Subsets of Human Origin Recognition Complex (ORC) Subunits Are Expressed in Non-proliferating Cells and Associate with Non-ORC Proteins*

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The origin recognition complex (ORC) in yeast is a complex of six tightly associated subunits essential for the initiation of DNA replication. Human ORC subunits are nuclear in proliferating cells and in proliferative tissues like the testis, consistent with a role of human ORC in DNA replication. Orc2, Orc3, and Orc5 also are detected in non-proliferating cells like cardiac myocytes, adrenal cortical cells, and neurons, suggesting an additional role of these proteins in non-proliferating cells. Although Orc2–5 co-immunoprecipitate with each other under mild extraction conditions, a holo complex of the subunits is difficult to detect. When extracted under more stringent extraction conditions, several of the subunits co-immunoprecipitate with stoichiometric amounts of other unidentified proteins but not with any of the known ORC subunits. The variation in abundance of individual ORC subunits (relative to each other) in several tissues, expression of some subunits in non-proliferating tissues, and the absence of a stoichiometric complex of all the subunits in cell extracts indicate that subunits of human ORC in somatic cells might have activities independent of their role as a six subunit complex involved in replication initiation. Finally, all ORC subunits remain consistently nuclear, and Orc2 is consistently phosphorylated through all stages of the cell cycle, whereas Orc1 is selectively phosphorylated in mitosis.

Homologs of the yeast ORC subunits have been identified in several higher eukaryotes. In Xenopus egg extracts, a complex of proteins containing known homologs of yeast ORC subunits was shown by immunodepletion to be essential for DNA replication (10–13). This experimental system was used to demonstrate that a critical function of Xenopus ORC is in the recruitment of the CDC6 protein to chromatin, which then permits the recruitment of minichromosome maintenance (MCM) proteins. A complex of six proteins was identified in Drosophila embryo extracts containing all six homologs of yeast ORC that supported DNA replication in vitro (14–16). The genes for at least two of these Drosophila subunits are essential for viability (16, 17). In a tissue-specific gene amplification event in Drosophila ovarian follicle cells, Drosophila Orc2 co-localizes to the sites of gene amplification and binds to some of the sequences required for the amplification process (18). Overall, this large body of work provides convincing evidence that, as in yeast, ORC from higher organisms is a multi-subunit protein complex that serves as the initiator protein to recognize putative replicator elements in the genome and initiate DNA replication.

With the aim of understanding the initiation of DNA replication in mammalian somatic cells, we (17, 20, 21) and several others (19, 22, 23) identify five of the human homologs of the six yeast ORC subunits based on sequence similarity and association with known ORC subunits. Orc6 is poorly conserved in amino acid sequence between S. cerevisiae, Schizosaccharomyces pombe, and Drosophila (24) and is dispensable both for the formation of a multi-subunit complex and for sequence specific DNA binding in S. cerevisiae (25).

Given the central role of the six subunit ORC in the initiation...
FIG. 1. Expression of ORC mRNAs in human tissues. cDNA probes specific for Orc1-Orc5, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin were hybridized sequentially to Northern blots (CLONTECH) of poly(A)+ mRNA from indicated tissues (peripheral blood leukocyte (PBL)). Glyceraldehyde-3-phosphate dehydrogenase and β-actin are references of the amount of RNA loaded in each lane. HeLa, Northern blot of total RNA purified from asynchronously growing HeLa cells to demonstrate mRNA levels of ORC subunits in tissue culture cells.

of DNA replication, it is important to examine the status of this complex in the mammalian somatic cells that give rise to cell proliferative diseases like cancers. All studies on the biochemistry of ORC have utilized cells that either proliferate rapidly or have the potential to do so, e.g. yeast cells, Drosophila embryo extracts, or Xenopus egg extracts. In this paper we examine for the first time the biochemical and cell biological properties of human ORC in somatic cells that proliferate at a far slower rate than the special cases utilized to date and come to the conclusion that a tight complex containing all subunits of ORC is difficult to detect in these cells. In addition, individual ORC subunits are selectively expressed in certain non-proliferating tissues, suggesting that isolated ORC subunits might have functions unrelated to replication initiation. Even in proliferating cells in culture several of the ORC subunits are associated with unique proteins that are not the other known ORC subunits, raising the possibility that either there are more than six ORC subunits in mammalian cells or that some of the subunits are in complexes unrelated to DNA replication initiation.

EXPERIMENTAL PROCEDURES

Cell Culture—Culture conditions for human HeLa, U2OS, and 293T cells as well as conditions for cell cycle synchrony with nocodazole and hydroxyurea have been described previously (20). 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 μCi) of [35S]methionine (PerkinElmer Life Sciences, 1175 Ci/mmol). For in vivo labeling with [32P]orthophosphate (PerkinElmer Life Sciences, 8500 Ci/mmol) for another 4 h before harvesting.

