

Identification and Characterization of the Human ORC6 Homolog*

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Suman K. Dhar‡ and Anindya Dutta§

From the Division of Molecular Oncology, Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

A new protein was cloned and identified as the sixth member of the human origin recognition complex (ORC). The newly identified 30-kDa protein hsORC6 is 28% identical and 49% similar to ORC6p from *Drosophila melanogaster*, which is consistent with the identities and similarities found among the other ORC members reported in the two species. The human ORC6 gene is located on chromosome 16q12. ORC6 protein level did not change through the cell cycle. Like ORC1, ORC6 did not co-immunoprecipitate with other ORC subunits but was localized in the nucleus along with the other ORC subunits. Several cellular proteins co-immunoprecipitated with ORC6, including a 65-kDa protein that was hyperphosphorylated in G₁ and dephosphorylated in mitosis. Therefore, unlike the tight stoichiometric association of six yeast ORC subunits in one holo-complex, only a small fraction of human ORC1 and ORC6 is likely to be associated with a subcomplex of ORC2, 3, 4, and 5, suggesting differences in the architecture and regulation of human ORC.

Initiation of eukaryotic DNA replication takes place at multiple sites in the genome. In yeast *Saccharomyces cerevisiae*, a six protein origin recognition complex (ORC)¹ binds to ARS consensus sequences in a sequence-specific manner (1, 2). All six ORC subunits are essential for cell viability and DNA replication (1–6). In *Xenopus laevis* and in *Drosophila melanogaster*, a six-protein complex similar to that found in *S. cerevisiae* has been reported (7, 8). *Xenopus* egg extracts depleted of the ORC subunits can regain replication activity when xORC preparations are added back into the replication reaction (7). Recombinant dmORC preparation can rescue the DNA replication activity of ORC depleted *Xenopus* and *Drosophila* egg extracts (9). Genetic experiments in *Drosophila* have shown that ORC2 and ORC3 are essential for DNA replication (10, 11). These results lead to the conclusion that ORC is essential for DNA replication in higher eukaryotes. However, in metazoans, the DNA sequences essential for replication initiation still remain elusive.

The eukaryotic DNA replication model derived from the evidence in yeast *S. cerevisiae* suggests that ORC remains bound to origins of DNA replication throughout the cell cycle (12, 13).

In G₁, CDC6 is loaded on chromatin in ORC-dependent manner that in effect facilitates the assembly of MCM2–7 (13–15). *In vitro* studies in *Xenopus* egg extracts also show that ORC recruits essential replication factors to the DNA (16, 17). Formation of this prereplicative complex is the key to initiate chromosomal DNA replication at the onset of S phase. Recently, in *Xenopus* and in *Schizosaccharomyces pombe*, a protein, called CDT1, essential for DNA replication has been reported to be associated with the prereplicative chromatin in an ORC-dependent manner (18, 19). Like CDC6, CDT1 is also required for MCM loading on chromatin. As replication proceeds the MCM and CDC6 proteins are removed from the chromatin to prevent rereplication.

Although conservation of structure and function is expected between yeast ORC and metazoan ORC, several differences have emerged. Five members of human ORC have been reported so far (11, 20–25). HsORC1, 2, 4, and 5 have considerable homology with their yeast and *Drosophila* counterparts. HsORC3, in contrast, shows poor homology with yeast scORC3 but significant homology with dmORC3 (11). HsORC2, 3, 4, and 5 physically interact with each other, but biochemical interaction of hsORC1 with other hsORC subunits has not been established so far under endogenous levels of expression. In *S. pombe*, the N-terminal half of the spORC4 contains an AT-hook region that is unique to fission yeast (26, 27). These discrepancies clearly suggest that the behavior of individual ORC subunits may not be conserved perfectly among different eukaryotic species, although collectively they may play a similar role in DNA replication.

Here we report the cloning and expression of the sixth member of human ORC, the hsORC6 molecule homologous to dmORC6. Identifying hsORC6 will lead us to our ultimate goal of reconstituting the human ORC *in vitro*. Like hsORC3, the homology between scORC6 and hsORC6 is poor. Like ORC1, ORC6 did not interact with other ORC subunits under our experimental conditions. Therefore, the architecture of human ORC may be different from its yeast and *Drosophila* counterparts. We also found that several unidentified cellular proteins interact with hsORC6, which may be useful to get an insight on the function of human ORC.

