Genetic Analysis of Human Orc2 Reveals Specific Domains That Are Required in Vivo for Assembly and Nuclear Localization of the Origin Recognition Complex*

Received for publication, April 21, 2006, and in revised form, June 7, 2006. Published, JBC Papers in Press, June 7, 2006, DOI 10.1074/jbc.M603873200

Ilian Radichev†1, Sung Won Kwon†, Yingming Zhao§, Melvin L. DePamphilis†, and Alex Vassilev†2

From the †NICHD, National Institutes of Health, Bethesda, Maryland 20892-2753 and the §Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9038

Eukaryotic DNA replication begins with the binding of a six subunit origin recognition complex (ORC) to DNA. To study the assembly and function of mammalian ORC proteins in their native environment, HeLa cells were constructed that constitutively expressed an epitope-tagged, recombinant human Orc2 subunit that had been genetically altered. Analysis of these cell lines revealed that Orc2 contains a single ORC assembly domain that is required in vivo for interaction with all other ORC subunits, as well as two nuclear localization signals (NLSs) that are required for ORC accumulation in the nucleus. The recombinant Orc2 existed in the nucleus either as an ORC-(2–5) or ORC-(1–5) complex; no other combinations of ORC subunits were detected. Moreover, only ORC-(1–5) was bound to the chromatin fraction, suggesting that Orc1 is required in vivo to load ORC-(2–5) onto chromatin. Surprisingly, recombinant Orc2 suppressed expression of endogenous Orc2, revealing that mammalian cells limit the intracellular level of Orc2, and thereby limit the amount of ORC-(2–5) in the nucleus. Because this suppression required only the ORC assembly and NLS domains, these domains appear to constitute the functional domain of Orc2.

In eukaryotic cells, initiation sites for DNA replication are determined by the binding of a six subunit origin recognition complex (ORC) to DNA. During the M to G1 phase transition in the cell division cycle, prereplication complexes are assembled at these ORC-DNA sites where they are subsequently activated during the G1 to S phase transition to unwind the DNA and initiate the assembly of replication forks (1). Although this process is highly conserved, significant differences do exist. For example, in both budding and fission yeast, ORC consists of a stable complex of six different subunits that remain bound to chromatin throughout the cell division cycle (Ref. 2 and references therein). Yeast two-hybrid analysis of mouse ORC subunit interactions leads to the same conclusion (8). The second is a less stable complex consisting of ORC-(1–5) that is essential for assembly of prereplication complexes and for initiation of DNA replication in vitro (5, 9) and in vivo (10). The third complex, ORC-(1–6), is presumptive, because Orc6 neither co-purifies with the other ORC subunits (4–7), nor is it required for DNA replication in an ORC-depleted frog egg extract (5, 11). In fact, there is little evolutionary relationship between fungal Orc6 and human Orc6 proteins (12), making Orc6 the least conserved of the ORC subunits.

Because the conditions under which these studies were carried out may not have accurately reflected those in mammalian cells, we endeavored to investigate ORC assembly and function under native conditions by constructing human cell lines that constitutively expressed a specific HsORC subunit to which two unique epitopes were added to one terminus. These epitope tags not only allowed the recombinant protein to be distinguished from the endogenous protein, but they also allowed the recombinant protein to be rapidly purified under non-denaturing conditions in order to identify proteins that associate with it in vivo (13). Thus, multiprotein complexes could be detected that were assembled from proteins synthesized and modified under native conditions. In addition, mutations could be generated in the recombinant protein and the effects of these mutations examined in vivo.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Present address: Inst. of Molecular Biology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria.
‡ To whom correspondence should be addressed: NICHD, National Institutes of Health, Bldg. 6/3A-02, 9000 Rockville Pike, Bethesda, MD 20892-2753. Tel.: 301-402-8222; E-mail: vassilev@mail.nih.gov.
§ The abbreviations used are: ORC, origin recognition complex; NLS, nuclear localization signal; PBS, phosphate-buffered saline; HA, hemagglutinin; CDK, cyclin-dependent kinase; wt, wild type.
Human Orc2 Activities in Vivo

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa-RR cells are a suspension cell line that was previously used for preparation of accurate transcription initiation by RNA polymerase II (19). HeLa-C9L cells are a monolayer cell line that was previously used to characterize Orc1 and kindly provided by Mendez et al. (20). Phoenix-A cells were obtained from the ATCC (no. 3443 with permission from Garry Nolan). All three cell lines were cultured routinely in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, non-essential amino acids (BIOSOURCE), 100 units/ml penicillin, 100 µg/ml streptomycin (Invitrogen) with 5% CO₂ at 37 °C. HeLa-RR cells in volumes >1 liter were cultured in spinner flasks under the same conditions, except that DMEM and 10% fetal bovine serum were replaced by DMEM/RPMI and 5% calf serum.

Recombinant Orc2 Mutants—A full-length human Orc2 open reading frame was isolated from HEK cell RNA using reverse transcriptase and PCR to produce a DNA copy that was then cloned into the XhoI/NsiI site in pCI (Promega). The cloned human Orc2 sequence was identical to that given under accession number BC014834 (National Center for Biotechnology Information). Orc2 deletions (Table 1 and Fig. 2A) and amino acid substitutions (Table 2) were generated by PCR amplification of the N- and C-terminal fragments flanking the deleted region using Tgo DNA polymerase (Roche). External primers contained either a XhoI restriction site for the N-terminal fragment or a NsiI site for the C-terminal fragment. The internal primers contained a SpeI restriction site so that the N-terminal fragment could be cloned into the XhoI/SpeI restriction site and the C-terminal fragment into the SpeI/NsiI site in pBS KSII+ (Stratagene). The two fragments were then sequenced, excised from pBS KSII+ ligated together at their SpeI ends, and cloned into the XhoI/NsiI site in pCI. For M28, M29, M30, M31, and M32, SacII was used as internal cloning site.

