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Identification and Functional Analysis of a Human Homologue of the Monkey Replication Origin ors8

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Abstract We previously isolated from African green monkey (CV-1) cells a replication origin, ors8, that is active at the onset of S-phase. Here, its homologous sequence (hors8, accession number: DQ230978) was amplified from human cells, using the monkey-ors8-specific primers. Sequence alignment between the monkey and the human fragment revealed a 92% identity. Nascent DNA abundance analysis, involving quantification by real-time PCR, indicated that hors8 is an active replication origin, as the abundance of nascent DNA from a genomic region containing it was 97-fold higher relative to a non-origin region in the same locus. Furthermore, the data showed that the hors8 fragment is capable of supporting the episomal replication of its plasmid, when cloned into pBlueScript (pBS), as assayed by the *Dpn*I resistance assay after transfection of HeLa cells. A quantitative chromatin immunoprecipitation (ChIP) assay, using antibodies against Ku, Orc2, and Cdc6, showed that these DNA replication initiator proteins were associated in vivo with the human ors8 (hors8). Finally, nascent DNA abundance experiments from human cells synchronized at different phases of the cell cycle revealed that hors8 is a late-firing origin of DNA replication, having the highest activity 8 h after release from late G_1.
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Key words: origin-enriched sequences (ors); human replication origin; episomal; nascent DNA; replication

DNA replication initiates at chromosomal sites called replication origins. Work from the bacteria *Escherichia coli* and the budding yeast *Saccharomyces cerevisiae* suggested that origins are specific DNA sequences to which initiator proteins bind to promote initiation of DNA replication [Ogasawara et al., 1991; Nasheuer et al., 2002]. Control over the timing and the frequency of initiation is exerted at the origin, where the pre-replication complex (pre-RC) assembles (reviewed by Bell and Dutta [2002]). Numerous origins have been isolated and characterized in several eukaryotes, including *S. cerevisiae, Schizosaccharomyces pombe, Drosophila melanogaster*, Chinese hamster ovary cells (CHO), murine, monkey, and human cells [reviewed in Todorovic et al., 1999; Zannis-Hadjopoulos and Price, 1999]. In *S. cerevisiae*, replication origins are well-characterized, consisting of an essential 11-bp consensus sequence plus several additional elements that contribute to the activity of the origins. The 11-bp consensus sequence is the binding site for the origin recognition complex (ORC) of proteins [Bell and Stillman, 1992], which serves as the landing pad for the proteins of the pre-RC that allow initiation of DNA replication to take place (reviewed by Bell and Dutta [2002]). Because of the homology and conservation of the initiator factors among eukaryotes, *S. cerevisiae* serves as a model of metazoan initiation of DNA replication. Metazoan replication origins, however, including human, are much less characterized than in *S. cerevisiae*. From the 30 human replication origins that have been identified, less than five have been characterized at the molecular level [Todorovic et al., 2005].

We previously isolated origin-enriched sequences (ors) from early-replicating CV-1 cells and showed that several of them are able to replicate autonomously, when cloned into plasmids (reviewed in Zannis-Hadjopoulos and

†In memoriam.

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Price [1999]). Furthermore, among those ors from CV-1 cells, ors8 and ors12, which have been characterized in detail, were also shown to act as *bona fide* chromosomal replication origins [Pelletier et al., 1999; Novac et al., 2001]. Here, using specific primers that amplified the monkey replication origin ors8, we identified its human homologue, hors8, and shown that it is able to, both, replicate autonomously and serve as a chromosomal origin of DNA replication. Furthermore, we showed that it is bound in vivo by the initiator proteins Ku, Orc2, and Cdc6.

MATERIALS AND METHODS

Isolation of Nascent Strand DNA

Isolation of nascent strand DNA from HeLa cells was performed as previously described [Sibani et al., 2005b]. Briefly, cells cultured from five 15-cm plates were lysed with 5 ml of Hirt lysis buffer each (50 mM Tris-HCl, pH 8.0, 0.6 M NaCl, 1 mM EDTA, 0.5% SDS) [Hirt, 1967]. The lysates were digested with 0.1 mg/ml proteinase K overnight at 37°C, and nucleic acids were extracted by the standard phenol–chloroform method [Sambrook et al., 1989]. Twenty micrograms of DNA was digested with 15 units of exonuclease overnight at 37°C. Subsequently, the digested DNA was heat denatured at 95°C for 10 min, subjected to electrophoresis on a native agarose gel, and visualized with methylene blue. DNA fragments 0.5–1 kb in size were isolated, resuspended in 200 μl of dH_2O, and used for quantification with real-time PCR.

Real-Time PCR Quantification

DNA was quantified using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics). Briefly, 5 μl of nascent DNA was combined with 2 μl of 10 μM of each specific primer (see Table I for primer information), 2 μl of 25 mM $MgCl_2$, and 2 μl of the LightCycler FastStart DNA Master SYBR Green I, in a total final volume of 20 μl. The cycling conditions consisted of an initial 10 min denaturation step at 95°C, followed by 35 cycles of the following cycling conditions: 95°C for 5 s, annealing temperature (as indicated in Table I) for 10 s, and 72°C for 15 s. In order to calibrate each run, a standard curve for each fragment was derived from non-replicating DNA of serum-starved cells. None of the primer sets produced non-specific PCR products.

