor speed was kept constant and 400 ml fractions were collected at increasing flow rates (35 ml per minute to 100 ml per minute). Individual fractions were counted and processed for the chromatin-binding assay as described below. For chromatin-binding experiments with Raji cells, 5×10^8 cells were prepared as described above and resuspended in 10 ml HBSS medium. Distinct cell cycle fractions were separated in the JE-6B elutriation system using a Sanderson chamber (Beckman). Rotor speed was kept constant at 2000 rpm and 150 ml fractions were collected with an increasing medium flow rate (9 ml per minute to 40 ml per minute).

For G0 experiments, A39 cells were grown to high density and kept in stationary phase for at least 3 days. Polyploid cells and cells with a sub-2C DNA content were separated from resting cells with the same centrifugal elutriation protocol as used for Raji cells. The DNA content of the different fractions was determined by flow cytometry (Becton Dickinson) using standard procedures.

Chromatin-binding assay

The separation of soluble and chromatin-bound proteins is based on a protocol by Mendez and Stillman (Mendez and Stillman, 2000) with modifications. 1×10^7 cells were harvested, washed with PBS and resuspended in 250 μl hypotonic buffer A [10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, protease inhibitor mix Complete© (Roche)]. Cells were lysed by adding 0.04% Triton X-100 and incubated for 10 minutes on ice. Samples were centrifuged (4 minutes, 1300 ***g***, 4°C) to separate soluble cytosolic and nucleosolic proteins from chromatin. The chromatin-enriched fraction was washed with 250 μl low-stringency buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT) and centrifuged (4 minutes, 1600 ***g***, 4°C). Chromatin-bound proteins were extracted with 250 μl ice-cold RIPA buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 50 mM Tris-HCl pH 8.0) by incubation on ice for 30 minutes and centrifuged (10 minutes, 16,000 ***g***, 4°C). The

protein concentration was determined (BCA, Pierce) and equal amounts of soluble and chromatin-bound proteins respectively were analysed by immunoblot analysis. To inhibit the 26S proteasome, all buffers were complemented with 25 μM MG132 (a specific proteasome inhibitor). A 10 mM stock solution was prepared in DMSO. Whole cell extract was prepared by lysing cells in ice-cold RIPA buffer as described above. The lysates were centrifuged (10 minutes, 16,000 ***g***, 4°C) and supernatants were supplemented with Laemmli buffer.

ChIP assay and PCR analysis

For ChIP experiments, 1×10^7 nuclei were prepared for each immunoprecipitation as described above. Nuclei were washed at a concentration of 1×10^8 nuclei ml^{-1} in ice-cold buffer A supplemented with 200 mM NaCl. After centrifugation (1300 ***g***, 5 minutes, 4°C) nuclei were carefully resuspended in 1 ml buffer A. Then, 9 ml pre-warmed buffer A supplemented with 1.1% formaldehyde were added and the nuclei cross-linked for 10 minutes at 37°C. Fixed nuclei were washed twice with PBS with 0.5% NP40, resolved in 2.7 ml LSB (10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$) and lysed by adding 300 μl 20% Sarkosyl. The chromatin was transferred onto a 40 ml sucrose cushion (LSB plus 100 mM sucrose) and centrifuged (10 minutes, 4°C, 4000 ***g***). Supernatant was removed and the chromatin was resuspended in 2 ml TE and sonicated (Branson sonifier 250-D, 35% amplitude, 2 minutes in 1 second intervals). For partial DNA digests, 2 mM $CaCl_2$ and 8 U micrococcal nuclease (MNase) (Roche) were added to the chromatin and incubated for 10 minutes at 37°C. The reaction was stopped by adding 5 mM EGTA.

For immunoprecipitation, the extract was adjusted with 1/10 volume of 11× NET (550 mM Tris-HCl pH 7.4, 1.65 M NaCl, 5.5 mM EDTA, 5.5% NP40). 10 μg affinity-purified polyclonal antibodies (HsOrc3p, HsMcm3/7p), 15 μl of polyclonal HsOrc1p antiserum or 50 μl supernatant of monoclonal antibodies (EBNA1, HsOrc6p) were added respectively. The immunoprecipitation and purification of co-precipitated DNA was performed as illustrated (Schepers et al., 2001). Real-time PCR analysis was performed according to the manufacturers instructions using the same parameters and primer pairs as described (Schepers et al., 2001). A detailed protocol for the ChIP experiments are available (http://haema145.gsf.de/).

Results

Chromatin association of EBNA1 and pre-RC components during the cell cycle

Studies in the yeast *Saccharomyces cerevisiae* have shown that ORC is associated with chromatin throughout the cell cycle, whereas the MCM2-MCM7 complex is recruited to chromatin during late mitosis and G1, and released from DNA with ongoing replication. Mendez and Stillman have recently reported that this general phenomenon also occurs at the chromatin of human cell lines (Mendez and Stillman, 2000).

To compare the cell-cycle- and sequence-dependent binding of pre-RC components to *oriP* and global cellular chromatin, we separated different cell cycle phases of logarithmically growing human lymphoblastoid B-cell line A39 (Schepers et al., 2001) by centrifugal elutriation. The advantage of this method is that cells have not been treated by drugs that might interfere with metabolism and cause pleiotropic effects. The chromatin-binding assay described by Mendez and Stillman (Mendez and Stillman, 2000) was modified and used to analyse the presence of proteins on global chromatin. Briefly, cells were lysed in a hypotonic buffer containing Triton X-100 and sucrose. Nuclei were collected by low-speed centrifugation and washed in a low-stringency buffer. Proteins remaining associated with the chromatin were extracted and analysed by immunoblot analysis. To test the quality of the separation and cell cycle progression, fluorescence-activated cell sorting (FACS) profiles of the different fractions were determined in parallel with the chromatin association of cyclins E, A and B1 (Fig. 1A). Cyclin E was chromatin bound during G1 until cells entered S phase (fractions 35-55) and was then released. Cyclins A and B1 accumulated during S phase. Cyclin A dissociated from chromatin when cells entered mitosis, whereas cyclin B1 remained associated (fraction 90). The expression pattern of the cyclins at the different stages of the cell cycle indicate that centrifugal elutriation is a suitable way to analyse cell-cycle-dependent changes on chromatin.

Fig. 1B shows the cell cycle behaviour of EBNA1 and several pre-RC components. Only a small proportion of EBNA1 protein was stably associated with chromatin throughout the cell cycle. EBNA1 is a very abundant protein (20,000-40,000 copies per cell), whereas each *oriP* contains only 24 EBNA1-binding sites (Sternas et al., 1990). Mini-EBV episomes are maintained with an average copy number of five to ten per cell (Schepers et al., 2001). The biochemical separation of soluble and chromatin-enriched fractions indicated that the great majority of HsOrc2 to HsOrc4p was constantly bound to chromatin throughout the cell cycle. No cell-cycle-dependent changes were monitored for HsOrc6p but only half of the total amount of this subunit was associated with chromatin (Fig. 1B). The largest subunit of HsOrc, HsOrc1p, changed its chromatin association during the cell cycle. It was chromatin bound during G1 phase and dissociated partly from chromatin as cells progressed through S phase (Fig. 1B,C). This indicates that human ORC is a dynamic complex with HsOrc1p as the temporally controlled component.

The chromatin association of HsMcm3p and HsMcm7p was tightly cell cycle regulated (Fig. 1B,C). The proportion of these proteins bound to chromatin kept increasing from early G1 phase until the G1-S transition, when cyclins A and B1 became activated. Subsequently, HsMcm2p-HsMcm7p were released from chromatin during S phase. Liberation of HsMcm2p-HsMcm7p paralleled the chromatin release of HsOrc1p, indicating that the temporal order of origin activation might be responsible for the disassembly of the origin complex. Fig. 1C summarizes in a polygon plot the proportion of protein chromatin association with cell cycle progression. The quantification of chromatin-bound HsOrc1p indicated a relative stable, even chromatin association during G1 phase, whereas the HsMcm2p-HsMcm7p kept accumulating until the onset of S phase. Both HsMcm2p-HsMcm7p and HsOrc1p were released during S phase, although ~10% of the HsMcm2p-HsMcm7p and 25-30% of HsOrc1p remained associated with chromatin during G2 and mitosis, relative to maximal levels seen in association with chromatin at earlier times.

