Assembly of the Human Origin Recognition Complex

Sanjay Vashee‡, Pamela Simancek‡, Mark D. Challberg‡, and Thomas J. Kelly‡**

From the ¶Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205 and ∗Laboratory of Viral Diseases, NIAID, National Institutes of Health, Bethesda, Maryland 20892

Received for publication, March 20, 2001
Published, JBC Papers in Press, April 25, 2001, DOI 10.1074/jbc.M102493200

The six-subunit origin recognition complex (ORC) was originally identified in the yeast Saccharomyces cerevisiae. Yeast ORC binds specifically to origins of replication and serves as a platform for the assembly of additional initiation factors, such as Cdc6 and the Mcm proteins. Human homologues of all six ORC subunits have been identified by sequence similarity to their yeast counterparts, but little is known about the biochemical characteristics of human ORC (HsORC). We have extracted HsORC from HeLa cell chromatin and probed its subunit composition using specific antibodies. The endogenous HsORC, identified in these experiments, contained homologues of Orc1–Orc5 but lacked a putative homologue of Orc6. By expressing HsORC subunits in insect cells using the baculovirus system, we were able to identify a complex containing all six subunits. To explore the subunit interactions that are required for the assembly of HsORC, we carried out extensive co-immunoprecipitation experiments with recombinant ORC subunits expressed in different combinations. These studies revealed the following binary interactions: HsOrc2-HsOrc3, HsOrc2-HsOrc4, HsOrc3-HsOrc4, HsOrc2-HsOrc6, and HsOrc3-HsOrc6. HsOrc5 did not form stable binary complexes with any other HsORC subunit but interacted with sub-complexes containing any two of subunits HsOrc2, HsOrc3, or HsOrc4. Complex formation by HsOrc1 required the presence of HsOrc2, HsOrc3, HsOrc4, and HsOrc5 subunits. These results suggest that the subunits HsOrc2, HsOrc3, and HsOrc4 form a core upon which the ordered assembly of HsOrc5 and HsOrc1 takes place. The characterization of HsORC should facilitate the identification of human origins of DNA replication.

The initiation of DNA replication in lower eukaryotes is similar to that observed in bacteria in that it occurs at well defined origins of DNA replication that are recognized by specific initiator proteins (1–5). Saccharomyces cerevisiae origins of DNA replication are short, ~100-base pair (bp) segments that consist of two essential regions: the A domain, which contains a conserved 11-bp consensus sequence (ACS) that is required for origin function, and the B domain, which contains several stimulatory elements (6, 7). The ACS is recognized specifically by the S. cerevisiae initiator protein, the origin recognition complex (ORC) (8, 9). S. cerevisiae ORC (ScORC) is comprised of six proteins (Orc1–6), each of which is essential for viability in S. cerevisiae (10–13). A number of lines of evidence indicate that ScORC is required for initiation of DNA replication in vivo and functions in part to recruit other initiation factors, such as Cdc6 and the minichromosome maintenance proteins, to replication origins (13–15).

Homologues of ORC subunits have been identified in a variety of other eukaryotic species (2, 16), and ORC has been purified from the fission yeast Schizosaccharomyces pombe and the metazoans Drosophila melanogaster and Xenopus laevis (17–19). Genetic and biochemical data indicate that specific ORC subunits from the latter organisms, like those of S. cerevisiae, are required for the initiation of chromosomal DNA replication (20–22). The conservation of ORC as well as other initiation factors strongly suggests that there are common mechanisms for initiating DNA replication shared by all eukaryotes. However, despite this high degree of evolutionary conservation, the identification in other eukaryotes of ORC binding sequences comparable with the S. cerevisiae ACS elements has so far proved elusive. Origins of DNA replication in S. pombe are large, AT-rich elements that lack a common consensus sequence (23–25). The binding of S. pombe ORC to origins of DNA replication appears to be mediated in large part by a unique N-terminal domain of SpOrc4 that contains multiple copies of a motif that binds to AT tracts (26). Purified D. melanogaster ORC has been shown to interact with multiple sequences in ACE3, a cis-acting element that is required for the specialized DNA replication process that occurs during chorion gene amplification, but it is not yet clear that this interaction is relevant to initiation of DNA replication at origins of DNA replication in somatic cells (27, 28). These observations raise the possibility that there are functionally important differences in the way ORC mediates initiation of DNA replication in different eukaryotes.

