THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 31, Issue of August 3, pp. 29067–29071, 2001
Printed in U.S.A.

Architecture of the Human Origin Recognition Complex*

Received for publication, April 6, 2001
Published, JBC Papers in Press, June 6, 2001, DOI 10.1074/jbc.M103078200

Suman Kumar Dhar‡, Laurie Delmolino§, and Anindya Dutta¶

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

All the human homologs of the six subunits of *Saccharomyces cerevisiae* origin recognition complex have been reported so far. However, not much has been reported on the nature and the characteristics of the human origin recognition complex. In an attempt to purify recombinant human ORC from insect cells infected with baculoviruses expressing HsORC subunits, we found that human ORC2, -3, -4, and -5 form a core complex. HsORC1 and HsORC6 subunits did not enter into this core complex, suggesting that the interaction of these two subunits with the core ORC2–5 complex is extremely labile. We found that the C-terminal region of ORC2 interacts directly with the N-terminal region of ORC3. The C-terminal region of ORC3 was, however, necessary to bring ORC4 and ORC5 into the core complex. A fragment containing the N-terminal 200 residues of ORC3 (ORC3N) competitively inhibited the ORC2-ORC3 interaction. Overexpression of this fragment in U2OS cells blocked the cells in G_1, providing the first evidence that a mammalian ORC subunit is important for the G_1-S transition in mammalian cells.

Origin recognition complex (ORC)[1] was first described in yeast *Saccharomyces cerevisiae* (1). All six subunits, essential for cell viability, collectively bind to the ARS (autonomously replicating sequence) consensus sequence in a sequence-specific manner and lead to the chromatin loading of other replication factors like CDC6 and MCM (mini-chromosome maintenance) that are essential for initiation of DNA replication (2, 3). Similar six protein complexes have been discovered in *Xenopus laevis* (4), *Drosophila melanogaster* (5) and *Schizosaccharomyces pombe* (6), although a consensus DNA sequence that serves as an origin of replication and where ORC may bind has not been found in these species. Conservation of similar ORC subunits in mammals suggests that ORC has an equally important role in mammalian cells.

Although all six human homologs of yeast *S. cerevisiae* ORC subunits have been reported (7–14), purification of a six-protein human origin recognition complex remains elusive. Endogenous ORC2, -3, -4, and -5 subunits have been reported to interact with each other in extracts of cancer cell lines (14). ORC1 and ORC6 did not interact with other ORC subunits under these experimental conditions (14). Therefore it is possible that a functional human ORC exists only during a very short period of the cell cycle or in a specific sub-nuclear compartment, making it difficult to extract such a complex from human cell lines. In fact, in a recent study hamster ORC1 was reported to be easily eluted from chromatin during mitosis and early G_1 phase (15). It became stably bound to chromatin again during mid-G_1 phase with the appearance of a functional pre-replication complex at a hamster replication origin. In contrast, ORC2 was stably bound to chromatin throughout the cell cycle. Difficulties in obtaining six protein human ORCs may also be attributed to the fact that we are still missing some of the unidentified important components of the human ORC. Indeed, immunoprecipitation from [^{35}S]methionine-labeled HeLa cell lysate of ORC1, -2, -3, -4, and -6 showed many non-ORC proteins interacting specifically with the respective ORC subunits (14, 16).

A six protein ORC has been purified from *Drosophila* embryo extracts and possesses some demonstrated biochemical activities (5, 17, 18). All six *Drosophila* ORC subunits were expressed and subsequently purified to homogeneity from baculovirus-infected insect cells (17). Using an *in vitro* transcription translation reaction, a similar six-protein ORC has been reported in yeast *S. pombe* (6). With all the six human ORC subunits in our hand, we attempted to produce recombinant human ORC from the baculovirus expression system in order to dissect the activities and architecture of human origin recognition complex/subcomplex(s).

Because genetic experiments are difficult to perform in mammalian systems, the human ORC subunits have not been shown to have a role in replication or cell proliferation. Utilizing knowledge learned about the architecture of the human ORC, we created a dominant negative ORC subunit designed to disrupt the formation of endogenous ORC. Overexpression of this dominant negative ORC subunit blocked the cell cycle in G_1, providing the first evidence of the importance of ORC in cancer cell proliferation.