At least four independent experiments were performed. We found that the HsOrc1p was rapidly degraded within the soluble fractions. The amount was consistent within one experiment but slight variations were observed between independent experiments (data not shown). To determine, whether the degradation was 26S proteasome dependent, we analysed the total amount of HsOrc1p in the presence or absence of the specific proteasome inhibitor MG132 (Fig. 1B, bottom). Equivalent amounts of whole cell extracts were

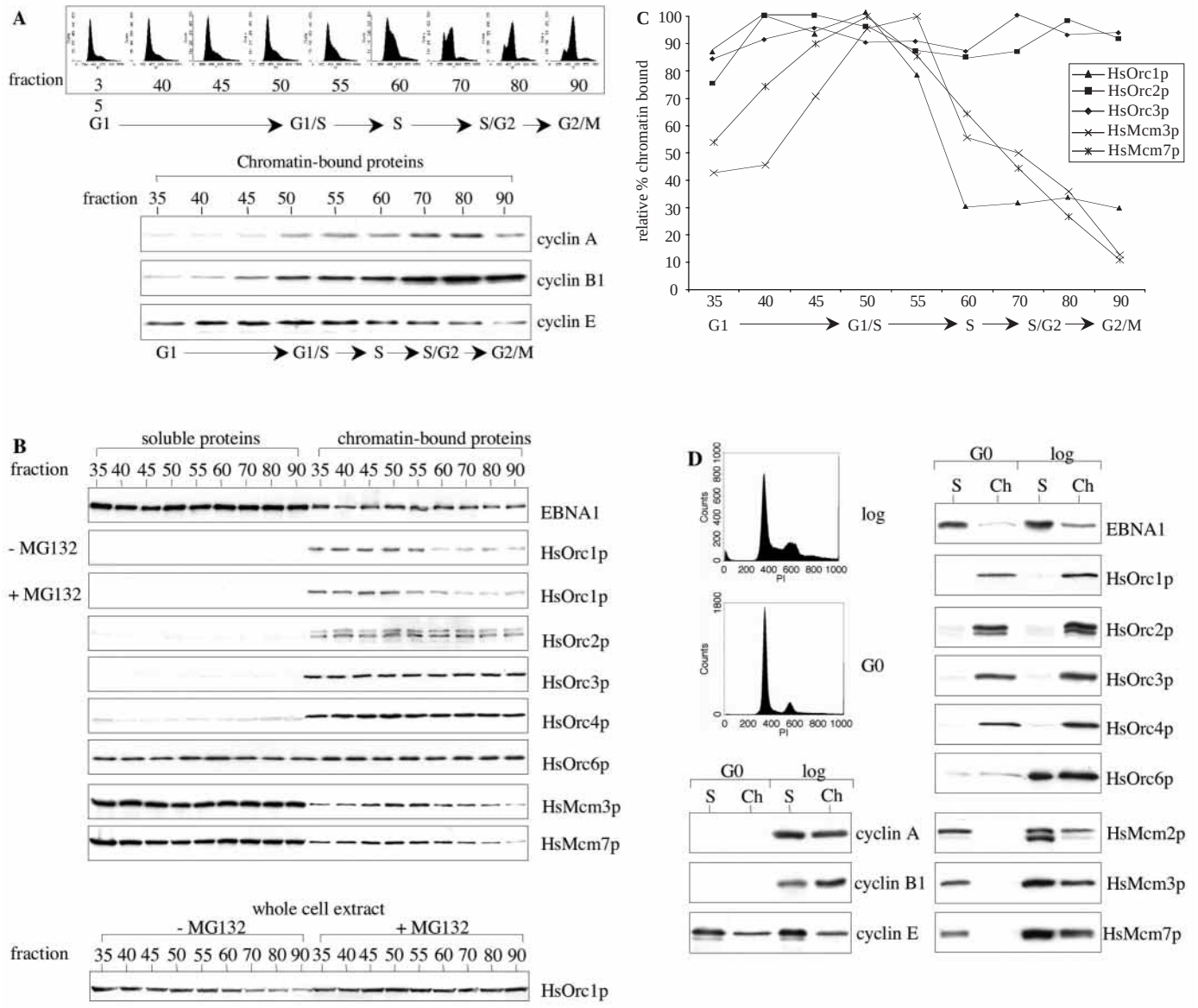


Fig. 1. Cell-cycle-dependent chromatin-binding of proteins in A39 cells. Cell cycle phases of logarithmically growing A39 cells were separated by centrifugal elutriation. The DNA content of the different fractions was determined by FACS. Soluble and chromatin-bound proteins of each fraction were separated by cell fractionation and investigated after SDS-PAGE by immunoblot analysis. (A) FACS profiles of the separated fractions (top) and immunoblot analysis of chromatin-associated cyclins A, B1 and E (bottom). (B) Immunoblot studies of EBNA1 and pre-RC components using antibodies as indicated on the right. Chromatin-binding experiments of hsOrc1 were performed in parallel in the presence or absence of 25 μM MG132 as indicated on the left. (Bottom) To analyse the relevance of a 26S-proteasome-dependent degradation, whole cell extracts (WCE) were prepared by lysing cells in RIPA buffer in the absence (left) or presence (right) of 25 μM MG132. After SDS-PAGE of equivalent amounts of WCE, the presence of HsOrc1p was detected by immunoblotting. (C) Relative ratios of chromatin bound proteins analysed in (B). Signal intensities of the respective autoradiograph were quantified using NIH Image and plotted against the flow rate (ml minute^{-1}) corresponding to cell cycle progression. The highest intensity of each individual factor was set to 100%. HsOrc4p and HsOrc6p are not shown for the clarity of the figure. (D) For G0 experiments, A39 cells were grown to high density and arrested for 3 days. G0-arrested and logarithmically growing A39 cells (FACS profiles on the top left) were fractionated using the chromatin-binding protocol. Soluble (S) and chromatin-bound (Ch) proteins were separated by SDS-PAGE and immunoblots were probed with antibodies as indicated on the right.

separated and analysed by immunoblotting. HsOrc1p was sensitive to the proteasome after the G1-S transition in the absence of MG132 but was stabilized if the inhibitor was added to the lysis buffer. This indicates that the degradation of HsOrc1p occurred after cell lysis and not in vivo, because the proteasome inhibitor was only added to the RIPA lysis buffer and not to the cell culture medium. The cell-cycle-dependent chromatin association was not affected by MG132, indicating that only the soluble portion of HsOrc1p was susceptible to proteasome-dependent degradation. All cell cycle experiments were repeated with the Burkitt's lymphoma cell line Raji (data not shown). The cell cycle behaviour of the investigated proteins in Raji cells corresponded in principle to our results obtained with A39

Table 1. Cell-cycle-dependent enrichment of EBNA1 at *oriP* and the remote reference site I3

A. EBNA1

		Cell cycle phases (Cp difference)					Average	
Primer	$E_{const.}$	G1	G1/S	S	S/G2	G2	Cp	Enrichment
sc2	1.74±0.01	5.6±0.14	4.8±1.56	5.7±0.78	5.3±1.2	4.7±1.0	5.2±0.93	18.2±4.3
sc3	1.84±0.01	8.5±0.85	8.0±1.06	9.0±0.35	9.1±1.34	8.1±2.05	8.5±1.13	185±55
sc4	1.83±0.03	9.6±0.07	9.9±0.42	10.0±1.13	9.9±0.35	8.6±1.1	9.6±0.78	348±82
sc5	1.97±0.03	9.7±0.21	9.2±1.48	9.9±0.28	9.8±0.99	9.2±1.7	9.5±0.93	659±149
sc6	1.95±0.03	10.0±0.42	8.4±1.20	9.5±0.14	9.4±0.71	8.6±1.27	9.1±0.71	506±199
sc7	1.92±0.03	9.0±0.21	8.7±1.20	9.3±0.71	9.6±0.49	8.7±0.71	8.9±0.71	371±96
sc8	1.94±0.04	9.4±0.57	8.4±1.2	8.8±1.06	8.9±0.21	8.1±1.56	8.8±0.93	340±90
sc10	1.88±0.02	3.4±0.57	3.6±0.38	3.4±0.35	4.4±0.42	4.3±0.71	3.8±0.47	11.5±3.7
I3	1.98±0.01	3.0±0.64	3.3±0.49	2.8±0.35	3.5±0.35	3.3±0.42	3.2±0.28	8.8±1.6