EXPERIMENTAL PROCEDURES

Cloning of ORC6—The ORC6 clone in expressed sequence tag 177464 contains the entire open reading frame except the first ATG. The CCATGG sequence was reconstituted by polymerase chain reaction primer mutagenesis, and the cDNA from the ATG to the 3' end were cloned between *EcoRI* (5') and *XhoI* sites of pBluescript SK(–), to give pSK-ORC6. The entire sequence was confirmed by automated sequencing of appropriate deletion constructs and use of a few synthetic primers.

Raising Antibody and Immunoprecipitation—ORC6 cDNA from pSK-ORC6 was cloned in pET14b plasmid between *NdeI* and *BamHI* to express ORC6 protein fused to a His₆ epitope tag. The protein was produced in bacteria followed by purification on a nickel resin column. The purified protein was used to raise antibodies in rabbits (Co-calico

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§ To whom correspondence should be addressed. Tel.: 617-278-0468; Fax: 617-732-7449; E-mail: adutta@rics.bwh.harvard.edu.

¹ The abbreviations used are: ORC, origin recognition complex; MCM, mini-chromosome maintenance; dmORC, *Drosophila melanogaster* ORC; PBS, phosphate-buffered saline.

TABLE I
Sequences of exon-intron boundaries of ORC6

Sequences of the introns are given in lowercase letters, and those of exons are in uppercase letters. The numbers in the parentheses are nucleotide coordinates of the exon sequences assuming that the start of the cDNA (GenBank™ accession number NM 014321) is nucleotide 9. First eight nucleotides of the cDNA are adapter sequences. A of ATG is nucleotide 49.

Exon I (9–113)	tctggtgaccCGCGCGTTC. . . . ACATGCTGAGgtgagttcgg . . .
Exon II (114–243)	ttttaaccagGAAAGCAGAG. . . . CTTGGACAGGtaagtaggt . . .
Exon III (244–407)	cctttcacagGCTTATTAA. . . . TACTAAAAAGgtatggggca . . .
Exon IV (408–497)	tctccttttagCTATGAGTCC. . . . CAGCATGCAAgtaggtattt . . .
Exon V (498–610)	ttgtttttagGATTCTAAAG. . . . CAGGTCGACagtaagtattc . . .
Exon VI (611–679)	cttccttaagGAGAACCTGG. . . . CCAGCAAAGGtaagtttta . . .
Exon VII (680–1621)	ttctgtcagAAATGGAGAA. . . . ATGCATTCAGgtgtacttg.

Biologicals). Antibodies against human hsORC1, hsORC2, hsORC3, hsORC4, and hsORC5 have been described elsewhere (11, 22, 23, 28). Cyclin A antibodies were purchased from Santa Cruz Biotechnologies. Immunoprecipitation and immunoblotting of cell lysates were carried out using standard protocols.

Cell Synchronization and [³⁵S]Methionine and ³²PO₄ Labeling of Cellular Proteins—Exponentially growing HeLa cells were arrested for 24 h with 40 ng/ml of nocodazole at M phase, with 10 mM hydroxyurea at early S phase, or with 5 μg/ml of aphidicolin at the G₁/S transition point. For synchronization at G₁, asynchronous cells were arrested for 36–48 h with mevastatin (10 μM). For metabolic labeling with [³⁵S]methionine, cells were incubated for 2 h in medium lacking methionine and then incubated overnight in medium supplemented to a final concentration of 3 mM cold methionine plus 200 μCi (34 pM) of [³⁵S]methionine (PerkinElmer Life Sciences; 1175 Ci/mmol) in the presence of respective drugs. For [³²P]orthophosphate labeling of cellular proteins, synchronized cells were first incubated in phosphate-free medium for 2 h and then in medium containing 350 μCi (80 pM) of [³²P]orthophosphate (PerkinElmer Life Sciences; 8500 Ci/mmol) for another 3 h in the presence of respective drugs before harvesting them.