MATERIALS AND METHODS

Plasmid Constructions—Cloning of ORC1–6 cDNAs are described elsewhere (7, 9–11, 14). Coding sequences of ORC1, ORC4, ORC5, ORC6, and ORC3N200 were cloned into pFastBac (Life Technologies, Inc.), and coding sequences of ORC2 and ORC3 were cloned into pFastBac Dual. ORC2, -4, and -5 were also cloned in pFB-GST vectors to express GST fusion proteins. ORC3 and all the related constructs for *in vitro* transcription and translation reactions were made into T7T3DPAC vector (GenBank™ accession number U13871). Full-length and C-terminal ORC2 fragments were cloned into pGEX-5X-3 (Invitrogen) to produce bacterial fusion proteins. Additional information regarding the constructs will be made available upon request.

Expression of ORC Subunits in Insect Cells, Purification, and Gel Filtration—Baculoviruses were produced from the recombinant pFastBac or pFB-GST plasmids using the Bac-to-Bac expression system (Life Technologies, Inc.). Hi5 or Sf9 cells (Invitrogen) were infected with

* This work was supported in part by National Institutes of Health Grant CA60499 (to A. D.). 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.

‡ Supported by United States Army Postdoctoral Fellowship DAMD17-00-1-0166.

§ Supported by the American Cancer Society Postdoctoral Fellowship PF-99-328-01-CCG.

¶ To whom correspondence should be addressed: Dept. of Pathology, Brigham and Women's Hospital, Harvard Medical School, 75 Francis St., Boston MA 02115. Tel.: 617-278-0468; Fax: 617-732-7449; E-mail: adutta@rics.bwh.harvard.edu.

[1] The abbreviations used are: ORC, origin recognition complex; GST, glutathione S-transferase; FACS, fluorescence-activated cell sorter; GFP green fluorescent protein.

these baculoviruses according to the manufacturers' recommendations. Cells were harvested 48 h post-infection. The cell pellet was washed once in cold phosphate-buffered saline and subsequently resuspended in hypotonic lysis buffer (10 mM Tris/Cl, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$,1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM dithiothreitol). The cell suspension was homogenized in a Dounce homogenizer using a B-type pestle followed by centrifugation at 3000 rpm for 7 min. The pellet containing the nuclei was lysed in buffer H/0.15 (50 mM HEPES/KOH, pH 7.5, 150 mM KCl, 0.02% Nonidet P-40, 5 mM magnesium acetate, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM dithiothreitol). The resulting suspension was subjected to ammonium sulfate precipitation (starting with 10% followed by 30% and finally 50%). The pellet after the 50% ammonium sulfate cut was resuspended in buffer H/0.0 (no salt) and then dialyzed overnight against buffer H/0.15. The dialyzed sample was then bound to GST beads (Sigma) and washed three times with buffer H/0.25. Proteins were eluted using reduced glutathione elution buffer (50 mM Tris/Cl, pH 8.0, 20 mM reduced glutathione, 0.01% Nonidet P-40, 100 mM NaCl). Gel filtration of glutathione eluate using a fast protein liquid chromatography Superose 12 (Amersham Pharmacia Biotech) column was performed as described previously (14).

Cell Culture, Transfection, Immunoblotting, Immunoprecipitation, and Silver Stain—Sf9 and Hi5 cells were maintained according to the manufacturers' protocol (Invitrogen). U2OS cells used for FACS analysis were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Life Technologies, Inc.). Plasmid DNA used for transfection were purified using Qiagen maxiprep kits. Cells were grown in 100-mm dishes and transfected using LipofectAMINE (Life Technologies, Inc.). Western blotting and immunoprecipitation techniques were carried out using standard protocols. Anti-GST polyclonal antibodies were purchased from Santa Cruz Biotechnologies. Antibodies against HsORC1–6 have been described previously (9–11, 14). The silver stain method has been described elsewhere (19).

In Vitro Transcription and Translation Reactions and GST Pull-down Assay—*In vitro* transcription and translation reactions to produce [^{35}S]methionine-labeled full-length and different deletions of ORC3 were performed using the Promega TNT system (Madison, WI). Pull-down assays on glutathione-agarose beads were done as described previously (20).

FACS Analysis—U2OS cells were transfected with farnesylated GFP (CLONTECH) alone or in combination with FLAGORC2, GFPC1-ORC3N, or GFPC1-ORC3C1. Forty-eight hours after transfection, cells were trypsinized, washed with phosphate-buffered saline, fixed with cold 70% ethanol, and stored until further use. Before analysis, fixed cells were resuspended in phosphate-buffered saline containing 50 μg/ml propidium iodide (Sigma), 10 μg/ml RNaseA (Sigma), and 0.05% Noniodet P-40 and then incubated for 1 h at 4 °C. Finally cells were washed in phosphate-buffered saline and analyzed by flow cytometry. The data were further analyzed using FLOWJO software to calculate the percentage of cells residing in different cell cycle stages.