B.

		x-fold enrichment above reference level (I3-fragment)					
Primer	$E_{const.}$	G1	G1/S	S	S/G2	G2/M	Average
sc2	1.74±0.01	2.6	1.6	2.6	2.1	1.6	2.1±0.5
sc3	1.84±0.01	24.6	17.4	32.7	34.8	21.7	26.2±7.4
sc4	1.83±0.03	36.9	45.6	48.4	44.2	24.9	40.0±9.5
sc5	1.97±0.03	79.8	56.9	94.6	88.4	58.8	75.7±17.1
sc6	1.95±0.03	91.4	37.1	65.4	61.2	35.9	58.2±22.9
sc7	1.92±0.03	39.4	32.4	49.6	58.3	33.5	42.7±11.11
sc8	1.94±0.04	52.5	30.6	41.5	44.4	26.8	39.1±10.4
sc10	1.88±0.02	1.0	1.1	1.0	1.8	1.7	1.3±0.4

(A) Summary of the histogram in Fig. 2B. E_{const} was determined from standard curves of tenfold dilutions as described (Schepers et al., 2001). The *Cp* differences and standard deviations of three independent experiments are shown for the five fractions analysed. The mean values of each DNA segment and cell-cycle phase were determined (*Cp* difference). Because EBNA1 is cell-cycle-independently bound, the mean value of each fragment was established (*Cp* average) and used as exponent to determine the enrichment by the equation $N=E_{const}^{\Delta Cp}$ (average enrichment). (B) To calculate the *oriP*-specific enrichment, we divided the mean value of the individual scanning fragments and the different cell cycle points with the accumulation of the reference fragment I3 (ΔC_p=3.2 cycles, E_{const}=1.98, enrichment=$1.98^{3.2}$=8.8 times). The average enrichments over the cell cycle are listed on the right.

cells and data published for other cell lines, although HsOrc1p appears not to be completely released from chromatin (Mendez and Stillman, 2000; Ritzi et al., 1998).

To complete the cell cycle analysis, we also investigated chromatin association of pre-RC components in G0-arrested cells. Therefore, A39 cells were grown to high density causing arrest after 3 days with a 2C DNA-content (Fig. 1D). Chromatin-binding assays indicated that neither cyclin A nor cyclin B1 were detectable, whereas cyclin E levels seemed to be unaffected. A similar result was observed by Ohtsubo et al. (Ohtsubo et al., 1995) and it is very likely that the CDK2/cyclin-E complex is masked by high levels of p27 (Hara et al., 2001; Nakayama et al., 2001; Reed, 2002). Chromatin association of EBNA1 appeared to be slightly reduced in quiescent cells compared with logarithmically growing cells.

HsORC is a dynamic complex during the proliferative cycle and so the levels of DNA-bound ORC constituents were different in G0-arrested and logarithmically growing cells. Levels of HsOrc2p-HsOrc4p were constant in arrested cells, and these proteins remained chromatin associated (Fig. 1D). By contrast, chromatin-bound and soluble HsOrc6p decreased in G0 cells (Fig. 1D). About 50% of HsOrc1p disappeared from global chromatin in quiescent cells (Fig. 1D). Again, no HsOrc1p could be detected in the supernatants. Similar results were obtained in the presence of MG132 (data not shown). In summary, our results prove that HsOrc1p is the only ORC subunit regulated at protein level of the proliferative cycle. Resting cells illustrated a reduced chromatin association of HsOrc1p. Chromatin-bound HsMcm2p-HsMcm7p and HsOrc6p were hardly detectable in G0-arrested cells. The total amount of Mcm2p-Mcm7p was reduced in quiescent cells, indicating reduced protein synthesis. In differentiated cells, HsMcm3p has a half-life of ~24 hours, whereas the amount of HsOrc2p does not change (Musahl et al., 1998).

EBNA1 is associated with *oriP* throughout the cell cycle

The second part of this study focuses on the complex protein-DNA dynamics at a specific origin of DNA replication, the latent origin of EBV (*oriP*). We used five of the nine cell cycle fractions obtained by centrifugal elutriation (40 ml minute^{-1}, 50 ml minute^{-1}, 60 ml minute^{-1}, 80 ml minute^{-1}, 90 ml minute^{-1}; Fig. 1A) to analyse the dynamics of protein complexes at *oriP* by ChIP combined with real-time PCR (an outline of the mini-EBV 1478.A and primer locations is given in Fig. 2A). Nuclei were prepared following the protocol for the chromatin-binding assay. Before fixation with formaldehyde, nuclei were washed with a buffer containing 200 mM sodium chloride. Preparing nuclei according to the chromatin-binding protocol allowed a direct comparison of the situation at the chromatin level with the dynamics site-specifically observed at *oriP*. ChIP experiments were performed with DNA fragments of average length 300-500 bp. The data shown in Fig. 2B confirmed that EBNA1 is bound to both *oriP* elements throughout the cell cycle. This result agrees with previous reports (Hsieh et al., 1993; Niller et al., 1995). The overall level compared with the isotype control was several-hundred-fold higher in the region of *oriP* (primer pairs sc3 to sc8) and reduced to 11-18 times 2 kbp up- and downstream of *oriP* (sc2 and sc10, Fig. 2B, Table 1A).

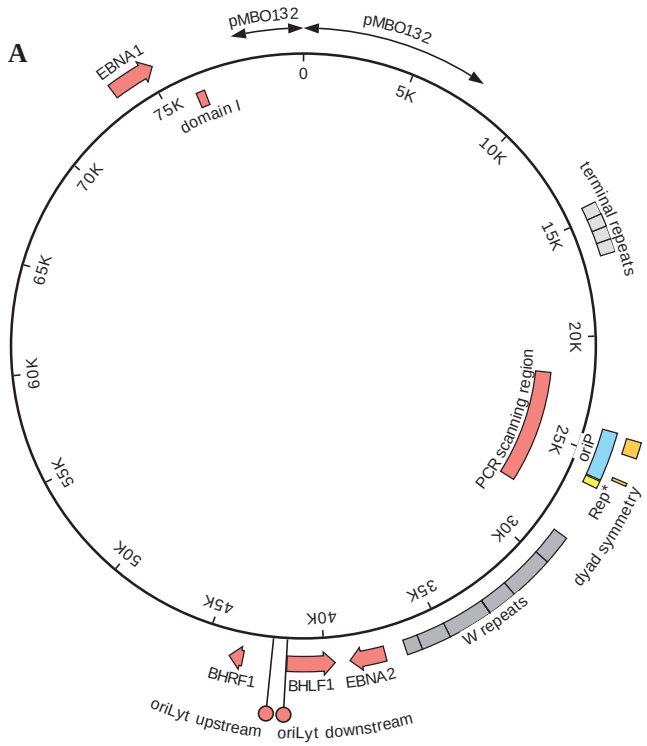
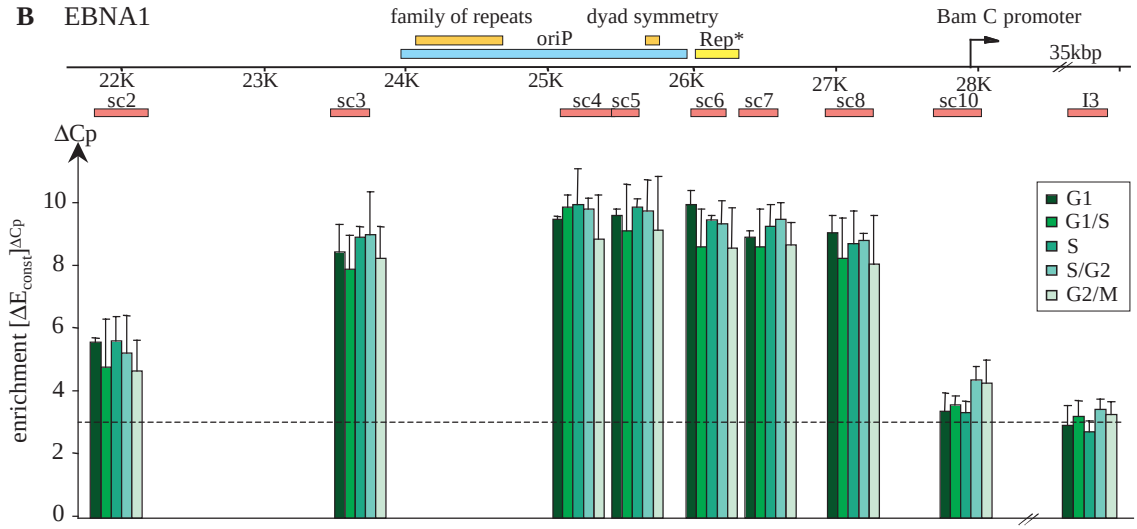