Human homologues of all six ORC subunits have been identified by sequence similarity searches (29–34). HsOrc1, HsOrc2, HsOrc4, and HsOrc5 exhibit considerable homology with their yeast and metazoan counterparts (28, 29, 31–33). Orc3 and Orc6 appear to have evolved faster than the other ORC subunits. HsOrc3 has significant similarity to metazoan and Orc6 is not required either for specific binding of ScORC to the ACS or for formation of multi-subunit complexes containing the ScOrc1-ScOrc5 (35). It has also been reported that HsOrc6 cannot be co-immuno-

* This work was supported by National Institutes of Health Grant CA40414. 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 a postdoctoral fellowship from National Institutes of Health Grant GM19675.

** To whom correspondence should be addressed: Dept. of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205. Tel.: 410-955-3292; Fax: 410-955-0831; tkelly@jhmi.edu.

The abbreviations used are: bp, base pair; ACS, ARS consensus sequence; ORC, origin recognition complex; ScORC, S. cerevisiae ORC; xORC, X. laevis ORC; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.
precipitated with HsOr2c, suggesting that HsOr6c may be only weakly associated with other human ORC subunits (34).

Although immunoprecipitation experiments from several laboratories have demonstrated physical interactions between several subunits of HsORC (29, 36, 37), a holo-complex of all six proteins has not been identified and characterized. Given the possibility of differences in ORC function among eukaryotes it will be important to identify and characterize HsORC. We report here that endogenous HsORC consisting of a complex of five subunits (HsOr1-HsOr5) can be extracted from HeLa cell chromatin. We have also expressed the HsORC subunits in insect cells using the baculovirus system and have identified a complex containing all six subunits. Using this system we have probed the protein-protein interactions between the subunits that are necessary for assembly of the complex. Our results suggest that the HsOr2, HsOr3, and HsOr4 proteins form the core complex to which HsOr5 and HsOr1 bind in an ordered manner. HsOr6 appears to be the most weakly bound subunit in the complex. The identification of human ORC should facilitate the identification of replication origins in human cells.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—To generate antibodies, the six HsORC subunits were expressed individually in bacteria as recombinant fusion proteins containing the GSH (glutathione) and GST (glutathione S-transferase) moieties. For HsORC1, the 1050-bp sequence that encodes the C-terminal 350 amino acids was amplified using Pfu polymerase (Stratagene), oligonucleotides 5'-GGGGTACCTCAAAGATCTCAAAATTTTTG-TGGAATCC-3' and 5'-CGGGGAATTTCCTCTGTCTTCCCGGACGAGA-C3' as primers, and the plasmid pGEX-HsORC1 as template. The PCR product was digested with BglII and cloned into the corresponding sites in the plasmid pET28a (Novagen). For HsORC2, the 810-bp sequence that encodes the first 270 amino acids was amplified using oligonucleotides 5'-GGAGATCTTAGTGAATACCAATCATGAAAATGA-ATTAAAGACAACCGAACTGGAATTTGTTTCCGAG-CTTAGTATATTAC-3' and 5'-ATAAAGATGCGCCCCCAAAATGGA-CTTTTAATGATCC-3' as primers, and the plasmid pMHC1H2 (kindly provided by Dr. B. Stillman, Cold Spring Harbor Laboratory) as template. The PCR product was digested with BglII and XhoI and cloned into the BamHII/XhoI sites of the plasmid pET28a (Novagen).