Episomal DNA Replication Assay

A full-length DNA fragments of 320 bp and 520 bp containing hors8 and an origin-lacking fragment located 2-kb downstream (hors8 + 2 kb), serving as negative control, respectively, were amplified, using Vent polymerase (New England Biolabs) (primers described in Table I). The PCR products were gel-purified, using the Qiagen kit (Qiagen, Mississauga, ON), and directly cloned into an EcoRV digested pBlue-Script (pBS). Mini-preparations of the plasmids were made using the Qiagen's quiaprep Spin Miniprep kit (Qiagen) and were sequenced to confirm the lack of mutations. pBS-A3/4 and pBS-CV-1ors8 are part of the laboratory plasmid stock and have been previously described [Todd et al., 1995; Price et al., 2003].

For transfection, HCT116 cells, at a density of 3×10^4, were seeded in 6-well plates (SARSTEDT) and approximately 16 h later were transfected with 3 μg of supercoiled plasmid DNA (2 μg of each constructs and 1 μg of pM1-SEAP vector (Roche Molecular Biochemicals, Indianapolis, IN) using FuGENE 6 transfection reagent (Roche Biochemicals), as per manufacturer's instructions. At 72 h post-transfection, low molecular weight DNA was isolated as described in [Hirt, 1967] and digested with *Dpn*I (New England Biolabs), as previously

TABLE I. Sequences and Annealing Temperatures of Primers Used for the Light Cycler

Primers	Sequence (5′–3′)	Annealing temperature (°C)
hors8F	TTGCACTTCACAGAGCAGTCAT	60
hors8R	GACCCACAAAGGCAAAAGTACC	
hors8 + 2 kbF	CCCTGAGGCAGGAGTGTTTGCC	66
hors8 + 2 kbR	GTATGCTCAATCTGCCCAACGG	
hors8 + 0.5 kbF	AGACAGGTCTGACAATGCTGTAGG	66
hors8 + 0.5 kbR	TTCAGTCACTGCCTCACATCCACA	
LB2F	GGCTGGCATGGACTTTCATTTCAG	66
LB2R (genbank: M94363)	GTGGAGGGATCTTTCTTAGACATC	
LB2C1F	GTTAACAGTCAGGCGCATGGGCC	66
LB2C1R (genbank: M94363)	CCATCAGGGTCACCTCTGGTTCC	

described [Landry and Zannis-Hadjopoulos, 1991; Matheos et al., 2002, 2003]. The *Dpn*I-digested as well as undigested DNA were used to transform DH5α strain of *E. coli* and the relative episomal replication efficiency of each plasmid was determined by counting the number of *Dpn*I resistant colonies, as previously described [Price et al., 2003]. The levels of secreted human placental alkaline phosphatase, determined by the SEAP Reporter Gene Assay kit (Roche Molecular Biochemicals) were used to normalize the transfection efficiency.

Cell Culture, Synchronization, and Flow Cytometric Analysis

HeLa, WI38, WI38 VA-13 subline 2RA (SV40-transformed WI38), or HCT116, were maintained in alpha MEM (Invitrogen) containing 10% FBS, penicillin, and streptomycin (hereafter termed complete media) at 37°C, 5% CO_2. Synchronization of WI38 VA-13 subline 2RA to late G_1 was done as described previously [Novac et al., 2001; Sibani et al., 2005b]. For synchronization to S-phase, cells were released from late G_1 arrest into complete media and harvested every 2 h. G_0 cells were obtained by serum-starvation during 48 h. For flow cytometric analysis, cells were washed with ice-cold phosphate-buffered saline (PBS) and resuspended in Vindelov's solution (3.4 mM Tris, 75 μM propidium iodide, 0.1% NP40, 0.01M NaCl, 700 U/L RNase A (Invitrogen)) overnight at 4°C [Vindelov, 1977]. They were analyzed using a Beckman flow cytometer and the CellQuest program.

Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay was carried out as previously described [Sibani et al., 2005b], using asynchronous cell cultures of WI38 VA-13 subline 2RA. Briefly, 10 plates (15 cm) of cells at 60–70% confluency were crosslinked with 1% formaldehyde for 10 min. Cells were washed and collected into ice-cold PBS and subsequently resuspended in lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1% Triton X-100, 2 mM EDTA, Complete protease inhibitors table, Roche Molecular Biochemicals). Upon passage through a 21G needle three times, the nuclei were harvested by centrifugation at 2,500*g* for 5 min at 4°C, resuspended in an equal nuclear volume of lysis buffer (i.e., 100 μl of buffer per 100 μl of nuclei), and sonicated ten times for 30 s each until fragments of DNA of less than 1 kb were obtained (the size of the fragments was monitored by electrophoresis). For the immunoprecipitation, sheared chromatin lysates (150 μg) were pre-cleared by incubation with 50 μl of protein G agarose (Roche Molecular Biochemicals) and subsequently incubated for 6 h with either 20 μg of anti-Ku70/86 antibody directed against the Ku heterodimer (clone162; NeoMarker), anti-Orc2 antibody [Quintana et al., 1997], anti-Cdc6 (H-304; Santa Cruz), or normal rabbit serum (NRS) at 4°C with constant shaking. Fifty microliters of protein G agarose was added and incubated overnight at 4°C. After centrifugation, pellets were washed twice with 1 ml of lysis buffer for 15 min each at 4°C, followed by 1 ml of washing buffer 1 (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% NP40, 0.05% sodium deoxycholate, complete protease inhibitors tablet, Roche Molecular Biochemicals), 1 ml of washing buffer 2 (50 mM Tris-HCl pH 7.5, 0.1% NP40, 0.05% sodium deoxycholate, complete protease inhibitors tablet, Roche Molecular Biochemicals), and 1 ml of sterile TE lacking any protease inhibitors. The beads were resuspended in 200 μl TE/1% SDS, incubated at room temperature for 15 min, and centrifuged at 1,000*g* for 1 min at room temperature. Finally, supernatants were incubated overnight at 65°C to reverse the crosslinks, followed by 100 μg of proteinase K at 55°C for 2 h. The DNA was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA), eluted in 100 μl 10 mM Tris-HCl pH 8.0, and used for real-time PCR quantification.

RESULTS

Sequence Similarity Between the Monkey (CV-1 Cells) ors8 and the Human ors8

A 320-bp fragment was amplified from several human cell lines (Fig. 1A; HeLa (lane 3), WI38 (lane 4), WI38 VA-13 subline 2RA (lane 5), and HCT116 (lane 6)), using the specific primers that had been previously used to amplify the monkey ors8 from CV-1 cells (lane2) [Novac et al., 2001, 2002]. The same primers failed to amplify this fragment when genomic DNA was not included in the reaction (not shown). Blasting the sequence of this fragment into the human genome localized it on chromosome 7, between nucleotides 112,323 and 112,044 (Fig. 1B). Sequence alignment of the human ors8 (hors8) homologous fragment (hors8, accession number: bankit743459) from HeLa

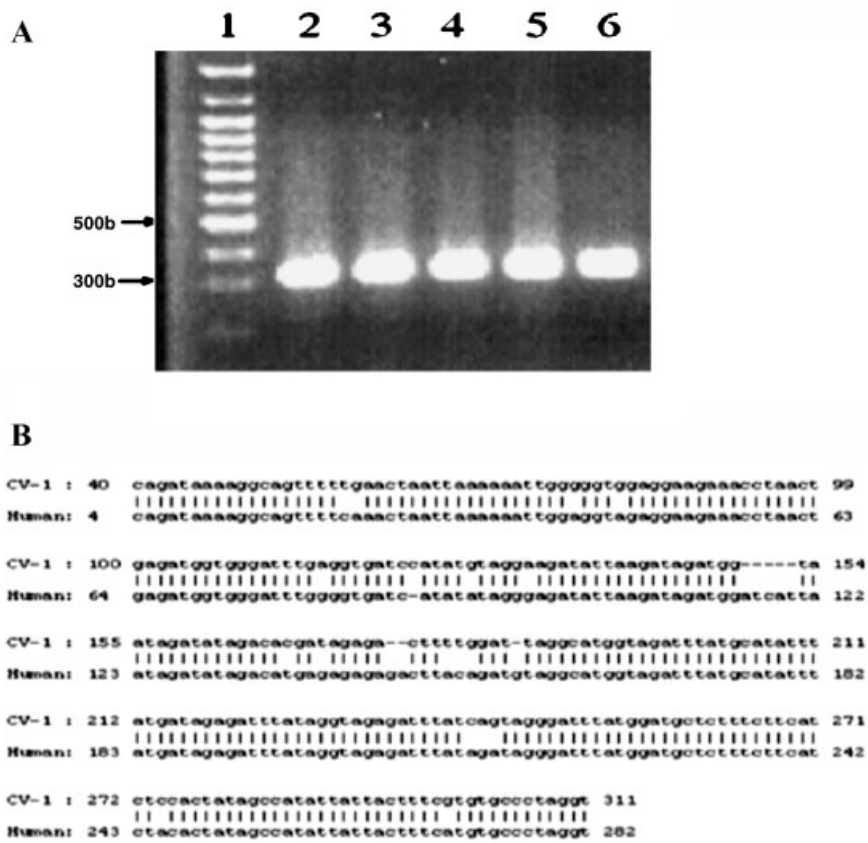

Fig. 1. Homology between the monkey (CV-1 cells) ors8 and the hors8. **A**: Genomic DNA from several human cell lines was used as a template for PCR amplification using the specific primers for the monkey cell (CV-1) ors8. **Lane 1** (DNA ladder), **lane 2** (CV-1), **lane 3** (HeLa), **lane 4** (WI38), **lane 5** (WI38 VA-13 subline 2RA), and **lane 6** (HCT116). **B**: Sequence alignment between ors8 from the African green monkey kidney cell line CV-1 (Genbank accession no. M26221) and the hors8 (Human chromosome VII). Identities = 254/280 (90%), Gaps = 9/280 (3%).

cells with the monkey (CV-1) ors8 revealed a 90% identity of the two sequences, with only 3% of gaps.