RNA Analysis—Northern blots containing RNA from 16 adult human tissues were obtained commercially (CLONTECH), and hybridizations using 32P-labeled cDNA probes were performed as suggested by the manufacturer. Blots were washed at 42 °C in 0.1× SSC, 0.1% SDS and exposed to film at −80 °C with an intensifying screen. Northern analysis of HeLa cells has been described previously (20).

Protein Analysis—Western blots containing 75 μg protein/lane from a panel of 9 tissues were obtained commercially (Geno Technology, Inc.) and immunoblotted with anti-ORC antibodies as directed by the manufacturer. Cell extracts were prepared in ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.1, 1% Nonidet P-40, 150 mM NaF, 1 mM Na3VO4, 0.1 mM phenylmethylsulfonyl fluoride, 10 units of DNase, 100 μg/ml RNase A, and 1 μg/ml each pepstatin A, leupeptin, and aprotinin). Lysates were precleared by incubation with pre- or non-immune antibody and protein A-Sepharose beads for 1 h, and the supernatants were transferred to new tubes before immunoprecipitation with specific antibodies. The beads were washed four times in RIPA buffer before the addition of sample buffer. For denaturation-immunoprecipitation experiments, immunoprecipitates were boiled in 1% SDS for 10 min, then diluted 10-fold in RIPA buffer that did not contain SDS and immunoprecipitated again.

Antibodies to Orc2, Orc3, Orc4, and Orc5 have been published (17, 20, 21, 26). Anti-Orc1 antibody was raised in rabbits (Cocalico Biologicals) against a bacteriologically expressed recombinant His6-tagged fragment of Orc1 from amino acids 579–861 created by cloning into the NdeI and BamHI sites of pET14b.

Gel Filtration Methods—5 × 10^6 293T cells were extracted in 0.1% Nonidet P-40 lysis buffer (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 centrifuged (15,000 × g for 20 min at 4 °C), and the clear lysate was filtered through a 0.22-μm filter. The filtrate was subjected to fast protein liquid chromatography Supersose 12 (Amersham Pharmacia Biotech) gel filtration column chromatography 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-ORC antibodies.

Immunostaining—For immunohistochemistry, 4-μm-thick paraffin sections of formalin-fixed tissue were used after deparaffinization and rehydration (27). Sections were blocked for 15 min at room temperature with 1.5% goat serum in PBS and then incubated with primary antibody at room temperature for 1 h in a humid chamber. Antibodies used were anti-Orc3 and anti-Orc5, with matched pre-immune sera. After rinsing with phosphate-buffered saline, sections incubated with anti-Orc3 or matched pre-immune serum were processed with the Vectastain Elite ABC Kit (Vector Laboratories, Inc.) as specified by the manufacturer and developed using 3,3′-diaminobenzidine (Sigma) as substrate. Sections incubated with anti-ORC or matched pre-immune serum were processed with the Vectastain ABC alkaline phosphatase kit (Vector Laboratories, Inc.) as specified by the manufacturer and developed using Vector alkaline phosphatase substrate kit I (Vector Red).

For immunofluorescence, U2OS cells were transfected with vectors expressing T7-tagged Orc1. For analysis of Orc2-Orc5, untransfected HeLa (Orc2 and Orc5) or U2OS (Orc3 and Orc4) cells were used. General conditions for immunofluorescence have been described previously (26) except that for Orc1 analysis, cells were fixed and permeabilized for 2 min at room temperature in 1:1 (v/v) methanol-acetone solution.

RESULTS

Expression of ORC mRNAs in Human Tissues—In every cultured human cell line we have examined to date (17, 20, 21, 26) all ORC subunits (Orc1–5) are expressed at reasonably high levels. To study ORC expression in normal human tissues, Northern blots of a panel of 16 tissues were hybridized sequentially with cDNA probes specific for Orc1-Orc5 (Fig. 1). Multiple RNA species were not observed; rather, a single transcript of the size predicted by the full-length cDNA was observed for each ORC subunit. The results suggest that the mRNA of individual ORC subunits is sometimes expressed in non-pro-
liferating tissues that do not always express all the other known subunits.

Orc1 expression alone correlated with the level of tissue proliferation. Highest levels of Orc1 mRNA were observed in tissues with high proliferative activity such as testis and colon mucosal lining. Orc1 mRNA is also high in a tissue like the placenta, which would have proliferated rapidly in its recent history and is moderately expressed in the thymus with proliferating T lymphocytes (after correcting for slight underloading of mRNA from the latter). Orc1 mRNA essentially is undetectable in primarily non-proliferating tissues such as heart, brain, lung, liver, skeletal muscle, kidney, spleen, prostate, ovary, small intestine, and peripheral blood leukocyte. The mRNA from the small intestine was obtained from the entire thickness of the gut and so was not enriched in the proliferating cells of the mucosa. Therefore, Orc1 mRNA levels reflect the proliferative activity in tissues consistent with regulation of Orc1 expression by the E2F transcription factor (28).