For HsORC3, the 808-bp region that encodes the terminal 350 amino acids of HsORC3 was amplified using oligonucleotides 5'-CGGGGATCCGCCAACTGGAATCTCAAAATTTTTGTTTCCGAGAGCAGA-ATTAAAGACAACCGAACTGGAATTTGTTTCCGAG-CTTAGTATATTAC-3' and 5'-TTCCCCCCGGTTTCCGAGCTTTTAATGATCC-3' as primers, and the plasmid pBluescript II SK+ as template. The vector encoding the HsORC3 gene was made by amplification of the full-length gene by PCR using oligonucleotides 5'-GGGGTACCTCAAAGATCTCAAAATTTTTG-TGGAATCC-3' and 5'-GGGGTACCTCAAAGATCTCAAAATTTTTG-TGGAATCC-3' as primers, and the plasmid pBluescript-HsORC3 as template. After digestion with BglII and EcoRI, the PCR products containing the HsORC3, HsORC4, HsORC5, and HsORC6 genes were individually cloned into the corresponding sites in the plasmid pFastBac1. The constructs were confirmed by DNA sequencing. All recombinant viruses were produced and amplified according to the manufacturer's instructions (Bac-to-Bac TM Baculovirus Expression Systems, Life Technologies, Inc.).

**Preparation of Nuclear Extracts**—Spinner cultures of HeLa cells were grown at 37°C in Spinner minimum Eagle's medium with glutamine (Quality Biological, Inc.) and supplemented with 5% fetal bovine serum and penicillin (100 units/ml), streptomycin (100 μg/ml). Nuclear extracts were prepared exactly as described by Challberg and Kelly (38). Nuclear extracts were prepared by first extracting the nuclei with OPB2 buffer (50 mM Hepes, pH 7.5, 5 mM MgCl2, 0.1 mM EDTA, 0.1 mM EGTA, 10% glycerol, 0.1% Triton X-100) at 4°C for 1 h. After centrifugation at 16,000 g for 15 min at 4°C, the resulting pellet was then extracted with OPB2 buffer plus 500 mM NaCl on ice for 1 h. The suspension was centrifuged at 140,000 g for 1 h at 4°C. The resulting supernatant was used in immunoprecipitation experiments.

**Immunoprecipitation and Immunoblotting Procedures**—For immunoprecipitation experiments, anti-HsORC2 and the anti-HsORC3 antibodies were separately cross-linked to rProtein A-Sepharose (Amer sham Pharmacia Biotech) as described in Harlow and Lane (39) with minor modifications. The solid phase was blocked at 0.1 M Na2BO3, pH 9.0, was used in all steps instead of 0.2 M Na2BO3, pH 9.0. Cross-linking was achieved with dimethyl suberimidate 2HCl (Pierce) at a final concentration of 20 mM.

Immunoprecipitation experiments were performed by incubating either HeLa cell nuclear extract or baculovirus-infected Sf9 insect cell nuclear extract with cross-linked antibody beads for 2–3 h at 4°C. The suspensions were then washed 3 times with 10× bead volume of OPB2 buffer plus 500 mM NaCl. The immunoprecipitated proteins were re-
leashed from the beads with 1% SDS and analyzed by 10% SDS/PAGE followed either by transferring separated proteins to nitrocellulose membranes and Western blotting or staining by silver.

For Western blotting, the following concentrations of antibodies were used: HsORC1 (1:1000), HsORC2 (1:3000), HsORC4 (1:1000), HsORC5 (1:3000), and HsORC6 (1:3000). Goat anti-rabbit IgG conjugated with horseradish peroxidase (Pierce) was used as the secondary antibody at a concentration of 1:6500.

**Gel Filtration Analysis**—A fraction of the baculovirus-infected Sf9 insect cell nuclear extract containing all six of the HsORC components was applied to a Superdex™-200 HR 10/30 gel filtration column (Amersham Pharmacia Biotech) equilibrated with OP2b buffer plus 500 mM NaCl. Fractions (500 μl) were collected and analyzed for HsORC subunits by 10% SDS/PAGE followed by transferring to nitrocellulose membranes and Western blotting.