Immunofluorescence—U2OS cells were fixed in 3% formaldehyde containing 2% sucrose at room temperature for 10 min, washed twice with phosphate-buffered saline (PBS) and permeabilized in Triton solution (3% bovine serum albumin, 0.5% Triton X-100 in PBS) for 5 min. Cells were then incubated at room temperature for 45 min with anti-ORC6 antibody (1:500 dilution) followed by three washes in 1× PBS. Finally cells were incubated with the secondary antibody (fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulin G) at room temperature for 30 min followed by three washes with PBS. Cells were stained briefly with 4',6-diamidino-2-phenylindole and photographed under a fluorescent microscope.

Gel Filtration—5 × 10⁶ 293T cells were lysed in 0.15 M Nonidet P-40 lysis buffer containing 50 mM Tris, pH 8.0, 0.1% Nonidet P-40, 150 mM NaCl, 50 mM NaF, 1 mM Na₃VO₄, 5 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, and 5 μg/ml aprotinin for 1 h at 4 °C. The lysate was then incubated at 37 °C for 15 min in the presence of 1000 units/ml DNaseI supplemented with 5 mM MgCl₂. The lysate was centrifuged (15000 × g for 20 min at 4 °C) and filtered through a 0.22-μm filter. The filtrate was passed through fast protein liquid chromatography Superose 12 (Amersham Pharmacia Biotech) column using lysis buffer at a flow rate of 0.2 ml/min. Alternate fractions were separated on 12% SDS-polyacrylamide gels and immunoblotted with anti-ORC2 and ORC6 antibodies.

RESULTS

Cloning and Sequence Analysis of Human ORC6—While searching the data base to get possible candidates for putative hsORC6 subunit, we found expressed sequence tags with considerable homology with *Drosophila* ORC6 subunit. The entire ORC6 sequence was reconstituted from two cDNA clones; IMAGE:199024 (H83116) and expressed sequence tag 177464 (AA306512). The entire sequence has been deposited in the data base by Dr. M. O'Donnell and co-workers (GenBank™ accession number NM014321), and the only difference is that the 5' end of ORC6 begins from +9 of the deposited sequence (Table I). Ours is the first published report on this new ORC6 subunit. The cDNA encoded an open reading frame corresponding to 252 amino acids with a predicted molecular mass of ~28 kDa. The predicted molecular mass is very close to that of dmORC6 (31 kDa) and a 29 kDa unidentified protein in xLORC (7, 8). An alignment of the ORC6 subunit in human, *Drosophila*,

S. pombe, and *S. cerevisiae* is shown in Fig. 1A. The polypeptide is significantly homologous to the ORC6 subunit of *Drosophila* (28% identical and 49% similar) over the entire coding region (Fig. 1, A and B). This degree of homology between hsORC6 and dmORC6 is in good agreement with the homology of other five human and *Drosophila* ORC subunits (Fig. 1B). The homology of the *Drosophila* and human ORC6 subunit with *S. cerevisiae* ORC6 is low (5% identical and 19% similar; 6% identical and 15% similar, respectively) (Fig. 1, A and C). The *S. pombe* ORC6 is also significantly different from *S. cerevisiae* ORC6 (27). These results suggest that ORC6 has evolved faster than the other ORC subunits in eukaryotes.

hsORC6 Is Located at Chromosome 16q12—BLAST search using the cDNA sequence of ORC6 showed a significant homology with a human BAC clone (accession number AC007225) located at chromosome 16q12. Comparing those two sequences we found the intron-exon boundaries of ORC6 (Table I). The mRNA is transcribed from 7 exons spanning over a 9-kilobase region in the genome. The nucleotide sequence analysis of genomic sequence upstream from the start site of the cDNA did not show any obvious E2F transcription factor binding site, suggesting that ORC6 mRNA level may not be cell-cycle regulated. This is consistent with the steady expression of other ORC subunits through the cell cycle but different from the promoters of ORC1 and CDC6, which have E2F binding sites and whose mRNA is cell cycle regulated (28, 29).

ORC6 Protein Level Is Unchanged throughout the Cell Cycle—To examine ORC6 protein in the cell extract, a polyclonal antiserum was raised against bacterially produced His₆-tagged ORC6p. It recognizes the bacterially expressed His₆-tagged antigen. The antiserum also recognizes a single 30-kDa protein in 293T cell extract (Fig. 2A) that is very close to the predicted mass of 28 kDa.