In contrast, mRNA levels of the other ORC subunits (Orc2–5) were not dependent on the proliferative activity in the tissue. As observed with Orc1, Orc2–5 are expressed at high levels in testis. However, high levels of Orc2–5 were observed in tissues with low proliferation such as heart, pancreas, and ovary. Intermediate expression of Orc2–5 was seen in brain, placenta, skeletal muscle, and kidney. The levels of Orc2–5 mRNA are high in heart and skeletal muscle even after the signal intensity is adjusted to reflect overloading (as judged from the control β-actin and glyceraldehyde-3-phosphate dehydrogenase probes).

In several tissues, only a subset of ORC subunits are expressed at significant levels. In lung, Orc3 and Orc4 mRNA are seen, whereas Orc2 and Orc5 mRNA are virtually absent. In thymus, only Orc1 and Orc5 show measurable levels, whereas in the small intestine only Orc5 exhibits a faint band. In all tissues the control β-actin and glyceraldehyde-3-phosphate dehydrogenase probes yield strong signals, suggesting that the low level of ORC subunit signal in some tissues is not due to drastic sample underloading.

In addition, note that even in proliferating tissues expressing Orc1, signal ratios between various ORC subunits are not constant. For example, thymus expresses Orc1 at much higher levels than Orc2–4, and Orc1 is expressed at a higher level than Orc2 in colonic mucosa. In testis, however, Orc1 appears underexpressed relative to most of the other subunits.

**ORC Subunit Proteins Are Expressed in Non-proliferating Human Tissue**—Since analysis of ORC subunit RNA levels in normal tissue suggested that abundance of transcripts does not correlate strictly with proliferation status, we investigated whether the same held true at the protein level. A commercially obtained blot containing protein from normal human tissue was immunoblotted sequentially with polyclonal antisera specific for Orc1-Orc5 (Fig. 2). Ponceau S staining of total proteins on the blot indicated that the brain extract was significantly underloaded. Orc1 is nearly undetectable in non-proliferating tissues, consistent with its mRNA being seen exclusively in proliferating tissues. Very low levels of Orc1 are observed in kidney and spleen when the immunoblot is overexposed. The approximately 90-kDa protein detected in heart also is recognized by pre-immune serum, and thus, we do not believe it to be Orc1. Orc2–4 also were detected in testis. The absence of Orc5 in the testis extract is explained by our earlier observation that Orc5 is associated with the nuclear matrix of cultured proliferating cells (21) and is expected to be extracted poorly under the conditions used to prepare these tissue samples. Immunohistochemistry (see below) indeed confirms that Orc5 protein also is abundant in the testis. Thus, a proliferating tissue like the testis contains all ORC subunit proteins.

Several non-proliferating tissues, however, expressed subsets of ORC subunits. A 70-kDa Orc2 protein was detected in non-proliferating tissues like pancreas, liver, brain, kidney, ovary, and heart. An 80-kDa Orc3 protein also was detected in non-proliferating tissues like the heart, but the levels in other non-proliferating tissues did not correlate with that of the mRNA. For example, non-proliferating tissues like pancreas, spleen, and ovary expressed Orc3 mRNA (Fig. 1), but the protein was undetectable (Fig. 2), suggesting regulation of expression at the post-transcriptional level. Orc4 protein of approximately the correct size (45 kDa) was detected in testis and at least three non-proliferating tissues (lung, kidney, and heart), consistent with the presence of Orc4 mRNA in these tissues.

Orc5 was detected in extracts from two non-proliferating tissues: brain and heart. In our hands Orc5 is poorly extracted from proliferating cells using conditions other than 8 M urea, and therefore, the lack of protein in the testis lane probably reflects poor extraction from proliferating cells. The ease of extraction of Orc5 from non-proliferating tissues like the brain and heart, on the other hand, is most likely because the protein is not in a holo complex interacting with the DNA replication machinery. We had earlier reported that the Orc5 locus produces an alternatively spliced mRNA that codes for an Orc5T protein containing the N-terminal two-thirds of Orc5 and a unique cytoplasmic tail of 31 amino acids (21). Specific anti-Orc5T antibody raised against this unique C-terminal peptide confirmed that the extractable Orc5 protein from brain and heart is not Orc5T (data not shown).

Slight variations in the size of ORC protein subunits in some
tissues are most likely due to tissue-specific post-translation modifications. For example, the Orc1 protein in the testis and spleen is about 20 kDa larger than the protein in HeLa cells or in the kidney. Orc2 in the liver is about 5-kDa larger that in other tissues. The Orc4 protein exhibits mobility shifts of 2–4 kDa in various tissues. In all cases, the bands shown in the figure were the sole polypeptides detected upon immunoblotting with the relevant antibody (in addition, the anti-Orc2 and anti-Orc4 antibodies had been affinity-purified on recombinant Orc2 or Orc4), leading us to believe that these reactions are specific and recognize either the authentic ORC subunit or a closely related variant. In addition, to eliminate cross-reactive proteins we immunoblotted with the relevant pre-immune control antisera (data not shown).