Hors8 Can Support In Vivo Episomal DNA Replication

The ability of hors8 to support the transient episomal replication of a bacterial plasmid containing it was examined using the *Dpn*I resistance assay (Materials and Methods), as previously described [Matheos et al., 2002, 2003]. The DNA fragments containing hors8, as well as the control region, were cloned into pBS. The plasmid containing A3/4, the 36-bp version of the mammalian consensus sequence [pA3/4; Price et al., 2003], was used as positive control, whereas the parental vector, pBS, without an insert, was used as negative control. The episomal replication capacity of each fragment, ascertained by *Dpn*I resistance [Matheos et al., 2002, 2003], was expressed relative to that of pA3/4, whose episomal replication activity was taken as 100%. As it is shown in Figure 2, the hors8 was able to support the in vivo episomal DNA replication of its plasmid (pBS-hors8) with the same efficiency as the previously tested pA3/4 [Price et al., 2003] and pors8 plasmids, the latter bearing the ors8 from CV-1 cells [Todd et al., 1995]. Thus, the *Dpn*I-digested pBS-hors8 produced 36 bacterial colonies, as compared to 38 colonies produced by pBS-CV-1ors8 and 35 colonies produced by pBS-A3/4. In contrast, pBS containing the hors8 + 2 kb region did not confer replication autonomy and behaved as the empty pBS vector.

Nascent DNA Abundance at the Human ors8 Region

To analyze whether hors8 served as a chromosomal replication origin, like its monkey cell

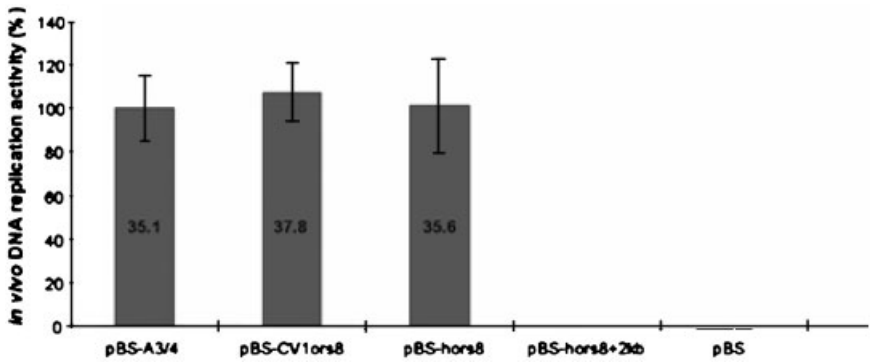

Fig. 2. Episomal replication activity of the hors8 fragment. Two micrograms of supercoiled pBS containing the A3/4, the monkey (CV-1) ors8, or the hors8 fragments were co-transfected with 1 μg of pM1-SEAP (reporter gene) into HCT116 cells. At 72 h post-transfection, the cells were lysed and plasmids were purified, digested with *Dpn*I, and used to transform DH5α bacterial cells. The average number of colonies per plate is indicated within the bars and it is normalized to the number of colonies obtained with pBS-A3/4 (positive control), which was taken as 100%. The data represent the average and one standard deviation of three independent experiments.

counterpart, ors8, an analysis of the nascent DNA abundance in that region was carried out, as previously described [Novac et al., 2001, 2002; Sibani et al., 2005a,b]. The sequence and annealing temperature of the real-time PCR primers used for each region is described in Table I. The human lamin B2 (LB2) origin and its control origin-lacking region (LB2C) were used as positive and negative control, respectively, as previously described [Sibani et al., 2005a,b]. As it is shown in Figure 3A, the nascent DNA abundance in the region containing hors8 was approximately 100-fold higher than in a region located 2-kb downstream from hors8 (hors8 + 2 kb) ($4.24 \pm 0.8 \times 10^4$ vs. $4.36 \pm 0.7 \times 10^2$ nascent molecules per 20 μg of genomic DNA). The activity decreased with the distance from hors8, since the activity of a region located 0.5-kb downstream was decreased to being approximately 50-fold higher with respect to hors8 + 2 kb ($2.3 \pm 0.5 \times 10^4$ vs. $4.36 \pm 0.7 \times 10^2$ nascent molecules per 20 μg of genomic DNA). By comparison, the abundance of nascent DNA at the region containing the LB2 origin, which was used as positive control, was approximately 70-fold higher with respect to its origin-lacking (control) region LB2C ($6.5 \pm 0.7 \times 10^4$ vs. $8.8 \pm 2 \times 10^2$ molecules per 20 μg of genomic DNA). The presence of the expected specific fragments amplified by each primer set was verified by subjecting each PCR sample to agarose gel electrophoresis (Fig. 3B): Hors8 (320 bp, lanes 2–4), hors8 + 0.5 kb (300 bp, lanes 7–9), hors8 + 2 kb (520 bp, lanes 12–14), hLB2 (208 bp, lanes 17–19), and hLB2C (240 bp, lanes 22–24). None of the primer sets produced non-specific PCR products. Furthermore, a single peak of amplified product was observed during the real-time PCR analysis (not shown).