RESULTS

GSTORC5, -2, -3, and -4 Forms a Complex—ORC2, -3, -4, and -5 subunits have been shown to interact with each other in human cell extracts (14). In an attempt to purify recombinant six protein human origin recognition complex, we infected Sf9 insect cells with baculoviruses expressing human ORC1–6 subunits. One of the subunits, ORC5, was GST-tagged. After pull down on glutathione beads, we found that GSTORC5, -2, -3, and -4 can be purified as a complex (Fig. 1*A*). ORC1 did not enter into the complex in a stoichiometric ratio, and the presence of very little ORC1 in Fig. 1*A* was not reproducible in different preparations. ORC6 did not enter into the complex at all. Both ORC1 and ORC6 were expressed at a high level. In a control experiment, we expressed GST alone with other ORC subunits. Pull down on glutathione beads purified only GST but none of the ORC subunits. Therefore, the results in Fig. 1*A* are due to the formation of a complex of ORC2, -3, -4, and -5 and not due to precipitation of the proteins on the glutathione beads. GST pull-down experiments using GST tags on different ORC subunits (GSTORC2 and GSTORC4) confirmed the previous result showing ORC2, -3, -4, and -5 form a core complex (data not shown).

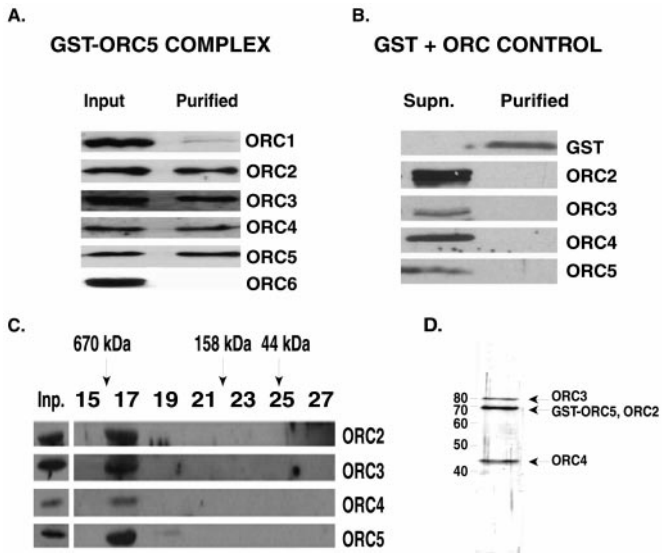

FIG. 1. **Human ORC2–5 co-purify in a complex.** *A*, Sf9 insect cells were infected with baculoviruses expressing ORC1, -2, -3, -4, -6 and GSTORC5. Proteins were purified as described under "Materials and Methods." Both purified proteins and crude lysate (*Input*) were immunoblotted using ORC1–6 antibodies. *B*, Sf9 insect cells were infected the same way as in *A* using baculoviruses expressing ORC2–5 and GST as control. The cell lysate was purified on GST beads and immunoblotted. *C*, Sf9 insect cells were infected with baculoviruses expressing ORC2, -3, -4 and GST-ORC5. Proteins purified on GST beads were fractionated on a Superose 12 gel filtration column. Alternate fractions were immunoblotted using anti-ORC2, -3, -4 and anti-GST antibodies. The positions of the molecular mass markers thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), and chicken ovalbumin (44 kDa) are shown on top. Input *lanes* were loaded with 5% of the total lysate passed through the column.

To further show that GSTORC5, -2, -3, and -4 subunits are in one complex, we analyzed the elution pattern of these proteins upon gel filtration. Proteins were eluted from the GST beads using reduced glutathione and subsequently passed through a Superose 12 gel filtration column. Upon Western blotting of different fractions with different anti-ORC antibodies, we found that GSTORC5, -2, -3, and -4 subunits were co-eluted in one fraction (Fig. 1*C*). The molecular mass of this complex is ~500 kDa, which is more than the combined molecular mass of the four ORC subunits. This may be because of the multimerization of the GST moieties to give a high molecular mass complex or because the complex has an atypical shape. Silver staining of the purified protein used for the gel filtration experiment indicated that GST-ORC5, ORC2, -3, and -4 were the only proteins present in the preparation in significant amounts (Fig. 1*D*).