**RESULTS**

**Identification of an Endogenous HsORC Composed of Five Subunits in HeLa Cells**—To begin biochemical characterization of HsORC, we first raised polyclonal antibodies against recombinant forms of each of the six subunits that had been individually expressed in bacteria and purified by affinity chromatography. The specificity of these antibody preparations was assessed in several ways. Western blotting experiments demonstrated that each antibody recognized an endogenous HeLa protein with the molecular weight expected for the corresponding HsORC subunit. In addition, antigen competition experiments were performed for five of the six HsORC subunits (HsOrc5 could not be tested in this manner because of insolubility of the antigen). In every case tested, the signal detected by Western blotting could be eliminated by preincubation of the antibodies with an excess of the appropriate antigen but was not affected by preincubation with an excess of control antigen (data not shown). Finally, each antibody specifically recognized the corresponding HsORC subunit when individually expressed in insect cells using the baculovirus system (see below).

To examine the localization of HsORC subunits in human cells we carried out subcellular fractionation experiments. HeLa cells were disrupted by Dounce homogenization at low ionic strength, and the cytoplasmic and nuclear fractions were separated by centrifugation. The nuclear fraction was then extracted sequentially with 100 and 500 mM NaCl to elute proteins bound to chromatin with different affinities. Western blot analysis revealed that the HsOrc2-HsOrc6 subunits were found almost exclusively in the 500 mM NaCl chromatin extract. A significant fraction of HsOrc1 was present in the same fraction, but HsOrc1 was also detected in the cytoplasmic and 100 mM NaCl chromatin extract (data not shown). This result suggests that a portion of cellular HsOrc1 is either not associated with the other HsORC subunits or is more weakly bound.

Preliminary experiments established that our polyclonal HsOrc2 antibody could be used to specifically and quantitatively immunoprecipitate the HsOrc2 present in the 500 mM chromatin extract. Fig. 1 shows an experiment in which we probed such immunoprecipitates for the other HsORC subunits. Western blot analysis revealed that HsOrc1-HsOrc5 were co-immunoprecipitated with similar efficiencies by the HsOrc2 antibody (Fig. 1A). None of the subunits was detected in control experiments with a preimmune antibody. HsOrc6p was not found in the immunoprecipitated fraction, suggesting that it is not part of the endogenous complex or is dissociated by washing the immunoprecipitates in 500 mM NaCl (Fig. 1A).

We also examined the complexity of the immunoprecipitated fraction by SDS/PAGE followed by silver staining. Fig. 1B shows that the immunoprecipitates obtained with the antibody against HsOrc2 contained five major proteins not present in control immunoprecipitates. The mobilities of these proteins were consistent with the calculated molecular weights of HsOrc1-HsOrc6 proteins and were the same as the corresponding bands observed by Western blotting. As previously observed, HsOrc2 protein appeared as a closely spaced doublet at ~72 kDa. To a first approximation, the bands corresponding to HsOrc2 and HsOrc5 stained with similar intensities, suggesting that they were present in roughly equal amounts. On the other hand, HsOrc1 consistently stained with lower intensity, suggesting that it may not be as abundant as the other HsORC subunits in the 500 mM chromatin extract. In fact, the amount of HsOrc1 protein in the immunoprecipitates varied somewhat from experiment to experiment, suggesting that the reduced level of HsOrc1 may be due in part to uncontrolled losses during fractionation. These conclusions are subject to the caveat that the relative intensity of silver staining is not always a reliable indicator of abundance, so further work will have to be done to establish the stoichiometry of the various subunits.

The pattern of bands in the HsORC immunoprecipitate is strikingly similar to that observed for X. laevis ORC (xI0RC) immunoprecipitated with antibody against xI0RC2 (40). Indeed, the similarity even extends to the fact that xI0RC3p, like HsOrc3, consistently stains more intensely than the other subunits. In the case of xI0RC it was demonstrated that xI0RC3 stains aberrantly with silver and is actually equimolar with the other subunits. Thus, our data indicate that HeLa cell chromatin contains tightly bound complexes comprised of HsOrc1-HsOrc5. Our data also suggest that some of these complexes may lack HsOrc1. Consistent with this possibility, we show below that complexes containing either HsOrc1-HsOrc5 or HsOrc2-HsOrc5 are stable.