Stable Cell Lines—The cloned mutants were excised from pCI and then ligated into pÖ, a derivative of the Moloney mouse leukemia virus derived pOZ retroviral vector developed by Bruce Howard (National Institutes of Health, Bethesda, MD) upstream of the puromycin-N-acetyl-transferase gene. Both genes are trans-

TABLE 1

Deletion mutants in Orc2 and their phenotype

| Name | Amino acids deleted | Replaced with | Subcellular localization | ORC assembly |
|------|---------------------|---------------|--------------------------|--------------|
| D1   | 1–123               | None          | N                        | +            |
| D2   | 124–226             | None          | N                        | +            |
| D3   | 227–363             | TS            | N                        | –            |
| D4   | 364–479             | None          | N                        | +            |
| D5   | 480–577             | None          | N                        | +            |
| D6   | 234–363             | TS            | N + C                    | –            |
| D7   | 364–390             | TS            | N                        | –            |
| D8   | 391–408             | TS            | N                        | –            |
| D9   | 409–435             | TS            | N                        | –            |
| D10  | 436–451             | TS            | N                        | –            |
| D11  | 452–479             | TS            | N                        | –            |
| D12  | 241–363             | TS            | N                        | –            |
| D13  | 241–261             | TS            | N                        | –            |
| D14  | 262–288             | TS            | N                        | –            |
| D16  | 289–314             | TS            | N                        | –            |
| D17  | 315–338             | TS            | N + C                    | –            |
| D18  | 339–363             | TS            | N                        | –            |
| D19  | 364–390             | TS            | N                        | +            |
| D22  | 227–261             | TS            | N + C                    | –            |
| D23  | 278–288             | TS            | N                        | –            |

* These amino acids resulted from the restriction site used to construct the deletion.

TABLE 2

Mutations that define nuclear localization signals in Orc2

| Deletions and mutations | Amino acid sequence* | Subcellular localization | ORC assembly |
|-------------------------|----------------------|--------------------------|--------------|
| wt NLS-A                | 227[^PSKRMKDRKTSSDLV^[240] | N                        | +            |
| D3                      | 227[^---]-[363] | C                        | –            |
| D7                      | 224[^363] | N + C                    | –            |
| D14                     | 241[^---]-[363] | N                        | –            |
| D18 region               | 315[^---]-[338] | N + C                    | –            |
| M28                     | 315[^GLGSKDRDLERFRTTLMQDSIHVV^[1388] | N                        | –            |
| M29                     | 315[^GLGSKDRDLERFRTTLMQDSIHVV^[1388] | N                        | –            |
| M30                     | 315[^GLGSKDRDLERFRTTLMQDSIHVV^[1388] | N                        | –            |
| M31                     | 315[^GLGSKDRDLERFRTTLMQDSIHVV^[1388] | N                        | –            |
| M32                     | 315[^GLGSKDRDLERFRTTLMQDSIHVV^[1388] | N                        | –            |

* The underlined sequence is the NLS predicted by PSORTII for animal and yeast sequences.

This strategy was applied first to the Orc2 subunit for four reasons. Orc2 is unique among the ORC subunits in that it is the only one that does not induce TP53-dependent apoptosis, suggesting that Orc2 may suppress the lethal effects of other ORC subunits during mammalian development (14). Orc2 plays a central role in the assembly of ORC-(2–5) core complexes in vivo (4, 6). Orc2 contacts DNA at replication origins in vivo (15–17), and remains with the chromatin fraction throughout cell division. Finally, Orc2 has been implicated in the regulation of chromosome structure and centromere copy number as well as in DNA replication (18).

Results presented here confirm previous studies the on mammalian ORC and extend them by demonstrating that the intracellular level of Orc2 is regulated, and that Orc2 contains a mammalian ORC and extend them by demonstrating that the underlined sequence is the NLS predicted by PSORTII for animal and yeast sequences.
Human Orc2 Activities in Vivo

lated from an internal ribosomal entry site as a bi-cistronic mRNA. The multicloning site in pOZ contains the sequence ACCATG-gactacagacagtagtgacagtacggtcatctctcagcgc-gcagctacgccGAGGACTCGAG-Orc2, which encodes FLAG and hemagglutinin (HA) epitopes (lower case). Translation begins at the Kozak consensus sequence (underlined) to produce M-DYKDDDK-LDGG-YPYDVPDYA-GGLE (2927 Da). LDGG and GGLE are spacers. Translated amino acid sequences were confirmed by sequencing the Orc2 DNA insert in all recombinant DNA molecules. Stable cell lines expressing recombinant FH-Orc2 molecules were established as previously described (13). Retroviruses containing the pOP constructs were generated after Ca2+-phosphate transfection of Phoenix-A cells, and the viral supernatant was used for transduction of HeLa-RR cells. Cells expressing the recombinant Orc2 protein were selected by their resistance to 1 μg/ml puromycin, pooled, and then expanded by culturing in the presence of puromycin.

Immunoblotting (Western Blotting)—Cells were washed with phosphate-buffered saline (PBS) and then lysed in SDS sample loading buffer, as previously described (13). Aliquots were subjected to electrophoresis through a 4–12% NuPAGE Bis-Tris polyacrylamide gel (Invitrogen), and the fractionated proteins were then transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell), according to the manufacturer’s instructions.