ORC2 and ORC3 Physically Interact with Each Other—We were interested in seeing which of the four interacting subunits interact directly. Sf9 insect cells were infected with six different combinations of baculoviruses expressing two ORC subunits in each case. One of the two viruses was GST-tagged. Affinity purification on glutathione beads showed that only ORC2 and ORC3 directly interacted with each other (Fig. 2). None of the other dual combinations of baculovirus showed any interaction under our experimental conditions. Therefore, we conclude that ORC2 and ORC3 form a core component of the ORC2, -3, -4, -5 complex.

ORC2 and ORC3 Recruit ORC4 and -5—The ORC2-3 complex is expected to recruit ORC4 and ORC5. We were interested in seeing whether ORC2-3 core complex can recruit ORC4 first, followed by ORC5, or vice versa. Sf9 cells were infected by baculoviruses expressing GSTORC4, -2, and -3 subunits or by viruses expressing GSTORC5, -2, and -3. GSTORC4 did not

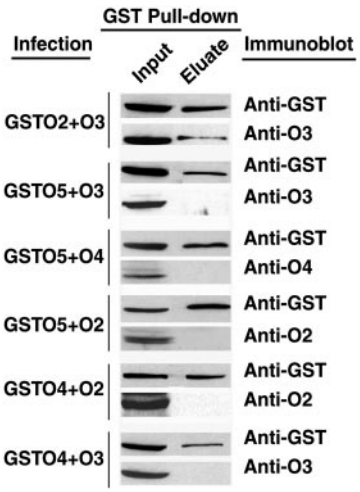

FIG. 2. **Direct interaction between ORC2 and ORC3 subunits.** Sf9 insect cells were infected with six different combinations of baculoviruses expressing two ORC subunits in each case (GSTORC2-3 (*GSTO2+03*), GSTORC5-3 (*GSTO5+03*), GSTORC5-4 (*GSTO5+04*), GSTORC5-2 (*GSTO5+02*), GSTORC4-2 (*GSTO4+02*), and GSTORC4-3 (*GSTO4+03*)). Proteins bound to GST beads were immunoblotted using either anti-GST antibody or respective anti-ORC antibodies. 5% of the total lysate was loaded in the input *lanes*.

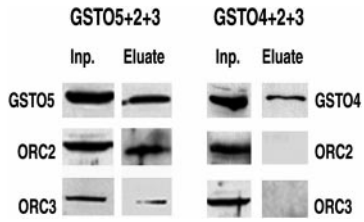

FIG. 3. **ORC2-3 subcomplex can load ORC5 but not ORC4.** Sf9 insect cells were infected either with baculoviruses expressing GSTORC5-2-3 (*GSTORC5+2+3*) or baculoviruses expressing GSTORC4-2-3 (*GSTORC4+2+3*). Proteins bound to GST beads were immunoblotted using either anti-GST antibodies or respective anti-ORC antibodies. Input (*Inp.*) *lane* contains 5% of proteins input on GST beads.