To examine whether ORC6 protein levels change through the phases of the cell cycle, HeLa cells were blocked at M phase using nocodazole and released over a time period of 24 h. Western blot analysis of protein samples at various stages of cell cycle clearly shows that the total level of ORC6p is not significantly changed throughout the cell cycle (Fig. 2B). Normal progression of the cell cycle was indicated by the periodic expression of cyclin A, which is normally induced at the G₁/S transition and degraded in M phase. The expression level of ORC2p is also not changed significantly over the same course of time (Fig. 2B). ORC1p, ORC3p, ORC4p, and ORC5p expression levels also remain unchanged through the cell cycle (11, 22, 23, 30).

ORC6 Protein Does Not Interact with Other hsORC Subunits—We wanted to examine whether the newly identified putative human ORC6 physically interacts with other hsORC subunits. Immunoprecipitation of 293T cell extract using anti-ORC2 antibody specifically co-precipitated hsORC3, 4, and 5 subunits (Fig. 3). ORC6 was not immunoprecipitated by anti-ORC2 antibody under these experimental conditions. Similarly, ORC1 was also not present in the anti-ORC2 immunoprecipitates. Conversely, immunoprecipitation using anti-

A

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hsORC6 : GSEELGRAPRLGIA-EFDMRRAEYRLSRVQVCGHS-----ARTTSTSSAACLALASWKKPPLR-----A : 67
dmORC6 : -MTTLEDTTKMAREEPNVLKNTLVRRLRSTNVP-----LILSYGKINLCALASCMIGIAFA-----S : 67
spORC6 : ---MERACLESRRRIPTSTLYNRRLSSGSLFLEWSK-----YKHLKATEELCRPYMAHLAELSLNDLNLGI : 70
scORC6 : ISMQQVHCVAEVLRLDPQKPDWSSGYKKKTNAISIIYNTSLNKVMKLKDESVARCHITRYTSQKKNNEKHMP---D : 79

hsORC6 : LKLSGLNRETQSCLKSPGCG-----LNSNIGIRHACQSCIPANMSKILKSYE : 122
dmORC6 : ALKLSGLRSQLNNKRMERLID-----LNKLSVNLHCQLDLNVAAYEELMTLF : 122
spORC6 : NLLATLPPKKIYKFTYQELSLPL-----TKSLAARDIMETITYLCTKLGGTALPIYA : 127
scORC6 : YIDSIETEPKAKHEMLNLRQSLSNSSPMKQFAWTSPKKNKRSPVKNGGRFTSSDPQLRNQLSGTPTKKNRQNDSEFT : 161

hsORC6 : SSLPQVQLLRLRLTSAALLSCKIQLRLVGRNFMVAAG-----VKKALDRCKKLL : 180
dmORC6 : G-PAEDMGITDSEQYATMAVFOCRLEKKNSSSLMPFPA-----LRSQLLEQWPE : 179
spORC6 : QLRAVVDNMRIERGGITLAAYLLVSAKAFKDELHINHERK-----KAQSVLQDTLSALQI : 187
scORC6 : PELTPMKNESPSITRKLAFEDFEDEDEEPPNGGSSKSHKNSITGTRNVDSDEYENHESDPTSEEEELGVQELRSGR : 243

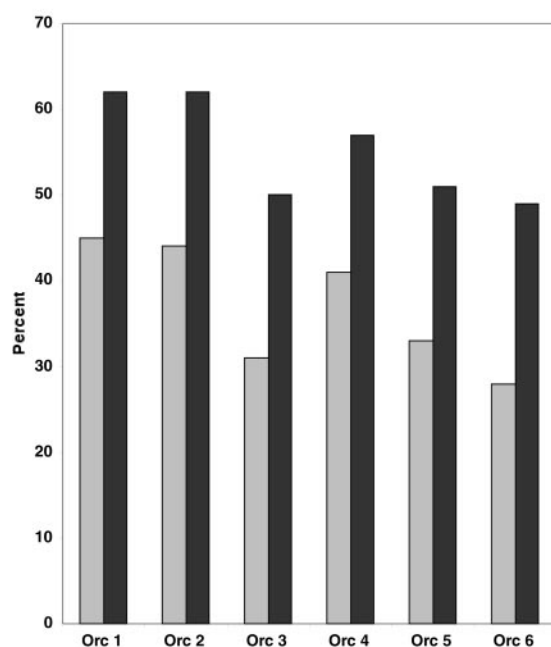
hsORC6 : IIGQQDRPGDRAIPRKKEKIVVEAPAKEMEKVEEMPHEFQKDE----- : 226
dmORC6 : RMIKHHKE-KKPSSTDMEGALKENNNENFGHDAKKAHKPP----- : 221
spORC6 : QYWMHLSWQYFEILNGIEYSSKQREKPSWSGIASMIQDYE----- : 233
scORC6 : QNKAKGEPQELAKAKLREERIPNLSLLVSKYCKMTTEEILCNDLFELPREVAYKIVDEYNINASRLVCPWQLVCGVLV : 325