To detect FH-Orc2 proteins, the membranes were blocked in 5% nonfat dry milk for 1 h and incubated with either anti-FLAG M2 (Sigma) or anti-HA 12CA5 (Roche) monoclonal antibody. With total cell lysates, both antibodies were used to increase detection sensitivity. Alkaline phosphatase-conjugated anti-mouse antibody (Amersham Biosciences) was the secondary antibody and Super Signal West Dura Extended Duration Substrate kit (Pierce) for detection according to the manufacturer’s instructions. Goat polyclonal anti-Orc1 (Santa Cruz Biotechnology), anti-Orc2 (Santa Cruz Biotechnology), anti-Orc3 (US Biological), and anti-Orc4 (Abcam) were used at a dilution of 1:700, followed by alkaline phosphatase-conjugated rat antigoat antibody (1:30,000, Pierce).

Affinity Purification—All steps were carried out on ice or at 4 °C. HeLa-RR cells (107) expressing wild-type FH-Orc2 were extracted for 30 min with 200 μl of buffer B (20 mM Tris-HCl, pH 7.9, 5 mM MgCl2, 10% glycerol, 0.1% Nonidet P-40, 10 μM leupeptin, 1 μM pepstatin, 1 μM phenylmethylsulfonyl fluoride, and 0.5 μg/ml aprotinin) containing 100 mM KCl. The lysate was then centrifuged for 20 min at 15,000 × g. The soluble fraction (B100) was recovered, and the pellet was extracted for 20 min with buffer B containing 300 mM KCl. The soluble fraction (B300) was recovered, and the protocol was repeated using buffer B containing 500 mM KCl. The B100 and B300 fractions were incubated with 1.3 μl and 0.3 μl, respectively, M2 anti-FLAG agarose beads (Sigma) for 4 h with rotation. The beads were then washed six times with 10 volumes of either B100 or B300. FLAG-tagged protein was selectively eluted by rotating the beads for 1 h with 0.2 mg/ml FLAG peptide (Sigma) in the appropriate buffer. From three to seven times as many cells were lysed with cell lines expressing FH-Orc2 mutants at amounts less than FH-Orc2(wt), but the same amount of M2 was used to collect the FH-Orc2 protein.

Immunofluorescence—Cells were cultured on coverslips, fixed for 10 min with ice-cold methanol (−20 °C), and then washed with PBS. The coverslips were then covered with anti-FLAG antibody (Sigma) diluted 1:900 in PBS, incubated at room temperature for 1 h, washed three times with PBS, and then incubated at room temperature for 1 h with Cy3-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch) diluted 1:400 in 1 μg of Hoechst per ml of PBS. The coverslips were then mounted on a glass slide in fluorescent mounting medium (Dako Cytomation) and viewed using an Nikon Eclipse E600 fluorescence microscope with a Nikon DXM1200F digital camera. Images were captured with ACT-1 software (Nikon) and merged using MetaVue software (Universal Imaging).

RESULTS

Recombinant Orc2 Can Replace Endogenous Orc2 in Vivo—To identify the functional domains of Orc2 protein in its native environment, HeLa cells were constructed that constitutively expressed an N-FLAG-hemagglutinin-Orc2 fusion protein (FH-Orc2) that was distinguished from endogenous Orc2 using either anti-FLAG or anti-HA antibodies (Fig. 1A). The steady-state level of FH-Orc2 in these cells was only 3-fold greater than the steady-state level of endogenous Orc2 in the parent cell line (Fig. 1, B and D). Surprisingly, constitutive expression of FH-Orc2 suppressed expression of endogenous Orc2 (Fig. 1, B and D, compare Orc2 with pOP). This suppression was specific for FH-Orc2, because cells that constitutively expressed other HsORC subunits (Fig. 1A) did not suppress Orc2 expression (Fig. 1B). These results revealed that mammalian cells limit the level of intracellular Orc2.

To determine whether or not the ability of ectopic Orc2 to suppress expression of endogenous Orc2 required a particular region of Orc2, deletions D1–D5 were constructed in Orc2 that spanned the entire molecule. These deletions systematically eliminated each of the three NLSs predicted from sequence analysis of Orc2, the two cyclin-dependent protein kinase (CDK) phosphorylation consensus sequences, and the six consensus sequences for binding cyclin proteins (Fig. 2). Like FH-Orc2(wt), each deletion mutant carried the FLAG-HA epitopes at its N terminus.

Immunoblotting with anti-HA and anti-FLAG antibodies revealed that deletion mutants D1, D2, and D5 were expressed at levels equivalent to FH-Orc2(wt) (Fig. 1C). Immunoblotting with anti-Orc2 antibody revealed that wt, D1, D2, and D5 each suppressed the expression of endogenous Orc2 (Fig. 1D). D5 was not detected by the anti-Orc2 antibody, because it recognizes an epitope in the C terminus of Orc2 that is absent in D5. In contrast, D3 and D4 were expressed to much lower levels than wt, D1, D2, and D5 (Fig. 1C), and the level of endogenous Orc2 in these cell lines was affected only slightly by the presence of the ectopic protein (Fig. 1D). These data suggest that the sequences deleted in mutants D1, D2, and D5 were not required for Orc2 function, whereas the sequences deleted in mutants D3 and D4 (the region from amino acids 227 to 479) were required. Nevertheless, changes were not detected either in the rate of cell proliferation or in the distribution of cells between the G1, S, and G2/M phases of the cell division cycle (fluores-
cence-activated cell sorting (FACS) analysis) among wt and mutant Orc2 proteins. Therefore, the ability of recombinant Orc2 to replace endogenous Orc2 required the region defined by deletions D3 and D4. Because the results described below showed that this region was required both for ORC assembly and for nuclear localization, it was termed the “Orc2 functional domain” (Fig. 2A).