**Reconstitution of HsORC Containing Six Subunits in Baculovirus-infected Insect Cells**—To facilitate the biochemical characterization of HsORC, we cloned cDNAs encoding each of the six HsORC subunits into baculovirus expression vectors and isolated the individual recombinant viruses. Sf9 insect cells were co-infected with all six recombinant baculoviruses, and after 48 h, 500 mM nuclear extracts were prepared by a method similar to that employed to isolate HsORC from HeLa cells (see “Experimental Procedures”). Recombinant HsORC was immunoprecipitated with the antibody against HsOrc2 and analyzed by SDS/PAGE. Examination of the silver-stained..
Experimental Procedures" to obtain a 500 mM NaCl nuclear extract. Ali-
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A, Sf9 cells were co-infected with baculoviruses encoding the six HsORC
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mass of 35 kDa was also immunoprecipitated (Fig. 2C). The
subunit complexes and is not due to nonspecific precipitation or
completely blocked the co-immunoprecipitation of the six
subunits in the immunoprecipitates (Fig. 2D). These results indicate that the observed
cross-reacting antibodies.
To estimate the size of the recombinant HsORC we carried out gel filtration chromatography on chromatin extracts of Sf9 cells co-infected with the six subunits (Fig. 3). Western blot analysis revealed that all six subunits co-eluted from the column in fractions corresponding to a molecular mass of ~400 kDa (fractions 19–21). This result is consistent with a holo-complex containing one molecule of each subunit. The distribution of HsORC1 was slightly broader than that of the other subunits and was skewed toward lower molecular mass fractions. We suggest that this may reflect some dissociation of HsORC1 from the complex during chromatography. Although HsORC6 was present in the same fractions as HsORC1-HsORC5, the peak of HsORC6 eluted slightly before the peak of the other subunits. One reasonable explanation for this behavior is that some of the complexes lack HsORC6 and that these elute slightly later than complexes containing the subunit. HsORC4, HsORC5, and HsORC6 were present in a second peak that eluted from the column in fractions corresponding to molecular mass of ~50 kDa. This peak presumably contains free, uncomplexed subunits. The presence of excess HsORC4, HsORC5, and HsORC6 subunits in the extracts probably reflects the fact that smaller proteins are generally expressed from recombinant baculovi-
ruses with greater efficiencies than larger proteins. Thus, our
gel filtration data together with the co-immunoprecipitation

FIG. 2. Reconstitution of recombinant HsORC in insect cells. A, Sf9 cells were co-infected with baculoviruses encoding the six HsORC subunits. After 48 h, the cells were processed as described under "Experimental Procedures" to obtain a 500 mM NaCl nuclear extract. Ali-
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FIG. 3. Gel filtration of recombinant HsORC. A 500 mM nuclear extract from insect cells co-infected with recombinant baculoviruses encoding the six HsORC subunits was applied to a Superdex 200 gel filtration column. Proteins from alternate fractions (fractions 15–39) were separated on SDS/PAGE, transferred to nitrocellulose mem-
branes, and probed with antibodies against HsORC1, anti-HsORC2, anti-
HsORC3, anti-HsORC4, anti-HsORC5, and anti-HsORC6. The positions of
molecular mass markers thyroglobulin (T; 670 kDa), γ globulin (G; 158
kDa), ovalbumin (O; 44 kDa), and myoglobin (M; 17 kDa) (Bio-Rad)
are marked by arrows.
data described above indicate that a holo-complex containing HsOrc1-HsOrc6 can be efficiently reconstituted from recombinant subunits.