hsORC6 : -----LTQDIEEGRRLNLA : 244
dmORC6 : -----PEDEIWKARMAKAC : 237
spORC6 : -----KRMNPFKWKACERLI : 251
scORC6 : NCTFIVFNERRRKDPRIDHFIVSKMCSLMLTSKVDVIECVKLVKELIIGEKWFRDLQIRYDDFGRIYDEIFRGLGSML : 407

hsORC6 : SAGQATAE----- : 252
dmORC6 : AKLLEPASSHMDSQLLA----- : 257
spORC6 : YYKSQELKGTAS----- : 264
scORC6 : TNLILMTDQTNWKKRLEMDLALTEPL : 435

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B

Human Orcs Vs. *Drosophila* Orcs in Identity and Similarity

C

Human Orcs Vs. Yeast Orcs in Identity and Similarity

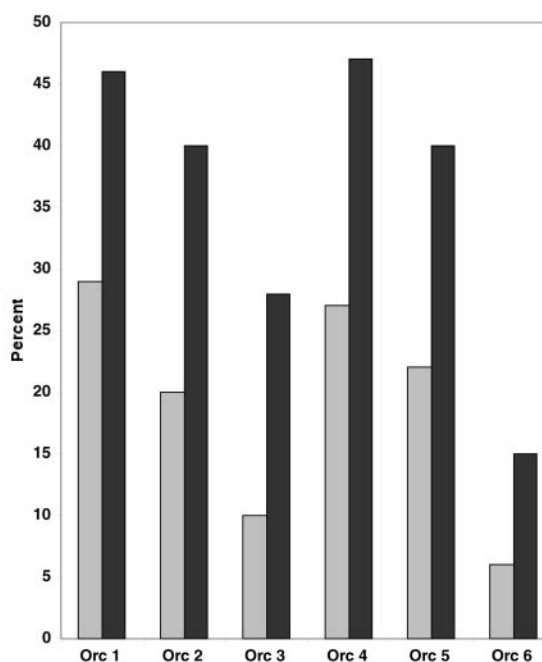


FIG. 1. Comparison of protein sequences of human ORC6 (hsORC6), *D. melanogaster* ORC6 (dmORC6), *S. pombe* ORC6 (spORC6), and *S. cerevisiae* ORC6 (scORC6). A, alignment of protein sequences of hsORC6, dmORC6, spORC6, and scORC6. The alignment and shading was performed using GeneDoc program. Identical residues are marked by dark shading, and similar residues are marked by light shading. The numbers on the right indicate the amino acid residues of ORC6 in each species. B, bar diagram, light bars indicate percent identity of hsORC6 subunits with those of dmORC6 counterparts, whereas dark bars show the percentage of similarity among them. C, bar diagram shows the identities and similarities between human and *S. cerevisiae* ORC subunits.

ORC6 antibody did not co-precipitate any of the other ORC subunits from 293T cell extract. Identical results were obtained from a HeLa cell line.