**ORC-(2–5) Does Not Bind Stably to Chromatin in Vivo**—As a prelude to identifying Orc2 regions required for Orc2 function in HeLa cells, optimal conditions were determined for recovering FH-Orc2 and its associated proteins. Cells expressing FH-Orc2 were lysed in buffer B containing 100 mM KCl (see “Affinity Purification”). The soluble fraction (B100) was recovered by centrifugation, and the pellet was resuspended in buffer B containing 300 mM KCl. The soluble proteins were recovered as the B300 fraction, and the process was repeated to obtain a B500 fraction. About 85% of the FH-Orc2(wt) was recovered in the B100 fraction, and the remainder in the B300 fraction (Fig. 3).

The results described above suggested that most of the ectopically produced FH-Orc2 was bound to the chromatin fraction weakly, if at all. To determine whether or not this was...
an artifact of ectopic expression of an epitope-tagged protein, cells that constitutively expressed FH-Orc2(wt), and their parental cell line, HeLa-RR, were extracted in parallel with buffer B containing either 0, 10, or 100 mM KCl. Remarkably, 80% of the FH-Orc2 protein (Fig. 4A) and 50% of the endogenous Orc2 (Fig. 4B) was soluble in buffer B alone, revealing that these proteins had little, if any, affinity for chromatin. Moreover, whereas endogenous Orc1 was bound to the chromatin fraction from HeLa-RR cells, about half of the endogenous ORC-(2–5) core complex in these cells was not.

To determine whether or not the experimental protocol used in this study was responsible for the large fraction of soluble ORC-(2–5) detected in HeLa-RR cells, a second HeLa cell line was examined. Mendez et al. (20, 21) have reported that all of the ORC subunits in their HeLa cells (designated HeLa-CSHL) were bound to the chromatin fraction. Therefore, HeLa-CSHL cells, which are anchorage-dependent, were examined in parallel with HeLa-RR cells, which proliferate in suspension culture. As previously reported (20, 21), Orc1, Orc2, Orc3, and Orc4 each required at least 300 mM salt to extract them from asynchronously proliferating HeLa-CSHL cells (Fig. 4C). Thus, the apparent differences in the affinity of ORC subunits for the chromatin fraction resulted not from differences in experimental protocols, but from differences inherent among HeLa cell lines. Furthermore, the surprising observation that the fraction of ORC-(2–5) tightly bound to the chromatin fraction varied among HeLa cell lines could not be attributed to differences in the concentrations of ORC subunits, because the relative amounts of ORC subunits 1–4 in HeLa-CSHL and HeLa-RR cells were essentially the same (Fig. 4C). These results suggest that retention of ORC-(2–5) with the chromatin fraction may require more than Orc1, and that the retention of Orc1 with the chromatin fraction may not require ORC-(2–5).

Recombinant Orc2 Is Assembled into ORCs in Vivo—To determine whether or not constitutively expressed FH-Orc2...
associates with other ORC subunits in vivo, cells were lysed in buffer B100, and the pellet was extracted in B300, as described above. The soluble proteins in each fraction were then bound to anti-FLAG antibody coupled to agarose beads, washed with the corresponding buffer (B100 or B300), and then eluted with FLAG peptide to selectively release FH-Orc2. This affinity-purified FH-Orc2 was then fractionated by SDS-PAGE.

Based on their apparent molecular masses in silver stained SDS-PAGE gels (Fig. 5) and their reactivity with appropriate antibodies (Fig. 6), FH-Orc2 in the B100 fraction was associated with Orc3, Orc4, and Orc5. Orc6 was not detected either in the B100 or the B300 fraction, consistent with previous reports that Orc6 is not associated either with purified HsORC or with HsORC subunits transiently expressed in insect cells (4–7). Orc1 was detected predominantly, if not exclusively, in the B300 fraction, along with Orc2, Orc3, Orc4, and Orc5. The identity of ORC subunits 2–5 was confirmed by two or more matches of tryptic peptides obtained from protein bands in SDS-PAGE gels to peptides predicted from the human genome (nano-HPLC/mass spectroscopic analysis for protein identification, Ref. 22). As previously reported (5, 23, 24), HsOrc5, whose predicted size is 50.4 kDa, migrated more slowly than HsOrc4, whose predicted size is 50.5 kDa. Reliable antibody against HsOrc5 was not available.

The relative intensity of the silver staining of the FH-Orc2, Orc3, Orc4, and Orc5 protein bands in both the B100 and B300 fractions (Fig. 5) was consistent with the presence of an ORC-(2–5) core complex. The apparent under representation of Orc1 reflects the fact that Orc1 is selectively degraded in S, G2, or M-phase cells (20, 25, 26). The appearance of Orc1 almost exclusively in the B300 fraction (Fig. 6), suggests that it required for stable binding of ORC to chromatin in vivo.

Orc2 Contains a Single Domain That Is Required for ORC Assembly in Vivo—To identify those regions of Orc2 that are required for ORC assembly in vivo, FH-Orc2 wild-type and mutant proteins were affinity-purified from both the B100 and the B300 fractions. Analysis of the ORC subunits that co-purified with FH-Orc2 revealed that mutants in the D3 and D4 region differed from wt, D1, D2, and D5 in their ability to
assemble ORCs. Neither D3 (which lacks NLS-3) nor D14 (which contains NLS-3) nor D4 were associated with other ORC subunits (Fig. 6A). In contrast, D1, D2, and D5 were comparable to wt in their ability to assemble ORCs (Fig. 6A). These results revealed that the region in Orc2 from amino acid 227–479 is required for ORC assembly in mammalian cells.