Besides small variations in the sizes of some of the ORC subunits there were two instances with a large variation in the size of the detected protein in a given tissue. A variant protein of 45 kDa reacted with the anti-Orc2 antibody in heart lysates. Similarly, brain expressed a 47-kDa protein that reacted strongly with the anti-Orc3 antibody (instead of the expected 80-kDa Orc3 protein). In both these instances, the cognate mRNA was abundant (Orc2 in heart and Orc3 in brain), so that one hypothesis is that the variant proteins are the products of alternatively spliced mRNA (as we have shown with ORC5T (21)). We cannot, however, rule out cross-reaction of the antibody to an unrelated tissue-specific protein.

**ORC Localization in Tissues by Immunohistochemistry**—The abundance of ORC subunit proteins in tissues that have not proliferated in decades such as heart and brain raise the possibility that the subunits are expressed by infiltrating cells from the blood rather than the non-proliferating cardiac myocytes or neurons. Immunohistochemistry on sections of tissues is another method by which one can confirm the presence of a protein and determine the specific cells expressing the protein of interest. Conditions for immunohistochemistry on paraffin sections were established using sections of testis as positive controls (Fig. 3). The anti-Orc3 and anti-Orc5 antibodies were the only antibodies that gave positive results on these sections, with pre-immune sera giving negative results. Positive staining with two out of the five antibodies tried is a reasonable success rate for immunohistochemistry on paraffin sections. In testis, with its high level of cell proliferation, both Orc3 and Orc5 proteins were detected in the nuclei of proliferating germ cells.

Orc3 protein is present in the nucleus of non-proliferating cells in at least three tissues: heart (myocytes), tonsil (lymphocytes outside germinal center), and adrenal (epithelial cells) (Fig. 4). We believe that the anti-Orc3 antibody stains Orc3 protein in the tissue section for the following reasons. In all tissues examined, the pre-immune serum exhibited no background staining in the nucleus. Of the tissues on the immunoblots (Fig. 2), nuclear staining with anti-Orc3 antibody was detected only in the testis and the heart (Figs. 3 and 4), the two tissues with an 80-kDa Orc3 protein. Tissues on the blot that did not have the 80-kDa protein (liver, brain, lung, kidney, spleen, and pancreas) did not show nuclear staining with anti-Orc3 antibody (not shown). Based on this correlation we are most certain that nuclear staining in paraffin sections with anti-Orc3 antibody detects the authentic 80-kDa Orc3 protein. Despite the expression of Orc3 mRNA in brain, kidney, spleen, and pancreas, the Orc3 protein is undetectable in these tissues by two independent methods (immunoblot and immunohistochemistry) confirming that the expression must be regulated at the post-transcriptional level. The 47-kDa protein detected by the anti-Orc3 antibody in immunoblots of brain extracts (Fig. 2) was not detected by immunostaining (not shown), indicating that epitopes recognized by the anti-Orc3 antibody in paraffin sections are not present in the 47-kDa variant.

Anti-Orc5 antibody recognized a nuclear protein in the testis, cardiac myocytes, brain, and tonsils, whereas the pre-immune serum gave negative results (Figs. 3 and 4). As discussed earlier, Orc5 protein is difficult to extract from proliferating cells in culture, so we were not surprised by the lack of correlation with the immunoblot from testis. The presence of Orc5 protein in cardiac myocytes was confirmed by two independent means (immunoblot and immunostaining). Anti-Orc5 antibody recognized the protein in extracts of brain but stained granules in the cytoplasmic compartment of neurons (Fig. 4). This result is consistent with our suggestion that ease of extraction of Orc5

**FIG. 3.** Nuclear localization of ORC in proliferating cells of the testis. Paraffin sections of normal testis were used for immunohistochemical analysis with anti-Orc5 (a) or anti-Orc3 (c) antibodies or with matched pre-immune control serum (b) and (d). Orc3 antibodies were detected with 3,3’- diaminobenzidine (stains brown), whereas Orc3 antibodies were detected with alkaline phosphatase (stains red). Nuclei were counterstained with hematoxylin (stains blue). Images were photographed at 200×.

**FIG. 4.** Localization of ORC in non-proliferating tissues. A, immunohistochemical analysis of heart, adrenal gland, and tonsil paraffin sections with anti-Orc3 antibody (a–c) or matched pre-immune control serum (d–f) as described in Fig. 3. B, immunohistochemical analysis of heart, brain, and tonsil paraffin sections with anti-Orc5 antibody (g–i) or matched pre-immune (Pre-imm.) control serum (j–l) as described in Fig. 3. j and l were photographed at 80×. Representative positive staining in (a, b, g, and h) is indicated by arrowheads.
HeLa cells were metabolically labeled with \[\text{[35S]}\]methionine associated with the ORC subunits. To examine this question, when overexpressed (17, 19–21). Such experiments do not, somatic cells can associate with each other under gentle extrac-


dition confirms that Orc2 (also 70 kDa) is co-precipitated (Fig. 5A, bottom) but not Orc1 nor Orc4. Additional proteins are detected, however, corresponding to sizes of 200, 160, 120, 85, and 33 kDa (Fig. 5A, top). Again, none of these proteins correspond to known ORC subunits.