Copy Number of Hors8

To verify that the nascent DNA data obtained above were not caused by multiple copies of hors8 being present or by an aberrant amplification of its sequence, the copy number of the hors8 origin-containing sequence and of the hors8 + 2 kb origin-lacking region was assessed by quantitative real-time PCR, by measuring the abundance of these regions in genomic DNA isolated from serum-starved HeLa cells relative to the single copy human B2 origin region. The results (Fig. 4) show that both the hors8 and its control region, hors8 + 2 kb, are present in single copy per haploid genome, indicating that the increased nascent DNA abundance observed in the hors8 region with respect to that of hors8 + 2 kb was due to the activity of hors8 as an origin of replication.

Association of Three DNA Replication Initiators Proteins, Ku, Orc2, and Cdc6 With the Human Hors8 Origin

Initiation of DNA replication requires the assembly of the pre-replication (pre-RC) complex at the origin, which involves several proteins including the ORC complex, Cdc6, and Ku [Sibani et al., 2005b; reviewed in Zannis-Hadjopoulos et al., 2004]. To determine whether these initiator proteins associated with the identified hors8 origin in vivo, quantitative ChIP analysis was employed (Fig. 5), as previously

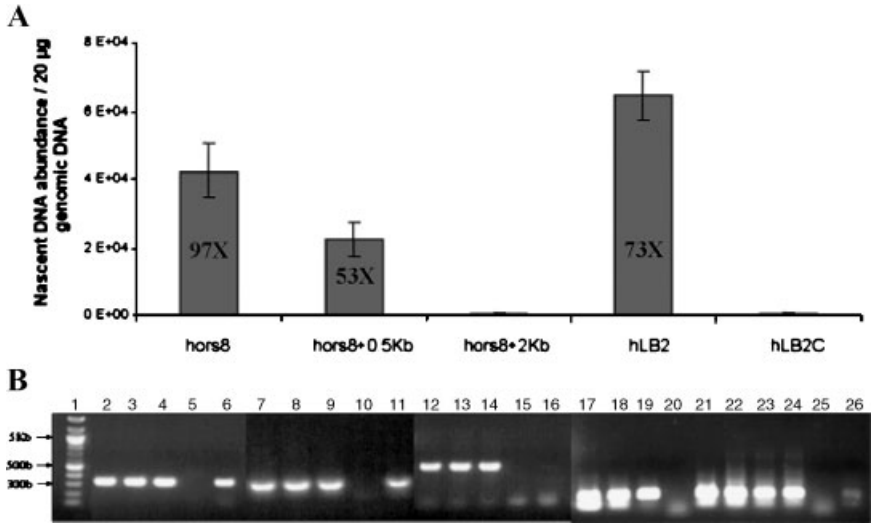

Fig. 3. Nascent DNA abundance at the human chromosomal region containing ors8. **A**: Nascent DNA from logarithmically growing HeLa cells was prepared as described in Materials and Methods. Briefly, nascent DNA was treated with λ-exonuclease, size fractionated, and abundance of each of the regions in DNA fragments of 0.5–1 kb in length was determined by real-time PCR. Histogram plots of the abundance of nascent strand DNA, expressed as number of nascent DNA molecules per 20 µg of genomic DNA, for the chromosomal regions containing the hors8, a region located 500-bp downstream from it (hors8 + 0.5 kb), an origin-lacking control region located 2-kb downstream from the ors8 origin (hors8 + 2 kb), the region containing the human LB2 origin (positive control) and its origin-lacking control region (LB2C) are shown. The error bars represent the average and one standard deviation of three independent experiments. **B**: The PCR products from each of the regions shown in panel **A** were subjected to electrophoresis on 1% agarose gel and visualized with ethidium bromide. Three concentrations of genomic DNA (2.5 ng, 5 ng, and 10 ng) were used to generate the standard curves for quantification of DNA abundance at the regions containing: hors8 (**lanes**: **2–4**), hors8 + 0.5 kb (**lanes 7–9**), hors8 + 2 kb (**lanes 12–14**), hLB2 (**lanes 17–19**), and hLB2C (**lanes 22–24**). No DNA was used as a negative control for the PCR reactions (**lanes**: **5**, **10**, **15**, **20**, **25**) and 5 µl of nascent DNA were used for each region: hors8 (**lane 6**), hors8 + 0.5 kb (**lane 11**), hors8 + 2 kb (**lane 16**), hLB2 (**lane 21**), and hLB2C (**lane 26**).

described [Novac et al., 2001, 2002; Sibani et al., 2005b]. DNA that was immunoprecipitated by antibody against the Ku heterodimer (Ku70/Ku80) was enriched on the average by approximately 200-fold in the hors8 sequence relative to that immunoprecipitated by NRS (1.08×10^4 vs. 50 molecules/150 µg of chromatin extract for Ku and NRS, respectively) (Fig. 5A). Likewise,