To further define the ORC assembly domain, a series of smaller deletions ranging from 15 to 27 amino acids were constructed throughout the D3 and D4 regions (D15–D19 and D8–D12, respectively, in Fig. 2A). These Orc2 mutants were then expressed constitutively in HeLa-RR cells and analyzed as above. The results revealed that any deletion within the region from amino acid 289 (D17) through 451 (D11) prevented Orc2 from associating with other ORC subunits (Fig. 6, B and C). Furthermore, deletion of amino acids 262–288 (D16) and 452–479 (D12) reduced, but did not eliminate, association of FH-Orc2 with other ORC subunits, thus marking the boundaries of the “ORC assembly domain” (Fig. 2B). These data further revealed that Orc1 was associated only with Orc2 molecules that were competent for ORC assembly and only with Orc2 that was bound to the chromatin fraction (i.e. the B300 fraction).

**NLS-3 Is Required For Orc2 Nuclear Localization**—Because all HsORC subunits are localized to the nucleus during cell proliferation (23), the fact that small deletions anywhere within a 162 amino acid region of Orc2 prevented its binding to any other ORC subunit raised the possibility that inactivation of ORC assembly was an indirect result of inactivation of one or more NLSs. Therefore, to determine whether or not the ability of Orc2 to associate with other ORC subunits depended on its subcellular location, established cell lines that constitutively expressed the recombinant protein were stained with anti-FLAG antibody to determine whether FH-Orc2 was localized to the nucleus, the cytoplasm, or both.

As expected, FH-Orc2(wt) was localized to the nucleus (Fig. 7). Moreover, since identical results were obtained with D1, D2, D4, and D5 (data not shown), none of these regions were required for nuclear localization of Orc2. Only the D3 deletion caused FH-Orc2 to localize to the cytoplasm (Fig. 7), suggesting that, of the three consensus NLSs predicted by sequence analysis (Fig. 2A), only NLS-3 was required for nuclear localization.

To determine whether or not the cytoplasmic localization of D3 resulted specifically from the absence of NLS-3, the amino acid sequence of NLS-3 was altered from a very basic sequence (pl = 12) to a slightly acidic sequence (pl = 5.5 and Table 2, M6) in order to eliminate the consensus of basic amino acids that are characteristic of known NLSs. Analysis of cells that constitutively expressed the altered NLS-3 mutant (M6) revealed that it was distributed throughout both the nucleus and cytoplasm (Fig. 7), confirming that NLS-3 does contribute to the nuclear accumulation of Orc2. Furthermore, M6 was indistinguishable from FH-Orc2(wt) in its association with other ORC subunits (Fig. 6A). Therefore, NLS-3 is not only required to localize Orc2 to the nucleus, but it is also required to localize ORC to the nucleus.

**Orc2 Contains a Second NLS within the ORC Assembly Domain**—In contrast to the deletion created by D3, the mutation created in M6 did not cause Orc2 to localize exclusively to the cytoplasm, revealing that NLS-3 was not the only sequence required for nuclear localization. To test this hypothesis, a deletion mutant was constructed (D7) that was identical to D3 except that it retained NLS-3 (Fig. 2A). The cellular localization of D7 was indistinguishable from that of M6 (data not shown). However, when the NLS-3 site was extended by 7 amino acids (D14), then complete nuclear localization was restored (Fig. 7), even though D14 was indistinguishable from D3 (Fig. 6A) in its inability to bind other ORC subunits. Therefore, the real NLS-3 consisted of amino acids 227–240 (Table 2), and this sequence was sufficient to localize Orc2 exclusively to the nucleus, at least in the absence of amino acids 241–363. However, when the complete NLS-3 was deleted (D22 and D23), Orc2 was distributed throughout the cell in both the nuclear and cytoplasmic compartments in a pattern that was again comparable to M6 (data not shown), suggesting that at least one additional sequence existed within the D3 region that was important for nuclear localization of Orc2. To locate this sequence, small deletions were constructed in Orc2 that spanned the region from amino acid 241 through 363 (Fig. 2A, D15–D19), and their effects on nuclear localization were determined. Only D18 affected Orc2 subcellular localization, and it mimicked the effects of M6 (Fig. 8A).

**FIGURE 7. Identification of nuclear localization signals (NLSs) in Orc2.** HeLa-RR cells that constitutively expressed either wild-type Orc2, Orc2 containing deletion D3, or Orc2 with a mutated NLS-3 (M6) were photographed with phase contrast (phase), stained with Hoechst (DNA), and stained with anti-FLAG monoclonal antibody to visualize FH-Orc2 proteins (FH-Orc2). The DNA and FH-Orc2 images were superimposed (merge) to facilitate subcellular localization of the FLAG-tagged protein. All other FH-Orc2 deletions or mutations exhibited one of these three subcellular localization patterns: nuclear, cytoplasmic, or both nuclear and cytoplasmic.
number of charged amino acids (Table 2). These mutations were then tested for their affects on Orc2 subcellular localization in the presence of the M6 mutation. Only the M6/H11001M31 and M6/H11001M32 double mutants were localized exclusively in the cytoplasm whereas double mutants M6/H11001M28, M6/H11001M29, and M6/H11001M30 remained distributed through both nucleus and cytoplasm (Fig. 8A). These results identified amino acids 324–336 as NLS-B, a site that facilitates the ability of Orc2 to localize to the nucleus (Table 2 and Fig. 2B).