Anti-Orc4 antibody immunoprecipitates Orc4 (45 kDa) and associated proteins of 150, 52, and 30 kDa. Co-immunoprecipitation of Orc2 and Orc3 (both of 70 kDa) or Orc1 (100 kDa) is not observed under these extraction conditions (Fig. 5A, bottom). Our anti-Orc5 antibody cannot be used for immunoprecipitation.

For each anti-ORC subunit antibody, denaturation of the immunoprecipitates in SDS followed by re-immunoprecipitation confirms this protein as Orc5.2 Because of interference from the 50-kDa IgH band in the immunoprecipitate in Fig. 5A, anti-Orc2 antibody cross-linked to protein A-Sepharose beads was used to immunoprecipitate Orc2 so that the precipitate could be blotted with anti-Orc5 antibody (Fig. 5B). A 48-kDa Orc5 protein is detected in the Orc2 immunoprecipitate, indicating that although the bulk of the cellular Orc5 protein can be extracted only with 8 M urea (21), a fraction of it is extracted from cells in RIPA buffer in a complex with Orc2. In contrast to Orc3 and Orc5, Orc4 (45 kDa) did not remain associated with Orc2 in RIPA buffer conditions (Fig. 5A, bottom). The 45-kDa metabolically labeled protein that co-immunoprecipitates with Orc2 is not recognized by the anti-Orc4 antiserum and is an unidentified protein. In summary, under RIPA extraction conditions Orc2 is co-immunoprecipitated with Orc3 and Orc5 but not with Orc1 or Orc4. Several other proteins present in the Orc2 immunoprecipitate in RIPA buffer conditions (180, 170, 145, 130, 120, 85, and multiple bands of 70 kDa and 45 kDa) do not correspond to known ORC subunits.

from certain tissues might indicate a role of the protein in something other than DNA replication.

Several Cellular Proteins That Are Not Known ORC Subunits Associate with Individual ORC Subunits with High Affinity—Previous immunoprecipitation/immunoblotting experiments indicate that several of the human ORC subunits from somatic cells can associate with each other under gentle extraction conditions (0.1% Nonidet P-40, 150 mM NaCl), especially when overexpressed (17, 19–21). Such experiments do not, however, reveal whether other cellular proteins are tightly associated with the ORC subunits. To examine this question, HeLa cells were metabolically labeled with \([\text{[35S]}\)methionine and the ORC subunits immunoprecipitated after extraction in RIPA buffer, a relatively stringent buffer that sustains only high affinity interactions (Fig. 5A, top). Similar immunoprecipitates from unlabelled cell extracts were immunoblotted with the various anti-ORC subunit antibodies (Fig. 5A, bottom).

We note first that the metabolically labeled, extractable, Orc1 (100 kDa) protein appears not to be associated with the other ORC subunits but is co-precipitated with unknown proteins of >200, 150, 80, 45, and 30 kDa (Fig. 5A, top). Immunoblotting shows that none of the associated proteins are recognized by antibodies to the other ORC subunits (Fig. 5A, bottom). Conversely, immunoprecipitation with anti-Orc2, anti-Orc3, and anti-Orc4 consistently failed to co-immunoprecipitate a band of 100 kDa that could be Orc1. Immunoblotting of these precipitates with anti-Orc1 antibody confirmed that Orc1 was not co-immunoprecipitated. We confirmed the recent report that the bulk of Orc1 is not extracted from cells even under RIPA buffer lysis conditions (29). Therefore the newly synthesized Orc1 that is extracted in Fig. 5A is most likely a sub-population that is not yet in the holo-ORC bound to DNA but is still tightly associated with cellular proteins that are not known ORC subunits.