**Binary Interactions of Recombinant HsORC Subunits**—We exploited the baculovirus system to explore in detail the subunit-subunit interactions that are required for the assembly of HsORC. For this purpose, HsORC subunits were expressed in various combinations, and chromatin extracts were prepared in the same manner as that used to isolate the HsORC holo-complex. Interactions among the subunits were detected by co-immunoprecipitation experiments carried out with antibodies against HsOrc2 or HsOrc3. In the initial experiments we sought to identify stable binary interactions (Fig. 4). For example, the first panel of Fig. 4A shows the results obtained when Sf9 cells were co-infected with HsOrc2 and either HsOrc3, HsOrc4, or HsOrc6. Western blotting revealed that both subunits were immunoprecipitated from extracts by antibody against HsOrc2 but not by control preimmune antibody. Moreover, the two subunits stained equally with silver, suggesting that they were present in the immunoprecipitates in similar amounts. We conclude that HsOrc2 and HsOrc3 form a binary complex that is stable enough to survive washing with buffers containing 500 mM NaCl. The data in Fig. 4 demonstrate that similar binary complexes can be formed between HsOrc2 and HsOrc4 and between HsOrc3 and HsOrc4.

We also detected interactions between recombinant HsOrc6 and either HsOrc2 or HsOrc3. In the case of the HsOrc2-HsOrc6 combination, only a small amount of HsOrc6 was recovered in the immunoprecipitates, as demonstrated by both Western blotting and silver staining. However, the co-precipitation of HsOrc2 and HsOrc6 was reproducible and was clearly dependent upon specific antibody. Significantly greater quantities of HsOrc6 co-immunoprecipitated with HsOrc3 (Fig. 4B), suggesting that the HsOrc3-HsOrc6 interaction may be stronger than the HsOrc2-HsOrc6 interaction. The HsOrc6 subunit stained weakly with silver even in the case of the HsOrc3-HsOrc6 immunoprecipitates, raising the possibility that not all HsOrc3 subunits were complexed with HsOrc6 subunits. However, estimates of the relative amounts of the two subunits from the intensity of staining are unlikely to be accurate given the large difference in size and composition between HsOrc6 and HsOrc3.

**Formation of Complexes Containing Recombinant HsOrc5**—Using the same general approach we next explored the requirements for assembly of HsOrc5 into the recombinant HsORC complex. As shown in Fig. 5, A and B, HsOrc5 did not efficiently form binary complexes with either HsOrc2 or HsOrc3. Thus, we tested various combinations of three subunits for complex formation. We observed three stable trimeric complexes: HsOrc2-HsOrc3-HsOrc5, HsOrc2-HsOrc4-HsOrc5, and HsOrc3-HsOrc4-HsOrc5. In all three instances, considerably more HsOrc5 was co-immunoprecipitated than when HsOrc5 was expressed with HsOrc2 or HsOrc3 alone. Complex formation by the combination of HsOrc2-HsOrc3-HsOrc5 was especially efficient, as the three subunits appeared to be present in roughly equal amounts in the immunoprecipitates (Fig. 5A). These data indicate that complex formation by HsOrc5 requires any two of the subunits HsOrc2, HsOrc3, and HsOrc4. There are two possible
explanations for these observations. HsOrc5 may interact weakly with several subunits, and the formation of stable complexes is a result of the additive effects of such interactions. Alternatively, binding of HsOrc5 may require conformational changes brought about by interactions of the other subunits. It should be noted that we were unable to directly test for a binary interaction between HsOrc5 and HsOrc4 because the corresponding antibodies are not suitable for immunoprecipitation studies. However, it is clear from the above data that HsOrc4 is not essential for assembly of HsOrc5 into a multi-subunit complex.

Assembly of Recombinant HsOrc1 into a Multi-Subunit Complex Requires HsOrc2, HsOrc3, HsOrc4, and HsOrc5—We extended our studies of HsORC complex formation to determine the requirements for the interactions of recombinant HsOrc1 with the other subunits. Fig. 6 shows some of the data that we obtained. Our initial experiments indicated that HsOrc1 did not form a binary complex with HsOrc2 (first panel, Fig. 6). Thus, we investigated the possibility that HsOrc1 might interact with various sub-complexes containing other HsORC subunits. Our data demonstrated that HsOrc1 did not co-immunoprecipitate with the HsOrc2-HsOrc3, HsOrc2-HsOrc3-HsOrc4, or HsOrc2-HsOrc3-HsOrc5 sub-complexes identified in the experiments described above (Fig. 6). Similar results were obtained with the combinations, HsOrc1-HsOrc2-HsOrc4, HsOrc1-HsOrc2-HsOrc5, HsOrc1-HsOrc2-HsOrc3-HsOrc4, and HsOrc1-HsOrc2-HsOrc4-HsOrc5 (data not shown). We only observed co-immunoprecipitation of HsOrc1 when...