Because NLS-B lay inside the ORC assembly domain, it was of interest to determine whether or not the site-specific amino acid changes in this region that interfered with nuclear localization of Orc2 also prevented ORC assembly by Orc2. Only M32 assembled detectable amounts of the ORC-(2–5) core complex (Fig. 8B), revealing that substitution of as few as two amino acids within the D18 region could interfere with ORC assembly, and in some cases (M31, M32) with nuclear localization as well.

CDK Phosphorylation Sites Are Not Required for ORC Assembly in Vivo—Human Orc2 contains two CDK consensus phosphorylation sites (Fig. 2) that are conserved among vertebrate Orc2 proteins. To determine whether or not phosphorylation at these sites are required for ORC assembly, the serine at each site was changed to an alanine in order to prevent phosphorylation. The mutated proteins were expressed constitutively in HeLa cells at the same level as FH-Orc2 wild-type, but they did not exhibit any detectable alterations in their ability to form ORCs, bind to chromatin, and localize to the nucleus (data not shown).

DISCUSSION

The results presented here (summarized in Fig. 2B and Tables 1 and 2) demonstrate a direct link between the ability of recombinant Orc2 to replace endogenous Orc2, and its ability to assemble ORC in vivo and localize it to the nucleus. Orc2 contained a single region of 252 amino acids (the “Orc2 functional domain”) that was required for recombinant Orc2 to replace endogenous Orc2 in vivo and thereby allow cells to continue to proliferate normally. This region encompassed a single 162 amino acid domain that was required for Orc2 to bind stably to any other ORC subunit (the “ORC assembly domain”), as well as two nuclear localization signals (NLS-A, NLS-B). Mutations that either reduced the ability of Orc2 to assemble ORCs or to localize to the nucleus also reduced the ability of recombinant Orc2 to replace endogenous Orc2. The Orc2 functional domain did not include the first 177 amino acids of Orc2 that have recently been shown not to be required for DNA replication (27). Because each of the small deletions and amino acid substitutions constructed within the ORC assembly domain eliminated association of Orc2 with all of the other ORC subunits, Orc2 appears to function in vivo only within the context of an ORC. The fact that NLS-B lies within the ORC assembly domain suggests that ORC assembly is linked to nuclear localization. Moreover, although ORC-(2–5) accumulated in the nucleus, only ORC-(1–5) was associated with chromatin fraction, suggesting that Orc1 is not only required for binding of ORC to DNA in vitro (5, 11), but it is also required for the assembly of ORC:chromatin sites in vivo.

Chromatin Association—Among previous studies on the association of ORC with chromatin, some found that essentially all of the Orc2 in HeLa cells was tightly bound to the chromatin fraction (20, 21, 28), whereas others found that a fraction of the Orc2 was only weakly associated with chromatin (10, 29). Results presented here reveal that these differences are likely caused by differences among cell lines rather than experimental conditions.
protocols. HeLa-RR cells and HeLa-CSHL cells proliferated at about the same rate, despite the fact that about half of the endogenous ORC-(2–5) core complex in HeLa-RR cells was not bound to the chromatin fraction, although all of it was localized to the nucleus. Moreover, the relative cellular concentrations of ORC subunits one through five in these two cell lines were essentially the same (Fig. 4). Therefore, the cellular concentration of ORC in HeLa cells must be in excess of what is needed for cell proliferation. In fact, the relative levels of ORC among human cell lines can vary significantly (30), and the level of chromatin bound ORC in a human colon carcinoma cell line can be reduced 3-fold with only a 30% decrease in the rate of cell proliferation (31). Therefore, binding of ORC-(2–5) to chromatin must require other factors in addition to Orc1.

These results are consistent with previous studies showing that Orc1 is required both for binding ORC-(2–5) to DNA in vitro, and for initiation of ORC-dependent DNA replication in Xenopus egg extract (5, 11). Moreover, Orc1 associates in vitro only with Orc-(2–5) (6). Therefore, because Orc1 does not bind to DNA in vitro in the absence of ORC-(2–5), and because most of the ORC-(1–5) was bound tightly in vivo to the chromatin fraction, Orc1 appears to be required in vivo for binding ORC-(2–5) to DNA.

ORC Assembly—Constitutive expression of a recombinant Orc2 in HeLa cells resulted in assembly of the recombinant protein into both ORC-(2–5) complexes that were localized in the nucleus, but not bound to chromatin, and ORC-(1–5) complexes that were bound to the chromatin fraction. Consistent with previous studies (5), Orc6 was not detected in these complexes. Analysis of 19 deletion mutants (Table 1 and Fig. 2) that spanned the entire Orc2 protein and six amino acid substitution mutants localized in the two NLSs (Table 2) revealed the surprising result that Orc2 contains a single 162 amino acid domain (289–451 (D11)) that is required for ORC assembly (Fig. 2B). No evidence was found for assembly of partial ORCs in which one or more of the other subunits were bound to a mutated Orc2. Remarkably, even small amino acid substitutions (M28 – M32 (Table 2)) within the ORC assembly domain inhibited ORC assembly. Therefore, Orc2 binds specifically to one of the other ORC subunits and the remaining ORC subunits then bind to the resulting Orc2 heterodimer, or ORC assembly is a concerted process that requires concomitant association of at least ORC subunits 2–5 followed later by association with Orc1 and possibly with Orc6.