Gel Filtration of ORC6 Subunit from 293T Cell Extract—ORC6 did not co-immunoprecipitate with any other ORC subunit under our extraction conditions. We were interested to see

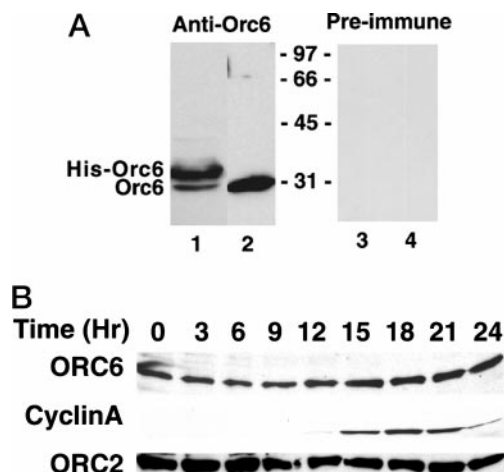


FIG. 2. Characterization of anti-ORC6 antibody and abundance of ORC6 through the cell cycle. A, bacterially produced His₆-tagged ORC6 (lanes 1 and 3) and 293T cell extracts (lanes 2 and 4) were immunoblotted with anti-ORC6 antibody (lanes 1 and 2) or with preimmune serum from the same rabbit (lanes 3 and 4). The positions of ORC6 and His₆-tagged ORC6 are indicated in the figure. B, HeLa cells were synchronized in mitosis using nocodazole (40 mg/ml) for 24 h and then released in drug free media. Whole cell extracts were prepared at indicated hours after release from mitotic block and immunoblotted using anti-ORC6 or anti-cyclin A or anti-ORC2 antibodies.

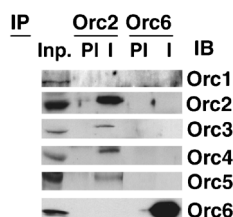


FIG. 3. Association of ORC6 with other ORC subunits. 293T cell lysate was immunoprecipitated (IP) either by using anti-ORC2 or anti-ORC6 antibodies (I) or their respective preimmune sera (PI) followed by immunoblotting using anti-ORC1, 2, 3, 4, 5, and 6 antibodies. 5% of lysate used for IP was loaded in the input lanes (Inp.).

whether the majority of ORC6p exists as monomers or multimers in the cell. ORC6 could also be present in a larger complex with other ORC subunits without any stable direct interaction among them. To investigate these issues, a 293T cell extract (150 mM NaCl, 0.1% Nonidet P-40) was passed through Superose 12 gel filtration column. Alternate fractions were immunoblotted with anti-ORC6 antibodies (Fig. 4). ORC6 peaked in fraction 21, consistent with a complex of 200 kDa, suggesting that either this protein is a multimer or associated with other cellular proteins. Although ORC2 level peaked at an earlier fraction (fraction 19) than ORC6, a reasonable quantity of ORC6 overlapped with ORC2 peak fraction. Therefore, there remains the theoretical possibility that small fractions of ORC2 and ORC6 proteins are part of a larger complex that is disrupted by immunoprecipitation with either anti-ORC2 or anti-ORC6 antibodies.

ORC6p Interacts with Unidentified Cellular Proteins—Because ORC6 did not co-immunoprecipitate with other ORC subunits but still eluted as a larger complex, we were interested to see whether this protein interacted with other cellular proteins to understand how the protein is regulated or even linked to the replication apparatus. [³⁵S]Methionine-labeled HeLa cell extracts prepared from asynchronous cells and cells blocked at the different stages of the cell cycle were immunoprecipitated using either anti-ORC6 antibody or preimmune serum. Besides the 30-kDa ORC6, polypeptides of 35, 40, 65, 68, 72, 80, and 115 kDa were present specifically in the im-



FIG. 4. Gel filtration of ORC6 subunit from 293T cell extract. 293T cell extract was fractionated over a fast protein liquid chromatography Superose 12 gel filtration column and alternate fractions (fractions 11–29) were immunoblotted using anti-ORC2 or anti-ORC6 antibodies. The positions of the molecular mass markers thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), and equine myoglobin (17 kDa) are shown on top. Input lanes were loaded with 5% of the total lysate passed through the column.

mune lane (Fig. 5A). None of these proteins were recognized by the anti-ORC6 antibodies in immunoblots, suggesting that these cellular proteins are not precipitated because of cross-reaction to anti-ORC6 antibody but are associated with hs-ORC6. To examine whether there was any change in ORC6 associated proteins in G₁ versus M, mevastatin blocked cells (G₁) were compared with nocodazole blocked cells (M). The same polypeptides were present in the immunoprecipitates in G₁ or M.