DISCUSSION

Although previous studies have identified individual subunits of HsORC and probed some of the interactions among them, a multi-subunit ORC from human cells has not been reported (29, 36, 37). We have identified a HsORC from HeLa cells that contains the five HsORC subunits HsOrc1-HsOrc5. The complex is tightly associated with chromatin and requires elevated salt concentrations (~500 mM NaCl) to dissociate it. Our results indicate that the general architecture of HsORC is probably similar to that of ORCs described in yeast and other metazoans (10, 17–19). Previous difficulties in identifying HsORC are probably consequences of the lack of good immunological reagents and inefficient extraction conditions. The endogenous HsORC lacks detectable HsOrc6, and a fraction of the complexes may also lack HsOrc1. This finding may be due to the partial (HsOrc1) or complete (HsOrc6) dissociation of these subunits during preparation and extraction of chromatin, but there are other possibilities as well (see below). In addition to the identification of the endogenous complex, we have achieved reconstitution of HsORC by co-expression of recombinant subunits in the baculovirus expression system. The recombinant HsORC is a holo-complex containing all six subunits but otherwise closely resembles the endogenous complex. We have made use of the baculovirus expression system to define the complexes that can be formed by subsets of the recombi-
human ORC subunits. These experiments have provided insight into the organization of human ORC and provided a rationale for the observed heterogeneity of the complexes extracted from human cells.

Assembly of Recombinant human ORC—The protein-protein interactions that we have observed in our studies of sub-complexes of recombinant human ORC are summarized in Fig. 7. Due to the many combinatorial possibilities, our analysis, although extensive, is not exhaustive. Nevertheless, the data illuminate some important features of the architecture of human ORC. The strong binary interactions among HsOrc2, HsOrc3, and HsOrc4 suggest that these three subunits represent the core of the complex. Formation of stable complexes containing HsOrc5 required the presence of at least two of the core subunits, whereas formation of complexes containing HsOrc1 required the presence of the four subunits HsOrc2, HsOrc3, HsOrc4, and HsOrc5. These data strongly suggest that the assembly of human ORC is an ordered process, i.e. the association of HsOrc5 or HsOrc1 requires the prior formation of specific sub-complexes. As noted above, there are two possible mechanisms to account for the dependencies that we have observed. One possibility is that the association of each subunit (HsOrc5 or HsOrc1) requires the additive effects of relatively weak interactions with two or more subunits present in a specific sub-assembly. The second possibility is that the association of each subunit is mediated by binding sites that are only uncovered by conformational changes that occur during formation of a specific sub-assembly. Further work will be required to distinguish between these two possibilities.

HsOrc6—Several observations strongly suggest that HsOrc6 is a bona fide human ORC subunit even though it was not observed in the endogenous complex. In the baculovirus expression system we observed binary interactions of recombinant HsOrc6 with both HsOrc2 and HsOrc3. Moreover, HsOrc6 was present in a high molecular mass complex containing all of the other human ORC subunits. Our control experiments demonstrated that the presence of HsOrc6 in this holo-complex was due to specific interactions with the other subunits. Thus, the absence of HsOrc6 in the endogenous complex was somewhat puzzling. One plausible explanation is that HsOrc6 binds only weakly to the other subunits and therefore dissociates from the endogenous complex under our extraction conditions. We would attribute our ability to detect HsOrc6 in the recombinant complex to the fact that HsOrc6 is expressed at very high levels in co-infected insect cells, thus increasing the probability of complex formation by simple mass action. An alternative, although perhaps less likely, explanation is that the absence of HsOrc6 from the endogenous complex is a consequence of post-translational modifications or specific interactions with other chromatin proteins that do not occur in insect cells. It has been observed that HsOrc6 is constitutively phosphorylated in human cells, but the possible biological consequences of the modification are unknown (34). The functional role of Orc6 is not known in any system. In S. cerevisiae it has been demonstrated that Orc6 is not required for assembly of the other five ScORC subunits into a complex that binds specifically to origins of DNA replication (35). However, mutants lacking ScOrc6 are inviable, indicating that the subunit does mediate some essential cellular function (12).