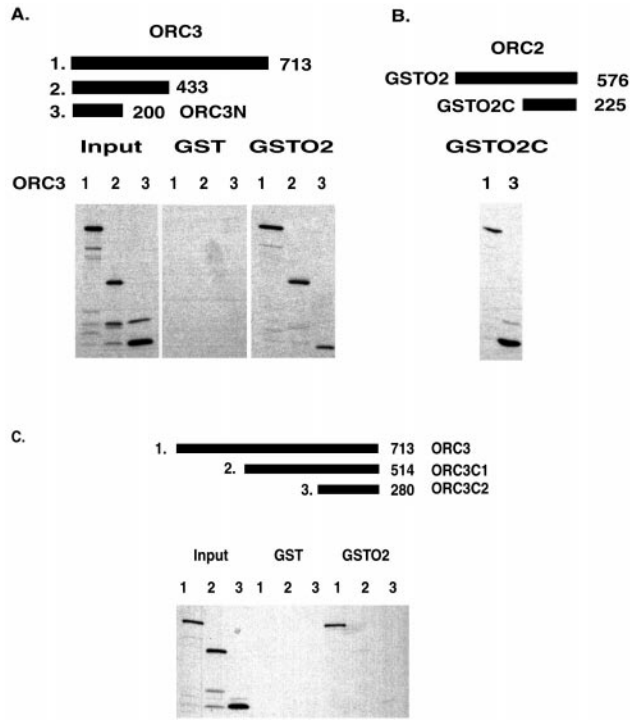

FIG. 4. **Mapping domains of interactions between ORC2 and ORC3.** *A*, full-length ORC3 or different N-terminal deletions of ORC3 were produced using *in vitro* transcription and translation reactions and tested for their ability to bind either GST or GSTORC2 in a pull-down experiment on glutathione-agarose beads coated with GST, GSTORC2 (*GSTO2*). In each case, the input *lanes* were loaded with 5% of the amount of the labeled protein incubated with the beads. The labeled proteins were visualized by SDS-polyacrylamide gel electrophoresis followed by fluorography. *B*, GST pull-down experiment as in *A* using GSTORC2C (C-terminal portion of ORC2) and *in vitro* transcribed and translated full-length ORC3 (*1*) or ORC3N200 (*3*). *C*, full-length ORC3 or different C-terminal deletions of ORC3 were produced using *in vitro* transcription translation reaction. GST pull-down experiments were performed as shown in Fig. 5*A* using either GST or GSTORC2.

interact with ORC2 and ORC3, whereas GSTORC5 interacted with ORC2 and ORC3 (Fig. 3). Therefore, ORC2-3 core complex is capable of recruiting ORC5, but it cannot recruit ORC4 by itself. The fact that ORC2, -3, -4, and -5 form a complex suggests that ORC2, -3, and -5 complex is necessary to load ORC4. It is also possible that ORC4 and ORC5 can be loaded on ORC2-3 core complex simultaneously independent of each other, but ORC5 is necessary to stabilize the association of ORC4 with the other ORC subunits.

N-terminal Portion of ORC3 Interacts with the C-terminal Portion of ORC2—Upon establishing the fact that ORC2 and ORC3 form a core complex, we mapped the interacting domains of ORC2 and ORC3. N-terminal fragments of ORC3 labeled with [^{35}S]methionine were produced by *in vitro* transcription and translation in rabbit reticulocyte lysate. The proteins were incubated with bacterially expressed and purified GSTORC2 protein. Three polypeptides derived from ORC3 were capable of binding GSTORC2, whereas the control GST protein did not bind any of them (Fig. 4*A*). The smallest fragment that bound to ORC2 contained 200 amino acids from the N terminus of ORC3 (*construct 3*, ORC3N). To map the portion of ORC2 involved in the interaction with ORC3, we expressed and purified GSTORC2C, containing the C-terminal 225 amino acids of ORC2. Both full-length ORC3 and ORC3N bound to GSTORC2C (Fig. 4*B*), whereas control GST alone did not bind to any one of them (data not shown). Therefore, the C-terminal 225 residues of ORC2 interact with the N-terminal 200 residues of ORC3 to form the ORC2-3 complex at the core of human ORC. In the reciprocal deletion, removal of the first 200 amino acids of ORC3 abolished its ability to bind to GSTORC2 (Fig. 4*C*). Based on these results we conclude that N-terminal 200 residues of ORC3 are necessary and sufficient to interact with ORC2.

N-terminal 200 Amino Acids of ORC3 Can Compete with the Full-length ORC3—If the N-terminal 200 residues of ORC3 are sufficient for binding ORC2, ORC3N might be able to compete with full-length ORC3 for binding to GSTORC2. [^{35}S]Methionine-labeled ORC3 was bound to GSTORC2 beads under conditions where the latter was limiting. These beads were then incubated with increasing amounts of ORC3N. We found that ORC3N protein could compete with the full-length ORC3 protein for association with GSTORC2 (Fig. 5).

ORC3N Cannot Form a Complex That Contains ORC4-5—Next we asked whether ORC3N is capable of mediating the interaction of ORC2 with ORC4 and ORC5. Sf9 insect cells were infected with baculoviruses expressing GSTORC5, ORC2, ORC4, and ORC3N. After affinity purification on glutathione beads and Western blotting, we confirmed the presence of GSTORC5 in the eluate from the beads. Interestingly, in contrast to the result in Fig. 1*A*, none of the other ORC subunits came down with GSTORC5, although they were all present in the input lane at reasonable quantities (Fig. 6*A*). We confirmed the physical interaction between ORC2 and ORC3N in the insect cell lysates by co-immunoprecipitation reactions. The lysate was immunoprecipitated using either anti-ORC2 or anti-

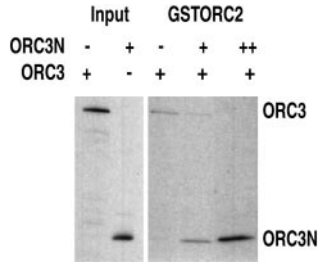

FIG. 5. **ORC3N can compete with full-length ORC3.** GSTORC2 beads were incubated with *in vitro* transcribed and translated full-length ORC3. After incubation, beads were thoroughly washed using binding buffer and incubated again with increasing amount of *in vitro* transcribed and translated ORC3N200. Beads were finally washed, and bound labeled proteins were visualized by SDS-polyacrylamide gel electrophoresis followed by fluorography.