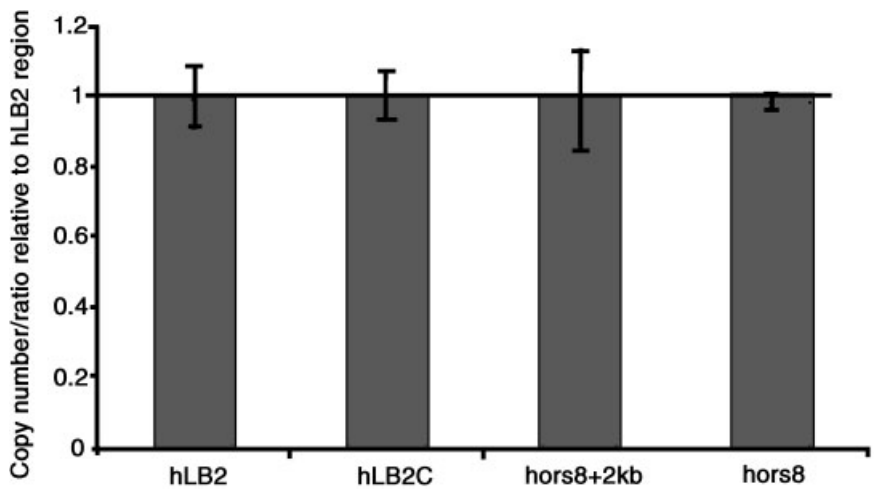

Fig. 4. Copy number of the hors8 region. Histogram plot of copy number/haploid genome of the abundance of hors8 origin and ors8 + 2 kb origin-lacking control regions. Total genomic DNA was isolated from serum-starved HeLa cells. The abundance of each region, measured by quantitative real-time PCR, is expressed as a ratio relative to the single copy human LB2 origin (hLB2). The dotted line represents a ratio of one, that is, single copy DNA in a haploid genome. The error bars represent the average of three experiments and one standard deviation.

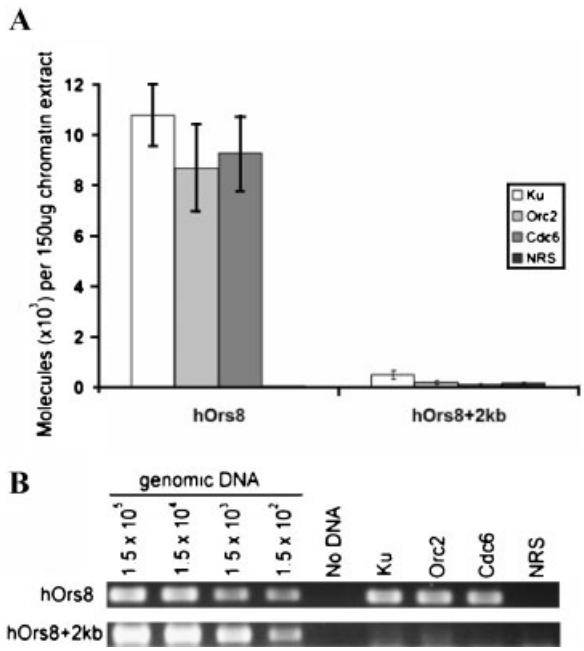

Fig. 5. In vivo association of Ku, Orc2, and Cdc6 with hors8. **A**: Quantitative ChIP assays (see Materials and Methods for details), measuring the association of Ku, Orc2, and Cdc6 proteins with the hors8 origin and hors8 + 2 kb origin-lacking regions in vivo. Real-time PCR (primers described in Table I) was used to measure the abundance of DNA from the samples immunoprecipitated with anti-Ku, -Orc2, -Cdc6 antibodies. The average and one standard deviation of three experiments are shown as histogram plots. **B**: The PCR products of the ChIP assays were subjected to electrophoresis on 1% agarose gel and visualized with ethidium bromide. The four standards of genomic DNA used for calibration, along with the ChIP DNA obtained with anti-Ku, -Orc2, -Cdc6 antibodies, are shown. NRS was used as a negative control for the ChIP and a no-DNA PCR reaction was used as a negative control for the PCR reaction.

immunoprecipitates of Orc2 and Cdc6 were enriched with hors8 sequence on the average by approximately 160- and 170-fold relative to those of NRS (8.7×10^3 and 9.3×10^3 vs. 50 molecules/150 μg of chromatin extract for Orc2, Cdc6, and NRS, respectively) (Fig. 5A). Comparison of the abundance of the hors8 sequence to the origin-lacking hors8 + 2 kb gives a measure of the specificity of association of the proteins to the origin. A representative gel showing the various PCR amplification products of the DNA obtained from the immunoprecipitates of hors8 and hors8 + 2 kb are shown (Fig. 5B). Known concentrations of genomic DNA were used to calibrate each real-time PCR run.

Ku immunoprecipitates were enriched by approximately 20-fold in hors8 relative to the control hors8 + 2 kb sequence (1.1×10^4 vs. 4.9×10^2 molecules/150 μg of chromatin extract for hors8 and hors8 + 2 kb, respectively). Similarly, Orc2 and Cdc6 immunoprecipitates were approximately 50- and 70-fold more enriched in hors8 relative to hors8 + 2 kb (8.7×10^3 vs. 1.8×10^2 molecules/150 μg of chromatin extract for hors8 and hors8 + 2 kb in Orc2 immunoprecipitates, respectively; and 9.3×10^3 vs. 1.3×10^2 molecules/150 μg of chromatin extract for hors8 and hors8 + 2 kb in Cdc6 immunoprecipitates, respectively). Thus, the ChIP experiments indicate that these three initiator proteins (Ku, Orc2, and Cdc6) bind specifically to hors8, suggesting that it is an origin of DNA replication.