Fig. 2. EBNA1 binds cell cycle independently at *oriP*. (A) Mini-EBV plasmid 1478.A used to immortalize human primary B cells. Some functional elements are shown on the outer circle. The plasmid backbone derived from the F-factor plasmid pMBO132 (arrows). EBNA1 is shown with some cis elements [*OriP*, *ori*Lyt (the lytic origin of DNA replication), the terminal repeats and the W repeats]. The inner circle of the map indicates the locations of the fragments that were analysed by PCR amplification after immunoprecipitation. (B) Enlarged view of *oriP* (top). The locations and designation of the PCR fragments used to scan the binding sites of EBNA1, HsOrc and Mcm2-Mcm7 proteins are shown below the ruler (sc2-sc10, I3). Different cell cycle phases were separated by centrifugal elutriation and five cell cycle fractions were subjected to ChIP (G1, 40 ml minute^{-1}; G1/S, 50 ml minute^{-1}; S, 60 ml minute^{-1}; S/G2, 80 ml minute^{-1}; G2/M, 90 ml minute^{-1}). Cross-linked chromatin of 1×10^7 cells was used for each immunoprecipitation. Co-precipitated DNA was isolated and 1/50 thereof was used for each PCR. The histogram shows the result of EBNA1-experiments. The difference between the crossing points of the EBNA1 immunoprecipitate and the isotype control is indicated on the logarithmic *y* axis (ΔC_p). The threshold level marked by the reference I3 is indicated as dotted line. The graph shows the mean values and standard deviations from three independent experiments.



To calculate the enrichment of a specific immunoprecipitation, the same analysis procedure was used as described before (Schepers et al., 2001). Briefly, the amplification efficiency E_{const} for each primer pair was determined with a series of tenfold dilutions. E_{const} is the basis of the enrichment equation $N = N_0 \times (E_{const})^n$, where N is the number of molecules, N_0 is the number of starting molecules and n is the number of cycles. The specific enrichment of a fragment is the difference between the crossing points (C_p) of the specific immunoprecipitate and the threshold level. The threshold level is defined as the enrichment of a fragment in an immunoprecipitate with an isotype antibody (for monoclonal anibodies) or the pre-immune control (for polyclonal antibodies).

The results indicated that the *oriP* flanking regions are 11- to 18-fold enriched (sc2 and sc10, respectively) in EBNA1-specific immunoprecipitates compared with the isotype control (Table 1A). This co-precipitation of remote DNA fragments can be caused by two different effects. First, some DNA fragments might be long enough to allow the amplification of more distal fragments. Second, a weak and non-sequence-specific DNA-binding activity of EBNA1 might cause co-immunoprecipitation of any DNA fragment. To distinguish between these possibilities, we used an additional remote DNA segment (primer pair I3, Fig. 2A). The reference fragment I3 contains no known functional element and is distal to any EBNA1-binding site. In EBNA1-specific immunoprecipitations, this region was ninefold more enriched than in the isotype control (*Cp* difference: 3.2 cycles, Table 1A). To take this non-sequence-specific DNA-binding activity of

EBNA1 into account, we divided the enrichment of all amplicons with the mean value of the remote I3 locus (Table 1B). The sequence-specific enrichment of EBNA1 at *oriP* (sc3-sc8) was up to 75-fold above this reference level, whereas the flanking regions showed no sequence-specific increase.

Chromatin-binding experiments with G0-arrested cells revealed a moderate decrease in the amount of chromatin-bound EBNA1 compared with a logarithmically growing culture (Fig. 1D). In contrast to this observation, the ChIP experiment indicated no detectable difference between cycling and resting cells (Fig. 5). It is likely that EBNA1 consensus motifs that are present in the cellular genome release EBNA1 in resting cells. These sites are probably non-functional (Kang et al., 2001). The ability to replicate is probably one of the last properties that is given up by EBV when entering G0.

Cell-cycle-independent *oriP* binding of HsOrc subunits

The chromatin-binding assay indicated that HsOrc1p is bound to chromatin in the G1 phase of the cell cycle and is at least partly released during S phase. All other HsOrc subunits examined remained associated with chromatin throughout the cell cycle. To test whether HsOrc subunits are bound to *oriP* sequences cell cycle dependently, we analysed the *oriP*-specific binding of HsOrc1p, HsOrc3p and HsOrc6p in ChIP experiments (Fig. 3). HsOrc3p, a member of the ORC core complex (Vashee et al., 2001), was constantly bound to *oriP*. The binding affinity peaked near the DS element, as has been described before with asynchronous cells (sc5 and sc6, Fig. 3A) (Schepers, 2001). Reference fragment I3 showed an accumulation that was also cell cycle independent and, on average, 2.3 times above the DNA amount co-precipitated with the pre-immune control. We used the I3 binding level as a reference to calculate the specific enrichment of the scanning fragments located at *oriP* (Table 2A). The DS elements flanking amplicons sc5 and sc6 were enriched 20-fold, whereas basically no accumulation was detected 2 kbp to both sides of this *oriP* element (Fig. 3A).

ChIP experiments confirmed a weak *oriP*-association of HsOrc6p. The enrichment levels obtained in HsOrc6p ChIP experiments were considerably lower than for HsOrc3p (three times versus 20 times above I3 level; Fig. 3B, Table 2B). In addition, the standard deviation was remarkably high even though seven independent experiments were carried out. There might be several reasons for this finding. It is possible that HsOrc6p is not constantly bound to the complex, which results in less efficient cross-linking and co-precipitation of specific DNA fragments. We also analysed whether the experimental set up might also influence the DNA-binding efficiency and cross-linked cells before and after preparing nuclei. No difference could be detected between the two protocols (data not shown), indicating that HsOrc6 has *per se* a low affinity to the complex. According to the chromatin-binding experiment, HsOrc3p remained bound to *oriP* in G0-arrested cells whereas HsOrc6p disappeared completely (Fig. 1D, Fig. 5). In summary, our findings indicate that HsOrc3p and HsOrc6p are not cell cycle regulated. Compared with HsOrc3, HsOrc6 is only weakly attached to the complex, and it will be interesting to find out whether any particular function is linked to this characteristic.