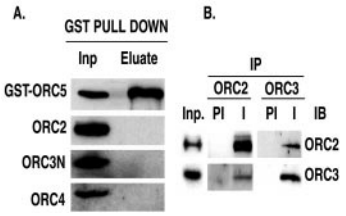

FIG. 6. **ORC3N does not form a complex with ORC2, -4, and -5.** *A*, Sf9 insect cells were infected with baculoviruses expressing GSTORC5, -2, -4 and ORC3N200. Proteins bound to glutathione-agarose beads were immunoblotted using either anti-GST or anti-ORC antibodies. *B*, immunoprecipitation using anti-ORC2 and anti-ORC3 antibodies. Insect cell lysate from *A* was immunoprecipitated (*IP*) using either anti-ORC2 or anti-ORC3 antibodies followed by immunoblotting (*IB*) with either anti-ORC3 or anti-ORC2 antibodies. In each case 5% of the lysate (used for immunoprecipitation) was loaded in the input (*Inp*) *lanes*. Sera used for immunoprecipitation: *I*, immune, and *PI*, preimmune.

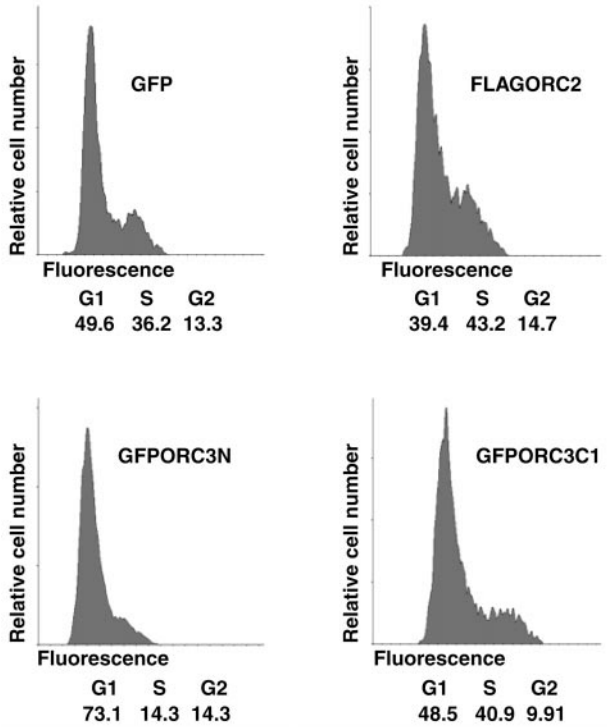

FIG. 7. **FACS analysis of U2OS cells transfected with different constructs.** U2OS cells were transfected either with farnesylated GFP (CLONTECH) or in combination with FLAGORC2, GFPORC3N, or GFPORC3C1. Transfected cells were fixed and stained with propidium iodide and then analyzed by FACS. The percentage of cell population present at different cell cycle stages in each transfection is shown at the bottom of the each *panel*.

ORC3 antibodies followed by immunoblotting with both the antibodies. ORC2 was detected in anti-ORC3 immunoprecipitate and vice versa (Fig. 6*B*). Therefore, ORC3N is capable of interacting with ORC2, but this interaction was not sufficient for further binding of ORC4 and ORC5. The C-terminal portion of ORC3 appears to be crucial for binding of ORC4 and ORC5 subunits to ORC2-3 subcomplex.

Expression of ORC3N in U2OS Cells Causes Cell Cycle Arrest—Because ORC3N did not form a complex with ORC4 and ORC5 but still could interact with ORC2, we reasoned that ORC3N might show a dominant negative effect on the cell cycle if overexpressed in a human cancer cell line. ORC3N and ORC3C1 were cloned into GFPC1 (CLONTECH) expression vector to produce non-farnesylated GFP fusion proteins. U2OS cells were transfected with plasmids expressing farnesylated GFP alone or in combination with FLAGORC2 or GFPORC3N or GFPORC3C1 (1:3 molar ratio) followed by FACS analysis after 48 h. Cells transfected with GFP alone, FLAG-ORC2, or GFP-ORC3C1 showed normal cell cycle progression, whereas cells transfected with GFP-ORC3N were blocked mostly in G_1 (73%) (Fig. 7). This is the first evidence for any cell cycle effect of any human ORC protein. Since ORC3N can still bind ORC2 but not ORC4 and ORC5, it is possible that over-expressed ORC3N interacts with ORC2 but prevents functional ORC formation. Consistent with this, over-expression of full-length ORC3, which interacts with ORC2 but allows functional ORC formation, did not block the cells in G_1 (data not shown).