Under similar stringent conditions anti-Orc2 antisera co-immunoprecipitated the 70-kDa Orc2 protein with several cellular proteins, only two of which were known ORC subunits: Orc3 and Orc5. The anti-Orc2 immunoprecipitate can be compared with the three other pre-immune lanes in Fig. 5A (non-immune controls). In addition, we have previously shown that the matched pre-immune serum (which is no longer available) did not immunoprecipitate any metabolically labeled protein from human cell extracts (20). Besides Orc2 (70 kDa) anti-Orc2 co-precipitated another protein of roughly the same size. The latter protein is Orc3 (Fig. 5A, bottom), which runs at 70 kDa under these gel conditions, suggesting that endogenous Orc2 and Orc3 remain associated with each other in RIPA buffer conditions. Peptide digestion and ion trap mass spectrometry of the 48-kDa polypeptide in the Orc2 immunoprecipitate identified this protein as Orc5.2 Because of interference from the 50-kDa IgH band in the immunoprecipitate in Fig. 5A, anti-Orc2 antibody cross-linked to protein A-Sepharose beads was used to immunoprecipitate Orc2 so that the precipitate could be blotted with anti-Orc5 antibody (Fig. 5B). A 48-kDa Orc5 protein is detected in the Orc2 immunoprecipitate, indicating that although the bulk of the cellular Orc5 protein can be extracted only with 8 M urea (21), a fraction of it is extracted from cells in RIPA buffer in a complex with Orc2. In contrast to Orc3 and Orc5, Orc4 (45 kDa) did not remain associated with Orc2 in RIPA buffer conditions (Fig. 5A, bottom). The 45-kDa metabolically labeled protein that co-immunoprecipitates with Orc2 is not recognized by the anti-Orc4 antiserum and is an unidentified protein. In summary, under RIPA extraction conditions Orc2 is co-immunoprecipitated with Orc3 and Orc5 but not with Orc1 or Orc4. Several other proteins present in the Orc2 immunoprecipitate in RIPA buffer conditions (180, 170, 145, 130, 120, 85, and multiple bands of 70 kDa and 45 kDa) do not correspond to known ORC subunits.

Immunoprecipitation with anti-Orc3 antibody precipitates Orc3 (runs as 70 kDa under these gel conditions). Immunoblotting confirms that Orc2 (also 70 kDa) is co-precipitated (Fig. 5A, bottom) but not Orc1 nor Orc4. Additional proteins are detected, however, corresponding to sizes of 200, 160, 120, 85, and 33 kDa (Fig. 5A, top). Again, none of these proteins correspond to known ORC subunits.

Anti-Orc4 antibody immunoprecipitates Orc4 (45 kDa) and associated proteins of 150, 52, and 30 kDa. Co-immunoprecipitation of Orc2 and Orc3 (both of 70 kDa) or Orc1 (100 kDa) is not observed under these extraction conditions (Fig. 5A, bottom). Our anti-Orc5 antibody cannot be used for immunoprecipitation.

For each anti-ORC subunit antibody, denaturation of the immunoprecipitates in SDS followed by re-immunoprecipita-

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2 S. Dhar, A. Dutta, and W. S. Lane, unpublished information.
**Human ORC**

**Fig. 6. Association of ORC subunits (Orc2–5) in human cell lysate.** 293T cell extract was fractionated over a fast protein liquid chromatography Superose 12 gel filtration column. Alternate fractions were immunoblotted using anti-Orc2, 3, 4, and 5 antibodies. Fraction numbers (11–29) are shown. Input (Inp.) lanes were loaded with 5% of the total lysate passed through the column. 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 indicated.

**TABLE 1**

| Fraction | Molecular Weight (kDa) |
|----------|------------------------|
| 11       | 670                    |
| 13       | 158                    |
| 15       | 44                     |
| 17       | 17                     |
| 19       | 23                     |
| 21       | 25                     |
| 23       | 27                     |
| 25       | 29                     |
| 27       | 31                     |
| 29       | Inp.                   |

**Antibody Immunoprecipitation**

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 indicated.

**Immunofluorescence of ORC Subunits in Cells**—We and others have reported changes in subcellular localization of CDC6 protein based on the position of the cell in the cell cycle. CDC6 is in the nucleus in G1 and is displaced to the cytoplasm in S phase such that in a culture of asynchronous cells the protein is detected in the nucleus in some cells and in the cytoplasm in others (Fig. 7). We can now examine the behavior of the ORC complex by immunofluorescence of asynchronous cells in culture. Affinity-purified antibodies to Orc2, Orc3, and Orc4 and anti-Orc5 antiserum each detected a single polypeptide upon immunoblotting extracts from cultured cells and detected the endogenous protein by immunofluorescence. Antibodies to Orc2 recognized a nuclear protein detectable throughout the nucleus but also concentrated in fine dots (Fig. 7, A and B). The nature of these dots is unclear. Orc2 was never detected in the cytoplasm. Antibodies to Orc3, Orc4, and Orc5 also recognized exclusively nuclear proteins. Anti-Orc5 antibody detected a protein with exclusively nuclear localization, which however, was like Orc2 in being distributed throughout the nucleus but also concentrated in discrete sub-nuclear foci (Fig. 7, A and B).

Antibodies to Orc1 did not give a positive result when used for immunofluorescence experiments, so epitope-tagged Orc1 protein (T7-Orc1) was expressed in cells by transfection, and the protein was detected by anti-T7 antibody. Untransfected cells served as negative controls. T7-Orc1 was detected only in the nucleus of asynchronously growing, transfected cells.

Thus, all the ORC subunits examined appeared to be in the nucleus in proliferating cells in culture regardless of their position in the cell cycle, suggesting that the subcellular changes in the localization of CDC6 occur independent of ORC.