ScORC6 is highly phosphorylated in the G₁ phase of the cell cycle by cyclin-Cdks (3). Sequence analysis of ORC6 did not, however, show any obvious Cdk phosphorylation sites. To find out whether ORC6 is phosphorylated in a cell cycle-specific manner, we performed immunoprecipitation experiments using [³²P]₄-labeled HeLa cell extracts prepared from cells blocked at the different stages of the cell cycle. ORC6 was very weakly phosphorylated, but its phosphorylation level did not change with the cell cycle (Fig. 5B). There were several phospho-proteins of 35, 45, 60, 65, 70, and 80 kDa co-immunoprecipitated with ORC6. One phosphoprotein of 65 kDa was particularly prominent in the G₁ phase of the cell cycle compared with M phase cells. A 65-kDa protein co-immunoprecipitated with ORC6 from [³⁵S]methionine-labeled cell extracts, and the abundance of this protein was not changed to the same extent between G₁ and M phase of the cell cycle (Fig. 5A). If the 65-kDa protein identified by [³⁵S]methionine and [³²P]₄ labeling is the same, then cell cycle-regulated phosphorylation of an ORC6-associated protein could be important for the regulation of ORC6.

Localization of ORC6 in Human Cells—Because one component of the replication initiation complex, CDC6, changes intracellular localization through the phases of the cell cycle (28), we examined by immunofluorescence whether ORC6 was similarly regulated. Asynchronous culture of human U2OS osteosarcoma cells was immunostained using preimmune or anti-ORC6 antibody (Fig. 6). ORC6 is localized predominantly in the nucleus consistent with the nuclear localization of other ORC subunits.² The fact that ORC6 was constitutively nuclear in all cells in the asynchronous culture suggests that its intracellular localization was not regulated through the phases of the cell cycle.

DISCUSSION

We report here the identification of a sixth member of the human origin recognition complex, ORC6. This protein is highly homologous to dmORC6. dmORC6 is part of the six-protein dmORC that is required for DNA replication in the *Drosophila* and *Xenopus* egg extract. Strong homology with *Drosophila* ORC6 and an equivalent mass of ~30 kDa suggests that this is indeed the hsORC6 subunit. We also found that

² K. C. Thome and A. Dutta, unpublished results.

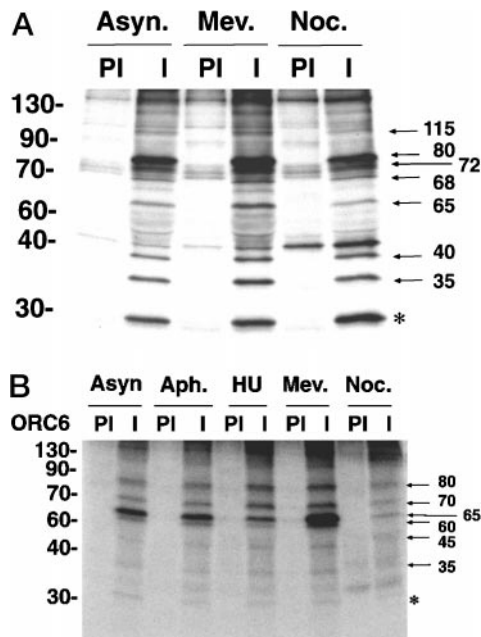


FIG. 5. ORC6 protein associates with other cellular proteins. A, HeLa cells were synchronized either in mitosis using nocodazole for 24 h (Noc.) or in G₁ using mevastatin for 36 h (Mev.). Synchronized cells were radiolabeled for 12 h using [³⁵S]methionine, and cell lysates were immunoprecipitated either using anti-ORC6 antibodies (I) or pre-immune sera (PI). Asynchronously (Asyn.) growing HeLa cells were also treated in the same way. Samples were loaded on 12% SDS-polyacrylamide gels and visualized by fluorography. The asterisk indicates the position of ORC6 molecule, and arrows indicate the cellular proteins specifically interacting with ORC6. Molecular mass markers are shown on the left. B, HeLa cells were synchronized at different stages of the cell cycle using the following drugs, e.g. aphidicolin or hydroxyurea (G₁/S boundary or S respectively), mevastatin (G₁), or nocodazole (M). Synchronized cells were then labeled with ³²PO₄ in the presence of respective drugs. Asynchronously growing HeLa cells were also treated the same way. Radiolabeled cell lysates were immunoprecipitated using either anti-ORC6 antibodies or pre-immune sera. Samples were then electrophoresed in a 12% SDS-polyacrylamide gel and visualized by autoradiography. The asterisk indicates ORC6, and arrows indicate associated phosphoproteins.