Temporal Activity of Hors8

The abundance of nascent DNA from the region containing hors8 was examined at different times after releasing synchronized WI38 VA-13 (sub line 2RA) cells from late G_1 arrest (see Materials and Methods). The distribution of the cells at each phase of the cell cycle was monitored by flow cytometric (FACS) analysis of cell cultures at either Log phase, late G_1, and at 2, 4, 6, and 8 h after releasing into S-phase (Fig. 6A). Quantification of the nascent DNA, indicated that hors8 is a late-firing origin of DNA replication, having the highest origin activity 8 h after release into S-phase (Fig. 6B).

DISCUSSION

We previously generated from early-replicating African green monkey kidney cells (CV-1) a library of ors and characterized them in terms of sequence and function [reviewed in Zannis-Hadjopoulos and Price, 1999]. The ors-containing plasmids that have been examined were capable of transient episomal replication in vivo, after transfection into mammalian (monkey and human) cells [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991], as well as in an in vitro replication system that uses HeLa cell extracts [Pearson et al., 1991]. Furthermore, ors8 and ors12 have been also shown to be active chromosomal replication origins [Pelletier et al., 1999; Novac et al., 2001]. ors8, which is 483 nucleotides long and is present at <5 copies per haploid CV-1 genome, is active in the early S-phase [Zannis-Hadjopoulos et al., 1985].

In this study, we identified in human cells a homologue of the monkey cell (CV-1) replication