HsOrc1—Our data suggest that HsOrc1 is consistently present in sub-stoichiometric amounts in endogenous human ORC extracted from HeLa chromatin. Thus, our extracts likely contain two forms of HsOrc1, a five-subunit complex containing HsOrc1 and a four-subunit complex lacking HsOrc1. Consistent with this observation, we recovered a significant fraction of HsOrc1 in the 100 mM NaCl chromatin extract, whereas most of the other human ORC subunits were recovered almost exclusively in the 500 mM NaCl chromatin extract. These findings indicate that not all of the cellular HsOrc1 is stably bound to the other subunits of human ORC. The reason for this behavior is not clear at this point, but one possible explanation is that the association of HsOrc1 with the other human ORC subunits is regulated during the cell cycle. It is known that the expression of HsOrc1, but not the other human ORC subunits, is under the control of the cell cycle-regulated transcription factor E2F (41). Moreover, two studies provide evidence that mammalian Orc1 is more easily dissociated from chromatin in M phase and perhaps early G (42, 43). This change in the properties of HsOrc1 is correlated with the hyperphosphorylation of the subunit that occurs during M phase, suggesting that the association of HsOrc1 with the other human ORC subunits may be regulated by post-translational modification (42). It has also been reported that human cells have two populations of HsOrc2 that differ in their association with HsOrc1 (37). One population of HsOrc2 can be extracted from chromatin at 250 mM NaCl and another at 450 mM NaCl. The majority of the latter population, but not the former, was shown to be associated with HsOrc1 in immunoprecipitation experiments. These results are consistent with our suggestion that HsOrc1 extracted from chromatin at 500 mM NaCl concentrations consists of two distinct complexes, one that contains HsOrc1 and the other which does not. It was also reported that the HsOrc1 dissociates from chromatin during S phase in HeLa cells and that it may be degraded at this time (37). If this result can be confirmed, it would provide a potentially attractive mechanism for preventing re-initiation of DNA replication within genomic segments that have already been replicated. However, it should be noted that several other reports have concluded that the total amount of cellular HsOrc1 does not significantly change during the cell cycle (42, 44). Thus, whereas it is not yet clear whether regulation of the association of HsOrc1 with the other HsORC subunits plays a role in controlling the initiation of DNA replication in human cells, several observations, including our finding that HsOrc1 can exist in at least two types of complexes, certainly raise this possibility (37, 43).

HsORC—Our data indicate that HsORC is generally similar to the previously purified S. cerevisiae, Drosophila, and Xenopus ORCs (10, 18, 19). However, there may be some subtle differences, including the propensity for dissociation of HsOrc1 and HsOrc6. Whether or not these apparent differences are simply due to differences in methods of extraction and characterization or whether they reflect some differences in function or regulation remains to be determined. To date only S. cerevisiae ORC has been shown to exhibit high affinity
binding to specific sequence elements within origins of DNA replication (8, 9). A small fraction of Drosophila ORC purified from embryos shows preferential association to a region of DNA implicated in gene amplification, but the basis for the specificity of this association has not yet been defined (27). Xenopus ORC appears to be capable of interacting directly or indirectly with many unrelated DNA sequences (45). Thus, it will be of great interest to define the interactions of HsORC with DNA. Such studies will be facilitated by the identification of HsORC complexes reported here.

Acknowledgments—We thank Drs. B. Stillman, A. Dutta, and J. Hurwitz for providing plasmids. We also thank Drs. L. Chretien, M. Davenport, M. Frattini, C. Houchens, and M. Taipale for helpful comments on the manuscript and all of the members of the Kelly lab for thoughtful discussion.

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