Table 2. Enrichment of HsOrc3p, HsOrc6p and HsOrc1p at *oriP* during the cell cycle

	x-fold enrichment above reference level (I3-fragment)					
Primer	G1	G1/S	S	S/G2	G2/M	Average
A. HsOrc3p						
sc2	0.9	1.6	1.9	2.4	2.0	1.8±0.5
sc3	1.3	1.6	2.0	1.9	2.0	1.8±0.3
sc4	8.5	7.9	10.2	10.2	11.1	9.6±1.3
sc5	18.3	20.1	24.1	21.5	26.9	22.2±3.4
sc6	22.7	14.7	20.3	19.4	18.1	19.0±2.9
sc7	11.7	11.5	12.5	14.3	13.1	12.6±1.1
sc8	6.3	6.6	8.7	9.7	10.0	8.3±1.7
sc10	0.7	1.1	1.0	1.3	0.9	1.0±0.2
B. HsOrc6p						
sc2	0.9	1.2	1.2	1.4	1.0	1.1±0.2
sc3	0.8	0.9	1.1	0.9	1.0	1.0±0.1
sc4	2.6	2.6	2.1	2.3	2.1	2.3±0.3
sc5	3.5	3.2	2.7	3.5	3.2	3.2±0.3
sc6	2.8	2.3	2.2	3.2	3.0	2.7±0.5
sc7	2.8	3.0	2.6	2.8	3.2	2.9±0.2
sc8	2.5	2.5	2.7	2.4	2.5	2.5±0.1
sc10	1.3	1.3	1.3	1.7	1.6	1.4±0.2
C. HsOrc1						
sc2	1.1	1.3	1.2	1.3	1.2	
sc3	0.9	1.4	1.6	1.3	1.2	
sc4	9.8	12.9	5.9	6.4	6.2	
sc5	23.4	24.3	9.2	6.1	9.4	
sc6	21.8	27.4	9.1	9.2	8.9	
sc7	16.6	15.9	7.9	5.8	7.2	
sc8	6.9	7.6	4.2	4.3	5.3	
sc10	1.9	1.8	1.4	1.3	1.4	

Table 2 summarizes the *oriP*-specific enrichment shown in the histograms in Fig. 3. E_{const} was determined from standard curves of tenfold dilutions as described (Schepers et al., 2001). The *Cp* differences and standard deviations of three independent experiments are shown for the five fractions analysed. The mean values of each DNA segment and cell-cycle phase were determined (*Cp* difference). Because EBNA1 is cell-cycle-independently bound, the mean value of each fragment was established (*Cp* average) and used as exponent to determine the enrichment by the equation $N=E_{const}^{\Delta Cp}$ (average enrichment). The mean value of the reference fragment I3 was determined and used as divisor to calculate the accumulation for the scanning fragments. The obtained numbers represent the enrichment at the particular fragment above the I3 level.

HsOrc1p *oriP*-binding is cell cycle regulated

Cell cycle ChIP experiments with HsOrc1p-specific antibodies demonstrated more enrichment of DS-proximal DNA fragments in early cell cycle phases than in later phases (Fig. 3C, Table 2C). Scanning fragments at the DS element (sc4 to sc7) indicated a cell-cycle-dependent DNA-binding pattern. More distal segments appeared to be cell cycle independent (sc2, sc3, sc8 and sc10) and similar to the reference I3. The HsOrc1p-specific enrichment of the I3 amplicon was 2.4 times and in the same range as co-precipitated with the HsOrc3p antibody.

Analysing the obtained results in more detail revealed that the *Cp* values of the sections sc4-sc7 were 1.0-1.5 *Cp* cycles higher in early cell cycle phases than in later phases, when most origins had already fired. This difference indicated that 50-70% of *oriP*-bound HsOrc1p are released during S and G2/M (Table 2C). The release of HsOrc1p was in the same range as observed with the chromatin-binding assay (Fig. 1C). These data are the

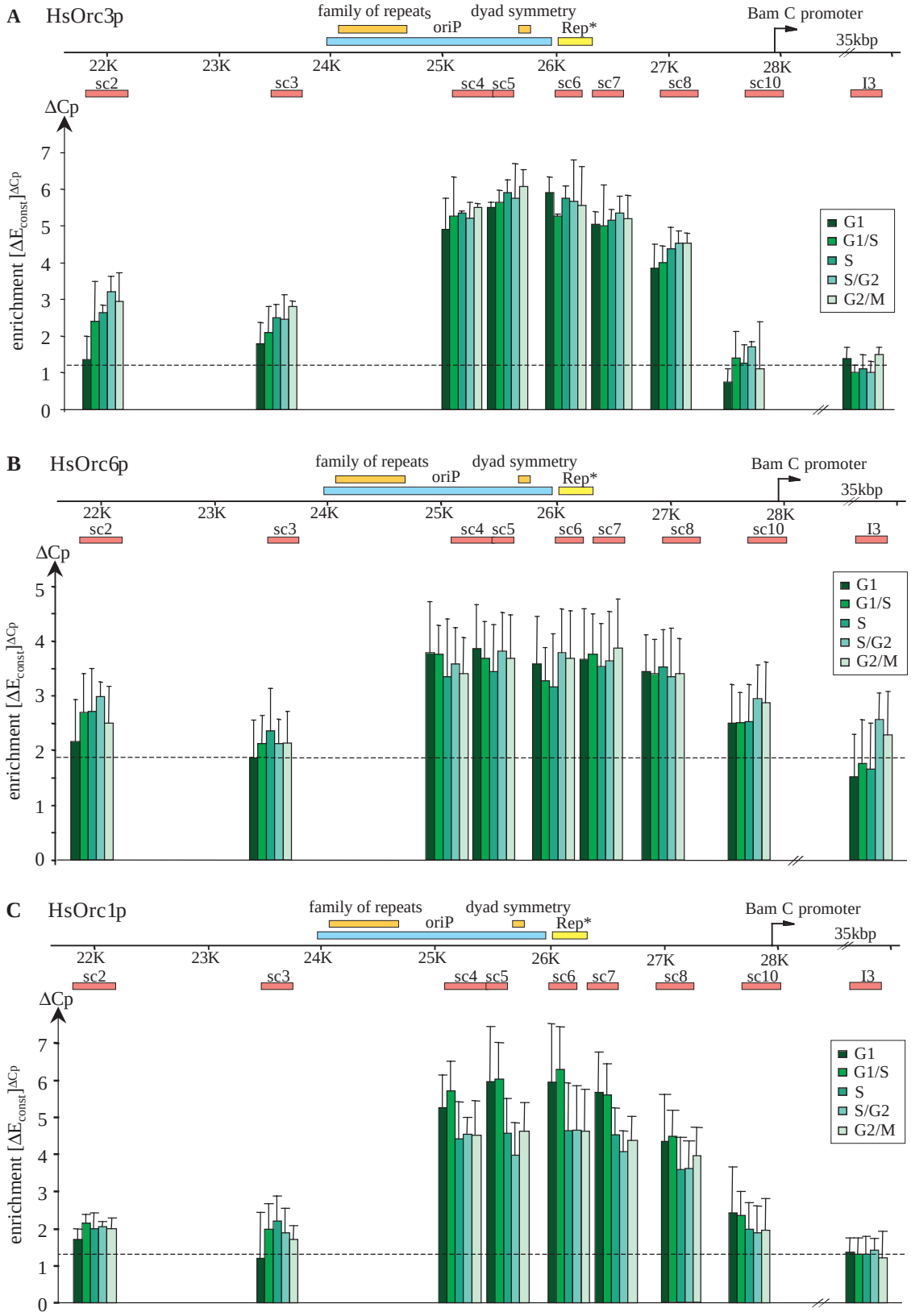


Fig. 3. HsOrc binding at *oriP* during the cell cycle. Different cell cycle phases were separated by centrifugal elutriation and five cell cycle fractions were subjected to ChIP (G1, 40 ml minute^{-1}; G1/S, 50 ml minute^{-1}; S, 60 ml minute^{-1}; S/G2, 80 ml minute^{-1}; G2/M, 90 ml minute^{-1}). Cross-linked chromatin of 1×10^{7} cells was used for each immunoprecipitation with antibodies directed against HsOrc3p (A), HsOrc6p (B) and HsOrc1p (C). Co-precipitated DNA was isolated and 1/50 thereof was used for each PCR. The mean values and standard deviations are calculated of four (A), and seven independent experiments (B,C).

means of seven independent experiments. Nevertheless, the standard deviations were relatively high, especially for the G1 fractions. To find out whether the *Cp* differences were statistically significant, we performed a Krustall-Wallis analysis of the nine independent groups (fragments sc2-sc10 and I3). For sc4-sc7, a statistic probability of $P \leq 0.05$ was determined, indicating statistically significant differences within each group (data not shown). We subsequently performed the Wilcoxon Rank Sum test for these four groups to determine which independent pairs within each population were significantly different. These methods were used because no normal distribution and equal variances could be presupposed. The independent Wilcoxon test evaluated that the differences between the early and late cell cycle phases are significant within the groups sc4-sc7 (data not shown). We would like to point out that, for the G1/S fraction, the significance was generally higher ($P \leq 0.05$) than for the G1 fraction ($0.05 \leq P \leq 0.10$), reflecting the higher variances in the G1 population. The results obtained with these ChIP experiments agree with the results of the chromatin-binding experiment (Fig. 1B), which indicated a similar decrease of chromatin-bound HsOrc1p levels. We postulate from these data that HsOrc1p shows a cell-cycle-dependent DNA-binding activity at the DS-element of *oriP*.