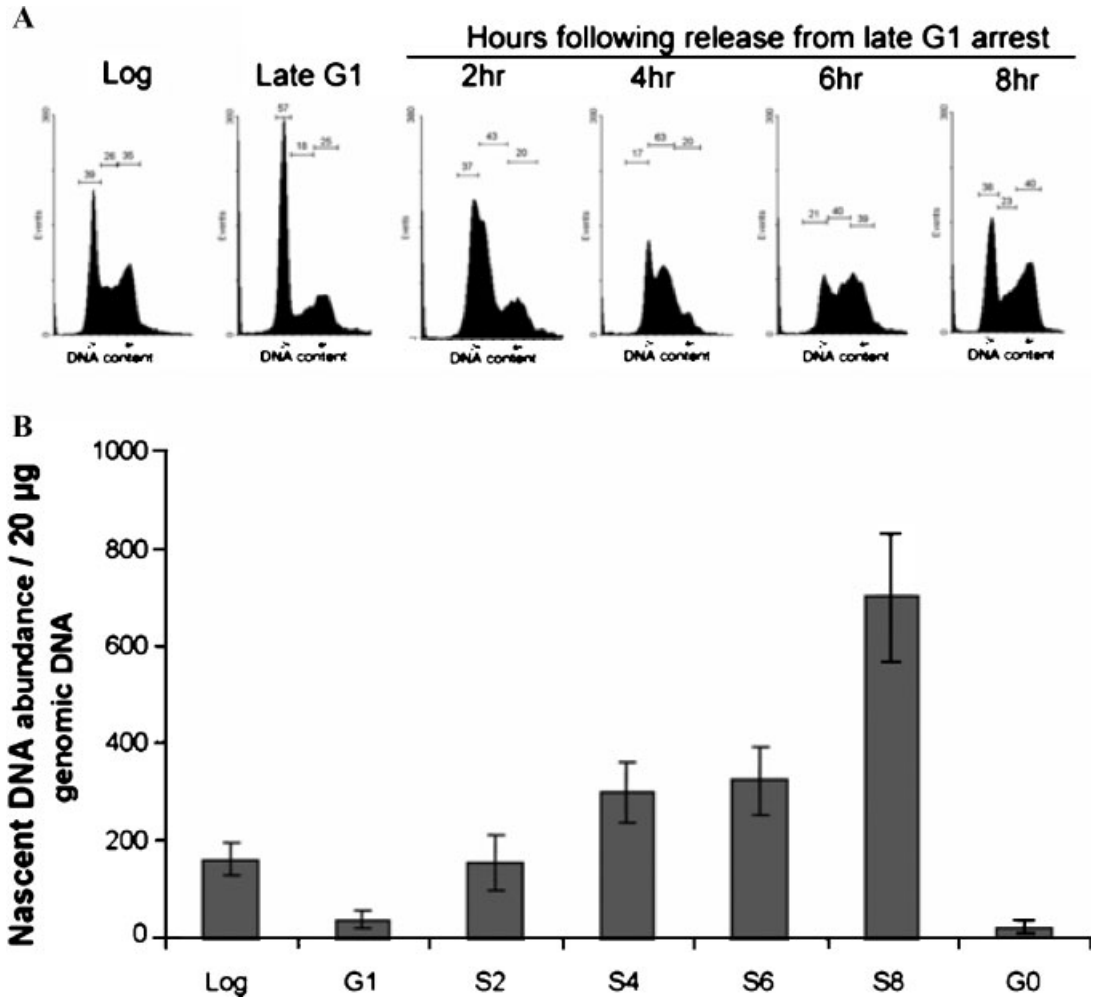

Fig. 6. Temporal activity for hors8. **A**: SV40-transformed WI38 cells were synchronized to late G_1 with mimosine and released into complete media (material and methods). They were harvested every 2 h, stained with propidium iodide, and analyzed by flow cytometry to monitor the cell cycle progression. The percentage of cells in G_0/G_1-, S-, and G_2/M-phases is indicated for each cell population. **B**: Nascent DNA abundance analysis of the hors8 origin region at different stages of the cell cycle was determined by real-time PCR. The data represent the average and one standard deviation of three different experiments.

origin, ors8. The hors8 had 90% sequence identity to the monkey ors8, and acted as a *bona fide* chromosomal replication origin, similar to the several metazoan origins that have been previously mapped, in which site-specific initiation has been observed, such as origins at the c-*myc* locus [Tao et al., 2000, 2001], the LB2 locus [Abdurashidova et al., 2000], b-globin locus [Kamath and Leffak, 2001], as well as at the DHFR intergenic region [Pelizon et al., 1996]. Two copies of a 20-mer-consensus sequence for mammalian DNA replication [Price et al., 2003] were found within the first 150 nucleotides of hors8 (data not shown). Episomal replication assays indicated that phors8, like its monkey cell counterpart, pors8, was capable of autonomous replication, as a similar number of *Dpn*I resistant colonies were obtained for both phors8 and ors8 as well as the positive control, pA3/4, when plasmid DNA isolated from HCT116 cells transfected with either phors8, pors8, or pA3/4 was used to transform *E. coli*. These colonies were the result of the replication conferred by the hors8 fragment, since DNA isolated from HCT116 cells that had been transfected with either the parental vector, pBS, or one containing the origin-lacking region failed to produce any colony. These data demonstrated that the novel human origin of replication, hors8, was as active as its monkey

counterpart or the mammalian consensus sequence, A3/4, in conferring episomal autonomous replication.

Analysis of nascent DNA abundance showed that hors8 was an active origin of DNA replication, containing approximately a 100-fold higher amount of nascent DNA than an origin-lacking region located 2-kb downstream from it. The abundance of nascent DNA at a region located 500-bp downstream of hors8 decreased from 100- to approximately 50-fold with respect to the same non-origin containing sequence. The origin activity of hors8 was approximately 1.5× lower than that of the human LB2 origin (hLB2, positive control), as indicated by the number of nascent DNA molecules quantified by real-time PCR ($4.24 \pm 0.8 \times 10^4$ vs. $6.5 \pm 0.7 \times 10^4$ nascent molecules per 20 μg of genomic DNA, respectively), while the abundance of nascent DNA for the hors8 control region (hors8 + 2 kb) was lower than the one of LB2C by approximately 2× ($4.36 \pm 0.7 \times 10^2$ vs. $8.8 \pm 2 \times 10^2$, respectively). The quantitative analyses of the nascent DNA for these two regions (hors8 and hLB2) were made from the same preparation of genomic DNA, ruling out the possibility that the above differences be the result of different DNA preparations. Furthermore, all the examined regions were found to be single copies per haploid genome.

The analyses of nascent DNA abundance at the hors8 chromosomal region as well as its capacity to support in vivo episomal DNA replication were reinforced by the results obtained from the ChIP assay, using specific antibodies against three initiator proteins, Ku, Orc2, and Cdc6, confirming that this region is an origin of DNA replication, where a pre-RC is assembled [Bell and Dutta, 2002; Zannis-Hadjopoulos et al., 2004]. We recently showed that Ku80 is an origin-binding protein [Novac et al., 2001] that binds to replication origins prior to the assembly of the ORC complex [Sibani et al., 2005]. Its deficiency led to a decreased ORC complex assembly at the LB2, β-globin and c-*myc* origins [Sibani et al., 2005], due to either decreased recruitment or stability of the ORC complex at these origins. The in vivo association of these DNA replication initiator proteins with hors8 was specific, since the immunoprecipitated material contained a lesser amount of the origin-lacking region hors + 2 kb.

Finally, even though the monkey and the hors8 origins shared high sequence and functional similarity, they differed in their temporal activity during the cell cycle. Thus, unlike the monkey ors8, which is an early-firing origin of replication [Kaufmann et al., 1985], hors8 showed the highest activity 8 h after release from a late G_1 block induced by mimosine. This difference in timing of activation between the two seemingly related sequences may reflect differences in chromatin structure in the regions that surround the origin between the two species.

In conclusion, we have identified a novel human replication origin, hors8, which shares 92% sequence identity to ors8, a replication origin that had been previously isolated from African green monkey (CV-1) cells. Hors8 is active late in S-phase and is associated with replication initiator proteins that are part of the pre-RC complex. Studies aiming at better characterizing hors8 are in progress.

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