Previous studies have revealed multiple, but conflicting, interactions among individual human ORC subunits. When insect cells were infected with baculovirus expressing all six epitope-tagged, recombinant HsORC subunits, only a stable ORC-(2–5) complex was recovered; Orc1 was weak but inconsistently bound and Orc6 was uniformly absent (4, 7). Furthermore, Orc2 and Orc3 could bind Orc5, but not Orc4, suggesting that Orc5 is required to incorporate Orc4. Incorporation of Orc1 required the presence of Orc2, -3, -4, and -5. In fact, only one dimeric interaction was detected: the C-terminal 225 amino acids of Orc2 associated with the N-terminal 200 amino acids of HsOrc3 (4). These results, together with those reported here, suggest that the ORC assembly domain in Orc2 binds specifically to Orc3 in vivo. However, the ORC assembly domain (289–451) only partially overlaps the C-terminal fragment of Orc2-(352–577) used in the study by Dhar et al. (4). Moreover, each the seven internal deletions (D17–D11) and five amino acid mutations within the D18 region (Table 2) that define the ORC assembly domain in vivo prevented Orc2 from interacting with any of the other ORC subunits. Because the 225-amino acid C-terminal fragment of Orc2 includes only 46% of the ORC assembly domain and is missing the critical D18 region, it is surprising that the C-terminal fragment alone would bind Orc3 at all. The answer to this paradox may lie in part with differences in the way various protein fragments fold, and in part with differences in the way these protein–protein interactions were assayed. For example, overexpression of ORC proteins in insect cells may result in unusually high concentrations of ORC subunits that may stabilize interactions that do not occur in vivo.

Comparable studies on ORC assembly also have been done using antibodies specific for various ORC subunits to immuno-precipitate ORC proteins, either from a high salt extract of HeLa cells, or from lysates of baculovirus-infected insect cells (6). Immunoprecipitation of Orc2 recovered a stable complex containing ORC subunits 2–5 that was deficient in Orc1 and devoid of Orc6, consistent with results reported here and elsewhere (4, 5). However, several binary complexes also were detected (Orc2-Orc4, Orc3-Orc4, and Orc2-Orc3), suggesting that Orc5 is not required for binding of Orc4 to other ORC subunits. Binary complexes between Orc5 and the other three members of the ORC core complex were detected, but they were much weaker than Orc5 trimeric complexes (Orc2-Orc3-Orc5, Orc2-Orc4-Orc5, and Orc3-Orc4-Orc5), suggesting that assembly of one or more binary complexes precedes incorporation of Orc5 into ORC.

The full scope of binary interactions between ORC subunits was revealed by a yeast two-hybrid screen of mouse ORC subunits (8). These results indicated that every ORC subunit binds to at least three other ORC proteins, although the intensity of the interaction varied significantly. Consistent with the results of Dhar et al. (31), Orc2 interacts strongly with Orc3 and Orc5, Orc3 interacts strongly with Orc2 and Orc5, and Orc5 interacts strongly only with Orc2 and Orc3. Orc4 interacts strongly only with Orc6, and Orc6 interacts strongly only with Orc4. Orc1 interacts only with Orc2, Orc4 and Orc6, and these interactions were all weak.

Thus, taken together, the studies reported here and elsewhere support the hypothesis that ORC assembly results from multiple, coordinate interactions between the subunits that comprise the ORC-(2–5) core complex, and that subsequent association with Orc1 results in binding of ORC to chromatin. One also can infer from the data that ORC assembly does not require association with DNA, although it is possible that ORC is assembled on DNA and then remains intact even after it has been eluted from the DNA. Moreover, it is possible that the pathway for ORC assembly, like that of SV40 T-antigen assembly, is dependent on DNA or even on specific DNA sequences.

Nuclear Localization—Mutational analysis of Orc2 revealed that NLS-3 (amino acids 227–233) is the only one of the three NLSs in Orc2 predicted by PSORTII that is required for nuclear localization. Therefore, NLS-3 plus the seven amino acids pres-
ent in D14, but absent in D7, is now referred to as NLS-A (Fig. 2B), a site that appears to be conserved among vertebrate Orc2 genes. Orc2 proteins that lacked NLS-A were no longer localized exclusively to the nucleus. Substitution of neutral amino acids for the basic amino acids in NSL-A (M6, Table 2) changed the distribution of Orc2 from nuclear to both nuclear and cytoplasmic. However, complete conversion of Orc2 from a nuclear to a cytoplasmic protein required elimination of both NLS-A and NLS-B, a region within the D18 deletion from amino acids 319–336 that was not predicted by PSORTII (Table 2). Both of these NLSs contributed to the nuclear localization of Orc2, because both had to be inactivated to localize Orc2 to the cytoplasm. One exception to this rule was deletion D14 which eliminated NLS-B but retained NLS-A. D14 localized to the nucleus. In contrast, deletion D18 also eliminated NLS-B whereas retaining NLS-A, but D18 was distributed throughout both nucleus and cytoplasm. The answer to this paradox is that NLS-A and NLS-B work together. Partial inactivation of NLS-A (M6, D7) led to incomplete cytoplasmic localization, but this phenotype could be converted to complete cytoplasmic localization by also inactivating NLS-B. Thus, it appears that NLS-A is a nuclear localization signal, but in the context of the Orc2 protein, nuclear localization also requires NLS-B. Deletion of the D14 region apparently altered the protein structure so as to relieve the requirement for NLS-B.

Orc2 NLS-A and NLS-B are critical to the nuclear localization of ORC. Loss of the Orc2 ability to localize to the nucleus did not result simply from the loss of its association with other ORC subunits whose own NLS may have facilitated nuclear localization of Orc2. M6, which contained a defective NSL-A but was equivalent to wtOrc2 in its ability to form an ORC, suggested that ORC-(2–5) would not assemble in the nucleus if the Orc2 NLS-A was disrupted. Conversely, D17, D19, and D8 to D11, which contained an intact NLS-A and NLS-B, but did not associate with any other ORC subunit, still localized to the nucleus. D18 was the only segment of the ORC assembly domain that was also required for nuclear localization.