We first assumed that the higher variances found in the G1-fraction of HsOrc1p experiments are caused by a resting (G0) subpopulation that cannot be separated from cells in G1 by elutrial centrifugation. To test this hypothesis, we performed ChIP and chromatin-binding experiments with cells arrested in G0. ChIP experiments with HsOrc1p antibodies indicated that, in G0 cells, the amount of DS-proximal bound HsOrc1p was reduced to the G2/M level (ΔCp=1.9 cycles), whereas the I3 levels remained similar (Fig. 3C, Fig. 5). These data also imply that the high variances of G1 cells cannot be explained by a G0 subpopulation. Logarithmically growing cells showed an intermediate enrichment. The chromatin-binding experiment indicated that ~50% of chromatin-bound HsOrc1p was released in G0 cells (Fig. 1D), which is consistent with the amount released from *oriP* (Fig. 5).

Cell-cycle-dependent association of the HsMcm2p-HsMcm7p complex with *oriP*

We used the same procedure to test whether members of the MCM2-MCM7 complex associate specifically at *oriP* or whether there are differences between the different MCM2-MCM7 subcomplexes. Therefore, we chose antibodies directed against Mcm3p for the Mcm3p/Mcm5p dimer, and against Mcm7p for the Mcm4p/Mcm6p/Mcm7p trimer (Burkhart et al., 1995; Musahl et al., 1995). HsMcm2p-HsMcm7p are expressed to high levels in mammalian cells and remain nuclear even during mitosis (Schulte et al., 1995). As for the EBNA1 ChIP experiments, nuclei were washed with a buffer containing 200 mM sodium chloride before the formaldehyde cross-linking step. This step should minimize nonspecific protein-DNA interactions. Fig. 4 shows the profile of the cell-cycle-regulated interaction of Mcm2p-Mcm7p with *oriP* sequences. The association of Mcm3p (Fig. 4A, Table 3A) and Mcm7p (Fig. 4B, Table 3B) with *oriP* was high during G1 until cells entered S phase. A decrease was observed during S phase and levels remained low during G2 and mitosis.

Table 3. Cell-cycle-dependent and *oriP* specific x-fold enrichment of HsMcm3p and HsMcm7p

	G1	G1/S	S	S/G2	G2/M
A. MCM3					
sc2	1.84	2.3	0.6	1.5	0.9
sc3	1.9	2.2	0.9	1.5	1.9
sc4	10.0	9.4	1.3	4.0	1.7
sc5	23.9	9.5	1.7	4.5	4.4
sc6	19.0	13.9	2.5	5.4	4.2
sc7	15.6	12.0	1.8	2.6	1.4
sc8	16.7	11.2	1.4	3.5	2.4
sc10	0.8	0.6	0.4	0.5	0.4
B. MCM7					
sc2	1.3	0.9	0.6	0.7	1.0
sc3	0.8	2.1	0.7	1.0	0.8
sc4	6.9	7.3	1.5	2.2	1.9
sc5	15.2	12.7	1.9	3.1	3.6
sc6	10.4	8.3	2.0	2.9	2.9
sc7	7.6	6.5	1.2	1.4	1.0
sc8	10.8	11.0	1.5	5.3	1.5
sc10	0.8	0.5	0.6	0.5	0.6

To verify the *oriP*-specific enrichment of HsMcm3p-HsMcm7p, E_{const} was determined from standard curves of tenfold dilutions as described (Schepers et al., 2001). The *Cp* differences and standard deviations of three independent experiments are shown for the five fractions analysed. The mean values of each DNA segment and cell-cycle phase were determined (*Cp* difference). Because EBNA1 is cell-cycle-independently bound, the mean value of each fragment was established (*Cp* average) and used as exponent to determine the enrichment by the equation $N=E_{const}^{\Delta Cp}$ (average enrichment). This table summarizes the data of the histograms in Fig. 4.

The general *oriP*-binding profile of Mcm2p-Mcm7p is similar to that known from *S. cerevisiae* and to the chromatin-binding experiment described above. Mcm2p-Mcm7p accumulated at origin-proximal sequences during G1 and were released from chromatin with ongoing replication (Fig. 4, Table 3). The spatial and temporal differences between the two MCM2-MCM7 subcomplexes were similar. Both Mcm proteins showed a broader distribution over the *oriP* locus than the ORC subunits investigated. PCR fragment sc8, for example, had a similar amplification rate to the DS element proximal scanning fragments. This might reflect the fact that several MCM2-MCM7 complexes are loaded per origin (Donovan et al., 1997; Edwards et al., 2002; Mahbubani et al., 1997). Elements sc2 and sc3, which are separated from all other *oriP* fragments analysed by FR, showed neither cell-cycle-specific nor sequence-specific enrichment. The binding pattern of sc10 was similar to the reference I3.

Both Mcm2p-Mcm7p ChIP experiments proved a cell-cycle-independent DNA-binding activity at I3, which was 5.9-fold (Mcm3p) and 6.3-fold (Mcm7p) above the pre-immune control respectively. Remnant amounts of Mcm2p-Mcm7p have also been observed by others (Alexandrow et al., 2002; Schaarschmidt et al., 2002). This could be due to the lower concentration of Triton X-100 (0.04%) used to lyse cells, with up to 0.5% being used for others (Kreitz et al., 2001). The more stringent conditions might disrupt interactions, which remain intact under our conditions. The association profiles of both Mcm proteins analysed displayed a slight increase at the DS element proximal fragments in the G2/M fraction, which might indicate that rebinding of the MCM2-MCM7 complex occurs in late mitosis. Although this hypothesis could, in principle, be