**Phosphorylation of ORC Subunits in Proliferating Cells**—Several cell cycle-regulated proteins are regulated by phosphorylation. For example, phosphorylation of cyclin E leads to its destruction, whereas cdk2 phosphorylation either activates or inactivates kinase activity (33, 34). Among the replication initiation factors, the phosphorylation of CDC6 by cdk2 is responsible for the displacement of the protein from the nucleus to the cytoplasm at the G1-S transition (30–32).

To examine whether any ORC subunits were phosphorylated in vivo, asynchronously growing HeLa cells were metabolically labeled with [32P]orthophosphate, and proteins were extracted in RIPA buffer and immunoprecipitated with antibodies to Orc1-Orc5. A 70-kDa phosphoprotein was present in the anti-Orc1 immunoprecipitate, but nothing was detected in the anti-Orc1, anti-Orc3, or anti-Orc4 immunoprecipitates (Fig. 8, left). Treatment with phosphatase removed all label from the Orc2, indicating that the label was due to phosphorylation (data not shown). To confirm that the 70-kDa phosphoprotein was in fact Orc2 and not merely an Orc2-associated protein, anti-Orc2 antibody was used to immunoprecipitate [32P]-labeled cell extracts denatured by boiling in SDS. The phosphoprotein was still immunoprecipitated by anti-Orc2. In addition, when these cells

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3 L. Delmolino and A. Dutta, unpublished information.
immunoprecipitates were transferred to nitrocellulose and immunoblotted with anti-Orc2 antibodies, the radiolabeled and chemiluminescent 70-kDa bands were coincident. We therefore conclude that the 70-kDa phosphoprotein is Orc2.

Consistent with the stable association of Orc2 with Orc3, immunoprecipitation with anti-Orc3 is expected to co-precipitate the 70-kDa phospho-Orc2. However, since most of the Orc2 in the cell is not associated with Orc3 in RIPA buffer (see Fig. 5A, bottom), we needed to immunoprecipitate Orc3 from 0.1% Nonidet P-40, 150 mM NaCl lysis buffer to visualize the co-immunoprecipitated 70-kDa phospho-Orc2 protein (Fig. 8, right).

To determine whether any of the ORC subunits were phosphorylated in any one phase of the cell cycle such that the phosphorylation was missed in the asynchronous cell extracts, cells were blocked in S and M phase (with hydroxyurea and nocodazole) and labeled with [32P]orthophosphate. These studies revealed that Orc2 was phosphorylated in both S and M phases of the cell cycle but did not indicate that any of the other ORC subunits were phosphoproteins. During the course of this work another group reported that Orc1 is phosphorylated (as measured by mobility shift) during the M phase of the cell cycle (29). Treatment with phosphatase eliminated the mobility shift. Using conditions suitable for Orc1 extraction (500 mM NaCl), we have confirmed by direct immunoblotting of the extracts that Orc1 is phosphorylated in M phase, as measured by mobility shift (data not shown). We were, however, not able to isolate 32P-labeled Orc1, but this could be explained by the fact that the isoform of Orc1 with slower mobility is poorly extracted and poorly immunoprecipitated with our antibody.

**DISCUSSION**

In higher eukaryotes, the origin recognition complex has been studied in very early developmental stages with unusual cell cycles: *Xenopus* egg extracts and *Drosophila* embryo extracts. In both these cases the cells are super-enriched in initiator protein factors in preparation for the very rapid S phases that occur during early embryonic stages of life, and the results are consistent in that all of the ORC subunits are in a stable complex required for DNA replication.

This report is the first paper to collectively examine five ORC subunits in mammalian somatic cells. The dominant theme that emerges is that, contrary to the situation in yeast or in early developmental stages, a holo complex of all ORC subunits...
is not easy to detect in mammalian somatic cells. Some of the difficulty might be due to technical problems in extracting Orc1, resulting in the breaking apart of the six subunit complex. In addition, Orc2, Orc3, Orc4, and Orc5 proteins are detected in several tissues that have very few proliferating cells. The abundance of these subunits are not strictly correlated with each other, and none of these non-proliferating tissues contain Orc1 mRNA and protein. Even in proliferating cultured cells individual ORC subunits are associated more tightly with unidentified non-ORC polypeptides than with known ORC subunits. Based on this, we conclude that unlike the situation in yeast, *Drosophila* embryos, or *Xenopus* eggs, in human somatic cells if the individual ORC subunits are in a tight holo complex containing all subunits, then such a complex is either not soluble or present in extracts at a level below the threshold of detectability in Fig. 6.