ORC6p does not co-immunoprecipitate with other ORC subunits under our experimental conditions. Although ORC subunits in yeast and early developmental stages of metazoa associate with each other in a stable holocomplex, the same is not true for mammalian ORCs from somatic cells. Endogenous hsORC2, 3, 4, and 5 subunits have been shown to interact with each other by co-immunoprecipitation experiments using human cell extract (11, 22). Interestingly, hsORC1 has not been shown to interact with other ORC subunits under normal conditions. This suggests that stable complex of six ORC may not be abundant in human somatic cells.

ORC6p expression level and subcellular localization is not changed through the cell cycle. This finding is consistent with the constitutive expression pattern of all other ORC protein subunits through the cell cycle in human somatic cells (22, 23, 28, 30). In yeast too ORC is constitutively expressed throughout the cell cycle. The G₁-specific formation of the prereplication complex in yeast is attributed to the appearance of CDC6, which is conceptually similar to the nuclear localization of human CDC6 in G₁.

Hs ORC6 interacts with many unidentified cellular proteins. Identification of these proteins may help us to understand the function of ORC6 in human DNA replication and other cellular processes. Based on the hypothesis that human ORC will be fundamentally similar to the yeast, *Drosophila*, and *Xenopus* counterparts, it is likely that a complex of six subunits is

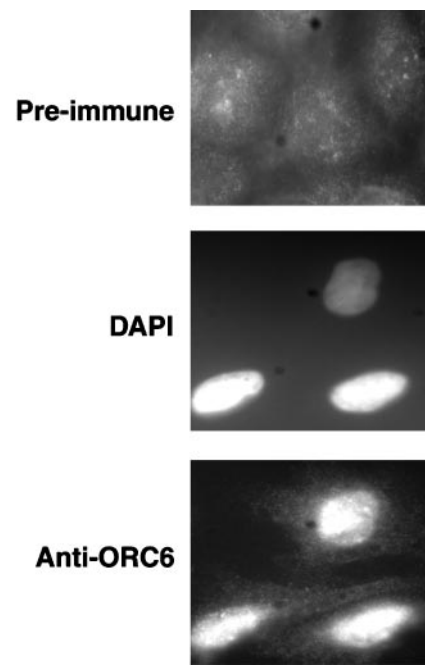


FIG. 6. Subcellular localization of ORC6 protein. U2OS cells were grown on chamber cover slides. Cells attached to slides were fixed and incubated with anti-ORC6 antibodies or preimmune sera followed by fluorescein isothiocyanate-conjugated swine anti-rabbit serum. Cells were washed and mounted with Vectashield containing 4',6-diamidino-2-phenylindole and visualized under a fluorescence microscope. The bottom two panels are of the same field photographed with separate filters for 4',6-diamidino-2-phenylindole (middle) or fluorescein isothiocyanate (bottom).

created in mammalian cells at origins of replication in G₁. The failure to isolate the complex may be due to the difficulties in extraction of some of the ORC subunits (23, 30). ORC6-interacting proteins may then be crucial for bridging ORC6 with other ORC subunits on the chromatin in G₁. One of the ORC6-interacting phosphoproteins is hyperphosphorylated during G₁ phase of the cell cycle and this may be important for ORC6 regulation.

The weak phosphorylation of human ORC6 contrasts with that of yeast ORC6. In addition, the latter is unphosphorylated in G₁ and gets phosphorylated by Cdks at the onset of S phase (3), whereas human ORC6 is constitutively phosphorylated through the G₁/S transition. Of the other ORC subunits, human ORC2 is constitutively phosphorylated through the cell cycle,² whereas human ORC1 is phosphorylated in M phase (30).

In summary, we have identified a putative hsORC6 molecule that is homologous to *Drosophila* ORC6. Although it does not interact with the other ORC subunits *in vivo*, it will lead us one step ahead to our ultimate goal of reconstituting the entire human ORC *in vitro*. The results so far indicate significant differences in the architecture and stability of human and yeast ORC and might predict differences in how replication is regulated in the two species.

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Suman K. Dhar and Anindya Dutta

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