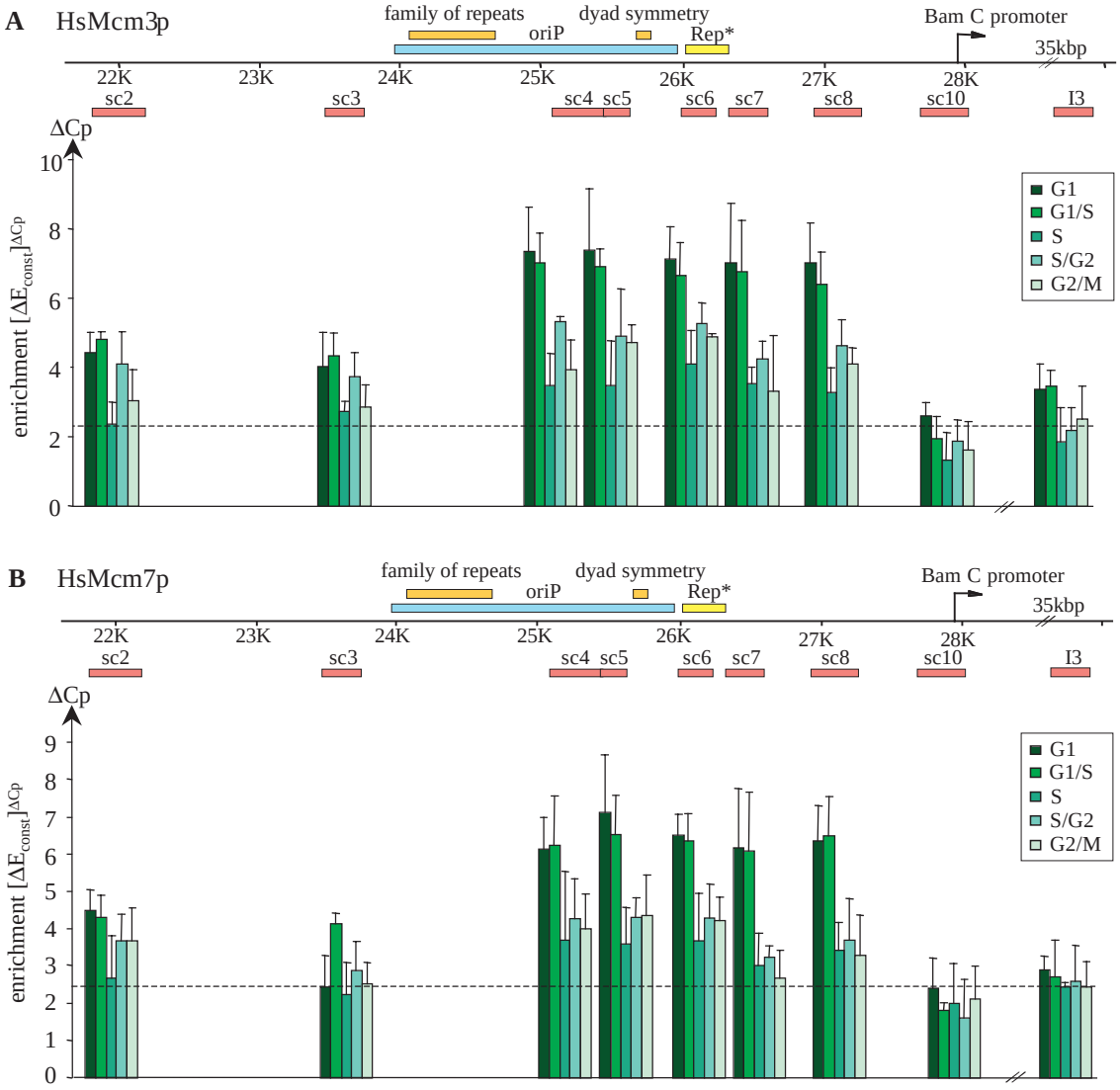


Fig. 4. Cell-cycle-dependent binding of HsMcm2p-HsMcm7p at *oriP*. Different cell cycle phases were separated by centrifugal elutriation and five cell cycle fractions were subjected to ChIP (G1, 40 ml minute^{-1}; G1/S, 50 ml minute^{-1}; S, 60 ml minute^{-1}; S/G2, 80 ml minute^{-1}; G2/M, 90 ml minute^{-1}). Cross-linked chromatin of 1×10^7 cells was used for each immunoprecipitation. Co-precipitated DNA was isolated and 1/50 thereof was used for each PCR. Antibodies directed against HsMcm3p (A) and HsMcm7p (B) were used to visualize the cell-cycle-dependent binding of this complex. The values are calculated from three independent experiments.

tested by nocodazole block and release experiments, such experiments are not feasible in immortalized LCL because these cell lines are not released synchronously after nocodazole treatment (data not shown).

Three independent ChIP experiments with Mcm3p- and Mcm7p-specific antibodies were sufficient to obtain statistically relevant data. As with the chromatin-binding experiment, the release of both Mcm2p-Mcm7p was more quantitative than of HsOrc1p (Fig. 3C, Fig. 4). The sequence-independent DNA-binding level of the Mcm2p-Mcm7p was relatively high compared with HsOrc binding. A possible explanation is the high abundance of Mcm2p-Mcm7p, which was also reflected by the observation that the immunoprecipitation of these proteins was not quantitative (data not shown). ChIP experiments with G0-arrested A39 cells confirmed that levels of HsMcm2p-HsMcm7p detected at the reference I3 remained bound to the *oriP* locus (Fig. 5). At present, we do not know whether this residual binding has any functional significance. In summary, both Mcm3p and Mcm7p showed a cell-cycle-dependent binding both site-specifically to *oriP* and to global chromatin.

Discussion

The activation of individual origins of DNA replication is limited to once per cell cycle and controlled by the sequential binding and release of replication initiation proteins in different phases of the cell cycle. The relevant regulatory event is the ordered formation of pre-RCs during the G1 phase, which culminates in the association of MCM2-MCM7 complexes at origins of DNA replication. Origin activation during S phase is associated with loss of the pre-RCs,

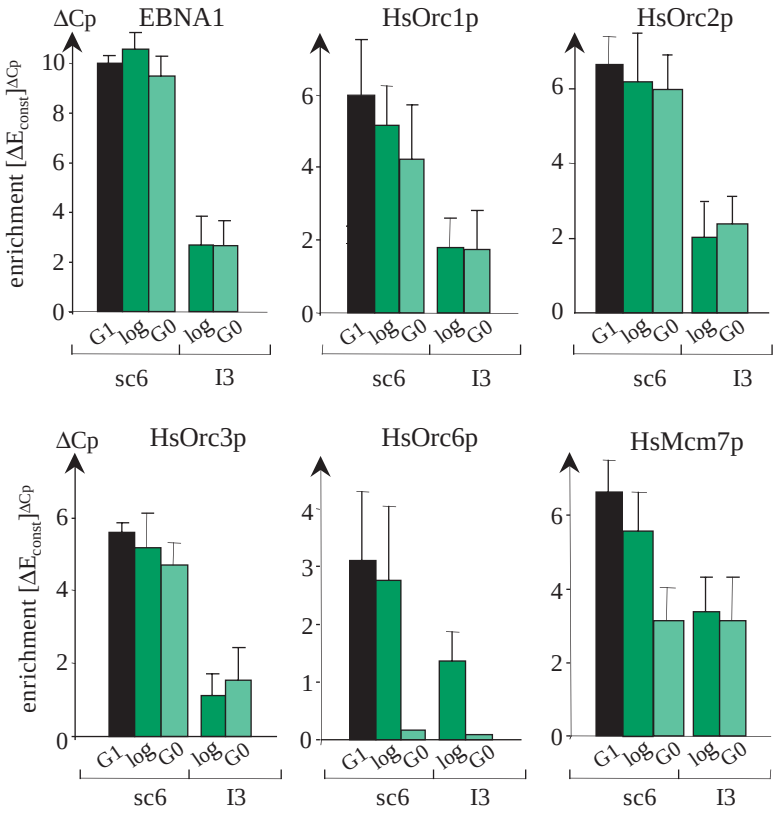


Fig. 5. *OriP*-bound proteins in G0-arrested cells. The same G0 and logarithmically growing cells as analysed in Fig. 1D were used to determine *oriP*-bound proteins. The ChIP experiments were performed and analysed using antibodies directed against HsOrc1p, HsOrc2p, HsOrc3p, HsOrc6p, HsMcm7p and EBNA1. The mean values and standard deviations are calculated from seven independent experiments.

preventing reactivation of each origin during the same cell cycle.

In a direct comparison with extracts from cell-cycle-fractionated cells, we analysed both global chromatin association and origin-specific binding of replication factors in parallel. We used the latent origin of EBV, *oriP*, as model system. In particular, we were interested to study the dynamics of replication initiation proteins such as the components of ORC and the pre-RC in a quantitative fashion in the course of the cell cycle. The composition of the complex components was also analysed in resting cells, which had acquired a G0 state with respect to chromatin-associated and origin-associated proteins. To our knowledge, no such detailed and thorough study has been carried out before in mammalian cells.