Faced with the overwhelming evidence that ORC acts as a holo complex in replication initiation in yeast, *Xenopus* eggs, and *Drosophila* embryos, we are not proposing that this is not the case in mammalian somatic cells. Instead, we propose that such a complex is formed in proliferating cells but only during a small window of time in the cell cycle or in a sub-nuclear fraction that is difficult to extract in lysates. The co-precipitation and gel filtration experiments in 0.1% Nonidet P-40 buffer indicate that at least a sub-complex of Orc2, Orc3, Orc4, and Orc5 can be detected in cell extracts. In addition several of the ORC subunits are detected in non-proliferating cells. Upon induction of the cell cycle, Orc1 is expressed from an E2F-regulated promoter (28). The newly synthesized Orc1 is better extracted than mature Orc1 but is not associated with other ORC subunits (Fig. 5A). We speculate that the new Orc1 protein subsequently associates with the other ORC subunits (most likely in a cellular fraction that is tightly associated with the chromatin) and renders ORC competent for initiating DNA replication. This suggestion is consistent with a recent report that in Chinese hamster ovary cells Orc2 remains firmly associated with chromatin in all phases of the cell cycle, whereas Orc1 selectively associates with chromatin in G1, thereby making the origins competent to replicate (35).

In several tissues the levels of mRNA and protein for individual ORC subunits do not appear to correlate (Figs. 1 and 2). For example, kidney and spleen do not contain detectable levels of Orc1 mRNA yet express low levels of Orc1 protein, and Orc2 protein is observed in liver in the absence of detectable Orc2 mRNA. Similarly, Orc3 mRNA is detectable in kidney and spleen, but no Orc3 protein is detected in these tissues. There are several explanations of these apparent discrepancies. First, the Northern and Western tissue blots were obtained from different sources, and thus, the tissue samples are not identical. Second, the stability of individual ORC subunit mRNA and polypeptides may vary between tissues. Particularly stable ORC subunit proteins could be produced from exceedingly low levels of mRNA not detectable by our analysis. Future studies should address this issue. Finally, the ease of extractability of individual ORC subunit proteins may vary between tissues. These results do, however, suggest that both mRNA and protein should be examined when studying expression of a given gene.

We reported earlier that an alternatively spliced variant of Orc5 results in the expression of a truncated Orc5 subunit (ORC5T) that does not encode the C-terminal one-third of the protein (21). Here we note the existence of a 47-kDa protein in brain that reacts to anti-Orc3 antibody and a 45-kDa protein in heart that reacts to anti-Orc2 antibody that are different in size from the authentic subunits seen in proliferating cells. These could be tissue-specific proteins that fortuitously cross-react with the antibodies but could also be tissue-specific ORC subunits produced by alternative splicing or post-translational processing and might indicate a role of these subunits in specialized tissue-specific functions.

Several ORC subunits associate with several cellular polypeptides with a higher affinity than with known ORC subunits. The unknown cellular proteins might be involved in DNA replication, indicating that ORC in somatic cells is a much larger protein complex than the six-subunit complex identified in other organisms. Alternatively, the other proteins might be involved in recruiting ORC subunits to functions other than initiation of DNA replication. For example, *Drosophila* Orc5 (Latheo) has been implicated in learning (17, 36, 37). Flies with a hypomorphic mutation in Latheo have a smaller than normal mushroom body (a portion of the fly brain) and demonstrate defects in synaptic transmission and defects in learning. Although the small size of the mushroom body can be explained by a role of Orc3 in cell proliferation, the defects in synaptic transmission implicate Orc3 in a function independent of cell proliferation. Similarly, *Drosophila* Orc2 associates with HP1 and decorates the heterochromatin of flies (38). Mutations in Orc2 result in defects in chromatin structure, as indicated by suppression of position effect variegation. In yeast, ORC has been implicated in transcriptional silencing (39). These results with *Drosophila* and yeast ORC lead us to suspect that the unique polypeptides associated with human ORC subunits are explained by a role of these subunits in functions other than replication initiation. Identification of the associated proteins will help determine whether this is indeed the case.

Regulation of replication initiation in the cell cycle could involve changes in phosphorylation or sub-cellular localization of ORC subunits. Our results, however, do not support such a point of view. In asynchronously proliferating cells in culture, no changes were detected in the sub-cellular localization of the ORC subunits. Similarly, no changes were detected in the phosphorylation state of the Orc2, 3, 4, and 5 subunits in the various stages of the cell cycle. As reported, Orc1 is phosphorylated in M phase, but the significance of this modification is unclear (29).

This is the first report of the status of ORC subunits in proliferating somatic cells and in non-proliferating tissues. The results indicate that unlike what has been so elegantly dem-
onstrated in yeast, *Drosophila* and *Xenopus*, a holo complex of just six ORC subunits is difficult to extract from mammalian somatic cells. Instead, sub-complexes of ORC exist in non-proliferating cells, which may become competent replication initiators only upon the appearance of Orc1 and its association with other subunits in a chromatin-associated sub-nuclear fraction. In addition a study of the associated proteins and of the expression of individual subunits in non-proliferating cells suggests that ORC might have additional components in mammalian somatic cells and that ORC subunits might be recruited to other functions not directly related to initiation of DNA replication.

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