Those Orc2 mutants that were localized exclusively to the cytoplasm (M6 + D18, M6 + M31, M6 + M32) could not assemble ORCs (Fig. 8B), suggesting that ORC assembly occurs predominantly, if not exclusively, in the nucleus. The same sequences may be involved in both nuclear localization and ORC assembly. Note that M6 inhibits nuclear localization (Fig. 7) but not ORC assembly (Fig. 6A). Moreover, given the demonstrable presence of two NLSs in Orc2, and the predicted presence by PSORTII of at least two NLSs in Orc3, but none in Orc4 and Orc5, one might predict that Orc4 and Orc5 are chaperoned into the nucleus by Orc2 and Orc3. Thus, the ORC-(2–5) core complex could assemble in the cytoplasm and then be transported into the nucleus. Orc1 and Orc6, which have a low affinity for ORC-(2–5), can be localized to the nucleus independent of ORC-(2–5). Orc1 has five putative NLSs (one of which has been confirmed, Ref. 25), and Orc6 has one. Still, we cannot exclude the presence of non-traditional NLSs, similar to NLS-B in Orc2, in other Orc proteins.

REFERENCES
1. Mendez, J., and Stillman, B. (2003) Bioessays 25, 1158–1167
2. Kong, D., and DePamphilis, M. L. (2001) Mol. Cell. Biol. 21, 8095–8103
3. DePamphilis, M. L. (2005) Cell Cycle 4, 70–79
4. Dhar, S. K., Delmolino, L., and Dutta, A. (2001) J. Biol. Chem. 276, 29067–29071
5. Giordano-Coltart, I., Ying, C. Y., Gautier, J., and Hurwitz, J. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 69–74
6. Vashee, S., Simancek, P., Chabolla, M. D., and Kelly, T. J. (2001) J. Biol. Chem. 276, 26666–26673
7. Ranjan, A., and Gossen, M. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 4864–4869
8. Kneissl, M., Putter, V., Szalay, A. A., and Grummt, I. (2003) J. Biol. Chem. 327, 111–128
9. Chesnakov, L., Remus, D., and Botchan, M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11997–12002
10. Ohta, S., Tatsumi, Y., Fujita, M., Tsutsumi, T., and Obuse, C. (2003) J. Biol. Chem. 278, 41505–41510
11. Vashee, S., Cvetic, C., Lu, W., Simancek, P., Kelly, T. J., and Walter, J. C. (2003) Genes Dev. 17, 1894–1908
12. Iyer, L. M., and Aravind, L. (2006) in DNA Replication and Human Disease (DePamphilis, M. L., ed) pp. 751–760, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
13. Vassilev, A., Kaneko, K. J., Shu, H., Zhao, Y., and DePamphilis, M. L. (2001) Genes Dev. 15, 1229–1241
14. Saha, T., Ghosh, S., Vassilev, A., and DePamphilis, M. L. (2006) J. Cell Sci. 119, 1371–1382
15. Keller, C., Ladenburger, E. M., Kremer, M., and Knippers, R. (2002) J. Biol. Chem. 277, 31430–31440
16. Ladenburger, E. M., Keller, C., and Knippers, R. (2002) Mol. Cell. Biol. 22, 1036–1048
17. Abdurashidova, G., Danailov, M. B., Ochem, A., Triolo, G., Djeliova, V., Radulescu, S., Vindigni, A., Riva, S., and Falaschi, A. (2003) EMBO J. 22, 4294–4303
18. Prasanth, S. G., Prasanth, K. V., Siddiqui, K., Spector, D. L., and Stillman, B. (2004) EMBO J. 23, 2651–2663
19. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
20. Mendez, J., Zou-Yang, X. H., Kim, S. Y., Hidaka, M., Tansey, W. P., and Stillman, B. (2002) Mol. Cell 9, 481–491
21. Mendez, J., and Stillman, B. (2000) Mol. Cell Biol. 20, 8602–8612
22. Zhou, G., Li, H., Gong, Y., Zhao, Y., Cheng, J., Lee, P., and Zhao, Y. (2005) Proteomics 5, 3814–3821
23. Thome, K. C., Dhar, S. K., Quintana, D. G., Delmolino, L., Shahsafaei, A., and Dutta, A. (2000) J. Biol. Chem. 275, 35233–35241
24. Saiioh, Y., Miyagi, S., Ariga, H., and Tsutsumi, K. (2002) Nucleic Acids Res. 30, 5205–5212
25. Ritzi, M., Tillack, K., Gerhardt, J., Ott, E., Humme, S., Kremmer, E., Hammerschmidt, W., and Schepers, A. (2003) J. Cell Sci. 116, 3971–3984
26. Tatsumi, Y., Ohta, S., Kimura, H., Tsutsumi, T., and Obuse, C. (2003) J. Biol. Chem. 278, 41528–41534
27. Takeda, D. Y., Shibata, Y., Parvin, J. D., and Dutta, A. (2005) Genes Dev. 19, 2827–2836
28. Fujita, M., Ishimi, Y., Nakamura, H., Kiyono, T., and Tsutsumi, T. (2002) J. Biol. Chem. 277, 10354–10361
29. Kreitz, S., Ritzi, M., Baack, M., and Knippers, R. (2001) J. Biol. Chem. 276, 6337–6342
30. McNairn, A. J., and Gilbert, D. M. (2005) J. Cell. Biochem. 96, 879–887
31. Dhar, S. K., Yoshida, K., Machida, Y., Khaira, P., Chaudhuri, B., Wohlschlegel, J. A., Leffak, M., Yates, J., and Dutta, A. (2001) Cell 106, 287–296