DISCUSSION

We report here that human ORC2, -3, -4, and -5 form a core complex in baculovirus-infected insect cells. ORC1 and ORC6 did not interact with this core complex under these experimental conditions. This was confirmed by using different tags on

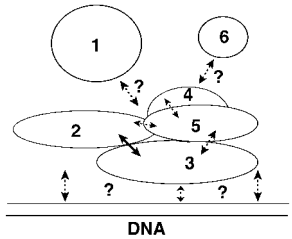

FIG. 8. **Model of human ORC2–5 subcomplex.** Human ORC subunits are showed as numbers 1–6. ORC2–5 subunits are shown to form a complex. N and C in ORC2 and -3 depict N- and C-terminal residues of the individual subunits. The *arrow* with the *bold line* shows a strong interaction, whereas *arrows* with *dotted lines* indicate weak interactions.

different ORC subunits and is consistent with our previously published data showing that endogenous ORC2, -3, -4, and -5 in a HeLa cell extract physically interacted with each other but not with ORC1 and -6(14). Recently ORC1 has been shown to interact with ORC2 by co-immunoprecipitation reaction using high salt nuclear lysate from HeLa cells (21). This study did not include ORC3-6 proteins. Unfortunately, we could not reproduce the co-immunoprecipitation of ORC1 with ORC2 in human cell lines using two different ORC1 antibodies, raised independently. Under the same conditions we still found strong interaction among ORC2–5 subunits. The data from HeLa cell extract and recombinant baculovirus proteins strongly suggest that ORC2–5 form a core complex with ORC1 and ORC6, joining the complex either at very restricted times or locations or in a very labile interaction that is easily disrupted upon cell lysis.

We further found that ORC2 and ORC3 form a tight complex essential for binding ORC4 and ORC5. Gel filtration of 293T cell extract showed that ORC2 and 3 were the only two subunits that

mostly co-eluted, consistent with the direct interaction between ORC2 and -3 reported here (16). Under our experimental conditions, none of the other ORC subunits interact with each other directly. The N-terminal 200 amino acids of ORC3 were enough to interact with C-terminal portion of ORC2 but not sufficient to allow association with ORC4 and ORC5, suggesting that the C-terminal of ORC3 is required for ORC4 and ORC5 loading on ORC2-3 subcomplex.

Recently, in yeast *S. cerevisiae*, ORC2 and ORC3 have been shown to interact directly (22). Insect cells were infected with baculoviruses expressing yeast ORCs, and recombinant yeast ORC was purified and tested for its DNA binding ability. ORC6 was found dispensable for DNA binding property. Elimination of ORC3 during baculovirus infection led to formation of ORC sub-complex without the presence of ORC2, suggesting that yeast ORC3 recruits ORC2 to the complex. Likewise, yeast ORC4 and ORC5 were shown to interact with each other. In addition, when yeast ORC was bound to yeast ARS1, ORC1, -2, -4, and -5 subunits were shown to directly contact ARS1 DNA by UV cross-linking (22). The human ORC2–5 sub-complex, however, did not show any sequence-specific DNA binding activity (data not shown).

Finally we showed that ORC3N has a dominant negative effect on cell cycle progression. U2OS cells expressing the same fragment were blocked in G_1, whereas the ORC3C1 or ORC2 protein did not prevent the cells from normal cell cycle progression. This can be explained by the fact that ORC3N titrates out ORC2 or an unknown cellular protein in the cell, thereby blocking G_1-S transition. Given that ORC2 and ORC3N form a very tight complex but the latter cannot support ORC2, -3, -4, -5 complex formation, we believe that ORC2 is the target that is titrated out by ORC3N. We cannot, however, overcome the effect of ORC3N by over-expressing ORC2 or ORC2C.[2] ORC2 or ORC2C might not be expressed at high enough levels to titrate out the ORC3N. Alternatively, the ORC3N targets an unknown cellular factor to cause the G_1-S block.

Based on these results we propose a molecular architecture of human ORC (Fig. 8). ORC2 and ORC3 interact directly with the C terminus of the former subunit, in close proximity with the N terminus of the latter. This binding favors the loading of ORC4 and -5 subunits via the C-terminal residues of ORC3. Although none of the other ORC subunits interacted with each other, we cannot rule out weak inter-subunit interactions among themselves. Further experiments will address how the ORC2–5 complex recruits ORC1 and ORC6 to form human ORC and study how the complex interacts with DNA.