Several aspects of this are noteworthy. (i) The data indicate that all proteins analysed in this study exhibited the same dynamics at *oriP* as at global chromatin. (ii) Orc constituents were chromatin- and *oriP*-bound throughout the cell cycle, except HsOrc1p. (iii) Mcm2p-Mcm7p as well as HsOrc1p were recruited to chromatin and *oriP* during G1 and simultaneously released with ongoing replication. (iv) During G0, the ORC 'core', consisting of HsOrc2, HsOrc3, HsOrc4 and HsOrc5 proteins, remains associated with the origin and with global chromatin, whereas HsOrc1p is released from both chromatin and *oriP*. By contrast, Mcm2p-Mcm7p and HsOrc6p are absent or extremely reduced.

The binding of replication factors to *oriP* follows the same kinetics as their association with global chromatin. This observation indicates that the binding of replication factors to chromatin probably reflects their specific binding to cellular origins of DNA replication. Although we have only limited information about the biochemistry and protein dynamics of a few cis-acting sequences that are bona fide origins of DNA replication in metazoan cells, it appears that lessons learnt from model organisms such as *S. cerevisiae* are valid even in complex metazoan systems. Moreover, detailed analysis of the chromatin-binding properties of various ORC subunits, Mcm2p-Mcm7p and EBNA1 proves that *oriP* follows the principles of the replication licensing system (Blow and Laskey, 1988; Diffley, 1996; Diffley et al., 1994).

This study confirms our previous finding that HsOrc binds at or near the DS element of *oriP* (Schepers et al., 2001). EBNA1, the only viral factor involved in latent DNA replication, seems to function as a recruiting factor for ORC. EBNA1 is bound to both essential elements of *oriP* throughout the cell cycle and remains bound even during G0. HsOrc3 and HsOrc6 proteins are present on chromatin throughout the cell cycle. Because HsOrc3p is a member of the ORC core complex, it is very likely that the other components of the core (HsOrc2, HsOrc4 and HsOrc5) are also present at *oriP*, because they are constantly bound to chromatin (Fig. 1B) (Bell and Dutta, 2002). In addition, this study clearly suggests that *oriP* is regulated like a mammalian origin of DNA replication. Our data provide evidence for the first time that HsORC and the HsMcm2p-HsMcm7p complex exhibit the same temporally modulated patterns at *oriP* as at global chromatin. HsMcm2p-HsMcm7p accumulate during G1 and most of these proteins is released during S phase, which is in line with a recent report by Schaarschmidt et al., who studied the cell-cycle-dependent binding of Mcm2p-Mcm7p and HsOrc2p at a potential origin at the *HsMcm4*-promoter region (Schaarschmidt et al., 2002). We found that Mcm2p-Mcm7p are recruited to chromatin and *oriP* while cyclin E is active, and are set free again as soon as cyclin A is recruited and starts to activate origins. The chromatin-binding experiments also indicate a cell-cycle-dependent chromatin association of cyclins. Because it is known that cyclin A and cyclin E interact with ORC and Cdc6p respectively (Furstenthal et al., 2001; Romanowski et al., 2000), it is tempting to speculate that cyclins are also associated with *oriP*, thus integrating latent DNA replication of EBV into the cell cycle.

In contrast to *S. cerevisiae*, there is growing evidence to suggest that metazoan Orc1p is not only crucial for the ATP/ADP-dependent origin binding but appears to be also cell cycle regulated (Asano and Wharton, 1999; Kreitz et al., 2001; Ladenburger et al., 2002; Li et al., 2000; Li and DePamphilis, 2002; Natale et al., 2000). Therefore, metazoan Orc1p might be a functionally limiting component for the formation of pre-RCs in the G1 phase of the cell cycle. It is controversial, whether Orc1p is completely or only partly released from chromatin during S phase. HsOrc1p was reported to be

completely released in S phase from chromatin (Fujita et al., 2002; Kreitz et al., 2001; Mendez et al., 2002) and a cellular origin of replication (Ladenburger et al., 2002) and degraded by the proteasome. Reports from other investigators challenge this idea (Okuno et al., 2001; Tatsumi et al., 2000). Our data support a model in which HsOrc1p is only selectively released from both chromatin and *oriP* in accordance with previous independent chromatin-binding studies (Asano and Wharton, 1999; Li and DePamphilis, 2002; Natale et al., 2000). The observation that only about half of HsOrc1p is released from chromatin as well as *oriP* allows several interpretations. (i) HsOrc1p might only be released from the 'parental' ORC that was involved in origin activation, whereas the 'new' ORC bound to the second allele contains HsOrc1p. (ii) HsOrc1p might change its affinity to the core complex (and/or the stability of this interaction) during the cell cycle and is less well associated when cells progress through S phase. This latter hypothesis is supported by the observation that HsOrc1p is biochemically only moderately attached to the Orc2 to Orc5p subcomplex (Mendez et al., 2002; Vashee et al., 2001). The rather high variation with HsOrc1p-specific antibodies in our ChIP experiments could reflect this scenario.

The data presented here are consistent with reports that HsOrc1p is sensitive to the 26S proteasome during S phase, G2 phase and mitosis. Our experiments, however, indicate that HsOrc1 is sensitive to the 26S proteasome after release from DNA but is not degraded in vivo (Fig. 1B). The observation that the total amount of HsOrc1p is stable over the cell cycle if a specific proteasome inhibitor is added to the lysis buffer indicates a modification of the protein that makes it sensitive to the proteasome. We have, however, been unable to detect increasing amounts in the soluble fractions in the course of the cell cycle, regardless of whether MG132 was present or not. This indicates that the protein is degraded by a cell-cycle-independent proteolytic activity probably contaminating the soluble fractions that is not inhibited by the common protease inhibitors but by RIPA. This proteolytic activity would explain the difficulty of detecting soluble HsOrc1p not only by us but also by other investigators. Possible modifications of HsOrc1 after chromatin release include ubiquitylation and/or phosphorylation by cyclin-A/Cdk2 (Laman et al., 2001). The data provided by our and other studies indicate that Orc1p might be the limiting subunit of metazoan ORC, a situation very different from *S. cerevisiae*, in which Orc1p is a stable constituent of the ORC complex throughout the cell cycle and is absolutely essential for the DNA-binding activity of ORC. The mammalian ORC core complex appears to be bound stably to both chromatin and *oriP* in resting cells, even with reduced amount of DNA-associated HsOrc1p. Nevertheless, it will be interesting to learn how the DNA-binding activity of metazoan ORC is regulated in the partial or complete absence of Orc1p or whether Orc1p can modulate the ORC-origin interaction.

HsOrc6p appears to be a special case with respect to its loose attachment to HsORC (Dhar and Dutta, 2000; Keller et al., 2002; Vashee et al., 2001). Our results indicate that only about half of all HsOrc6 molecules are associated with chromatin. The loose association of HsOrc6p to HsORC might indicate that Orc6p is less tightly involved in chromatin and origin recognition similarly to its *S. cerevisiae* homologue. It is also possible that this subunit is expressed at much higher levels than the larger Orc components because it is also involved in other cell processes (Prasanth et al., 2002). Following this hypothesis, the non-sequence-specific binding to DNA was remarkable in HsOrc6p ChIP experiments compared with all other ORC subunits analysed. The role of HsOrc6p in G0-phase cells seems to be peculiar for several reasons. HsOrc6p dissociates completely from chromatin and *oriP*, loses its nonspecific DNA-binding characteristics, and is expressed at low levels.

In conclusion, in this study, we survey the association of HsOrc and HsMcm2p-HsMcm7p constituents and EBNA1 with cellular chromatin and *oriP*. All analysed proteins show the same dynamics (qualitatively and quantitatively) at *oriP* as at global chromatin. Our data confirm the hypothesis of Dhar et al. that *oriP* is regulated like a chromosomal origin (Dhar et al., 2001). We provide direct evidence that *oriP* is a suitable model system for a bona fide mammalian origin. We demonstrate that ORC itself is a dynamic complex. It will be interesting to gain insight into the functional role of both HsOrc1p and HsOrc6p, which appear to be the two dynamic and presumably crucial ORC components. In G0-arrested cells, components of the ORC core complex remain associated with DNA, whereas Mcm2p-Mcm7p, HsOrc1 and HsOrc6 could not be detected. This scenario ensures that origins remain marked in resting cells and enable a rapid re-entry into the cell cycle.

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