[2] S. K. Dhar and A. Dutta, unpublished information.

Acknowledgments—We acknowledge David Garcia Quintana, Anjali Satoshkar, and Zhi Hui Hou for making the baculovirus constructs and J. Wohlschlegel for help with the analysis of the FACS data.

REFERENCES

1. Bell, S. P. & Stillman, B. (1992) *Nature* **357,** 128–134
2. Dutta, A. & Bell, S. P. (1997) *Annu. Rev. Cell Dev. Biol.* **13,** 293–332
3. Kelly, T. J. & Brown, G. W. (2000) *Annu. Rev. Biochem.* **69,** 829–880
4. Rowles, A., Chong, J. P., Brown, L., Howell, M., Evan, G. I. & Blow, J. J. (1996) *Cell* **87,** 287–296
5. Gossen, M., Pak, D. T., Hansen, S. K., Acharya, J. K. & Botchan, M. R. (1995) *Science* **270,** 1674–1677
6. Moon, K. Y., Kong, D., Lee, J. K., Raychaudhuri, S. & Hurwitz, J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 12367–12372
7. Gavin, K. A., Hidaka, M. & Stillman, B. (1995) *Science* **270,** 1667–1671
8. Takahara, K., Bong, M., Brevard, R., Eddy, R. L., Haley, L. L., Sait, S. J., Shows, T. B., Hoffman, G. G. & Greenspan, D. S. (1996) *Genomics* **31,** 119–122
9. Pinto, S., Quintana, D. G., Smith, P., Mihalek, R. M., Hou, Z. H., Boynton, S., Jones, C. J., Hendricks, M., Velinzon, K., Wohlschlegel, J. A., Austin, R. J., Lane, W. S., Tully, T. & Dutta, A. (1999) *Neuron* **23,** 45–54
10. Quintana, D. G., Hou, Z., Thome, K. C., Hendricks, M., Saha, P. & Dutta, A. (1997) *J. Biol. Chem.* **272,** 28247–28251
11. Quintana, D. G., Thome, K. C., Hou, Z. H., Ligon, A. H., Morton, C. C. & Dutta, A. (1998) *J. Biol. Chem.* **273,** 27137–27145
12. Tugal, T., Zou-Yang, X. H., Gavin, K., Pappin, D., Canas, B., Kobayashi, R., Hunt, T. & Stillman, B. (1998) *J. Biol. Chem.* **273,** 32421–32429
13. Ishiai, M., Dean, F. B., Okumura, K., Abe, M., Moon, K. Y., Amin, A. A., Kagotani, K., Taguchi, H., Murakami, Y., Hanaoka, F., O'Donnell, M., Hurwitz, J. & Eki, T. (1997) *Genomics* **46,** 294–298
14. Dhar, S. K. & Dutta, A. (2000) *J. Biol. Chem.* **275,** 34983–34988
15. Natale, D. A., Li, C. J., Sun, W. H. & DePamphilis, M. L. (2000) *EMBO J.* **19,** 2728–2738
16. Thome, K. C., Dhar, S. K., Quintana, D. G., Delmolino, L., Shahsafaei, A. & Dutta, A. (2000) *J. Biol. Chem.* **275,** 35233–35241
17. Chesnokov, I., Gossen, M., Remus, D. & Botchan, M. (1999) *Genes Dev.* **13,** 1289–1296
18. Austin, R. J., Orr-Weaver, T. L. & Bell, S. P. (1999) *Genes Dev.* **13,** 2639–2649
19. Dunn, M. J. & Crisp, S. J. (1994) *Methods Mol. Biol.* **32,** 113–118
20. Lin, Y. L., Chen, C., Keshav, K. F., Winchester, E. & Dutta, A. (1996) *J. Biol. Chem.* **271,** 17190–17198
21. Kreitz, S., Ritzi, M., Baack, M. & Knippers, R. (2000) *J. Biol. Chem.* **276,** 6337–6342
22. Lee, D. G. & Bell, S. P. (1997) *Mol. Cell. Biol.* **17,** 7159–68

Architecture of the Human Origin Recognition Complex

Suman Kumar Dhar, Laurie Delmolino and Anindya Dutta

J. Biol. Chem. 2001, 276:29067-29071.
doi: 10.1074/jbc.M103078200 originally published online June 6, 2001
