A structural role for ATP in the formation and stability of the human origin recognition complex

Anand Ranjan and Manfred Gossen*

Max Delbrück Center for Molecular Medicine, Robert-Rössle-Strasse 10, 13125 Berlin, Germany

Edited by Charles M. Radding, Yale University School of Medicine, New Haven, CT, and approved February 10, 2006 (received for review November 29, 2005)

The locally restricted recruitment of the multisubunit origin recognition complex (ORC) to eukaryotic chromosomes defines the position of origins of DNA replication. In budding yeast and metazoans the DNA binding activity of ORC is stimulated by ATP and requires an AAA+ type nucleotide binding domain in the largest subunit. Little else is known about the mechanisms behind the ATP requirement for ORC in its initiator function and, specifically, the relevance of nucleotide binding domains present on other subunits. Here we show that ATP is required for specific subunit interactions in the human ORC, with the Orc4 subunit playing a critical role in this dynamic process. ATP is essential for the maintenance of ORC integrity and facilitates complex formation. Thus, besides its previously identified role in DNA binding, ATP serves also as a structural cofactor for human ORC.

AAA+ protein | DNA replication | prereplicative complex

The origin recognition complex (ORC) was initially characterized in budding yeast, where its subunits ScOrc1–6 form a heterohexameric protein assembly that binds to origins of DNA replication. Despite a considerable variation in the architecture of the six elements constituting origins in Saccharomyces cerevisiae, one sequence element, the 11-bp autonomous replicating sequence consensus sequence (ACS), is found in all these origin sequences. The replicator ACS serves as the specific binding site for the initiator protein S. cerevisiae ORC (ScORC) (1). Subsequent to ORC’s binding to the origin, other replicating factors are recruited in a highly coordinate manner. This process results in the formation of a prereplicative complex that renders the replication origin initiation competent (2). Therefore, the binding of ScORC to the ACS is the first defined cis event in preparing any given origin for initiation of DNA replication. In vitro studies have shown that the interaction of ScORC with its cognate binding sequence is ATP-dependent. Two subunits, ScOrc1p and ScOrc5p, contain consensus ATP binding and hydrolysis motifs that, together with additional structural features, place them in the AAA+ family of NTPases (3). ATP binding by ScOrc1, but not ATP hydrolysis, was shown to be essential for the sequence-specific ScORC/ACS interaction in vitro (4). When analyzed in vivo, ScOrc1 defective for ATP binding or hydrolysis is not able to support cell proliferation (4). However, with respect to ScOrc1’s ATPase activity and its coordination by the different subunits involved, a mechanistic picture of its role in prereplicative complex formation is just emerging (5).

In many other eukaryotic species ORC genes homologous to those from S. cerevisiae have been identified (2). Where analyzed biochemically, these ORC subunits form multiprotein complexes, reminiscent of the findings for ScORC. Whereas ScORC appears to be a tight protein complex of six subunits in stoichiometric amounts, the composition of metazoan ORC is more variable. Regarding its uniformity as a heterohexamer, Drosophila ORC resembles the ScORC (6). In contrast, the composition and subunit dynamics of other metazoan ORCs seem less static. In comparison to the budding yeast paradigm, Orc1 and Orc6 subunits often show deviating patterns in their interactions with or localization apart from the ORC core complex, Orc2–5 (7–10). It is unclear to what extent these differences reflect distinct mechanisms of origin selection, replication initiation, and proliferation control. Especially the interaction of mammalian Orc1 to the ORC core assembly in the course of S-phase progression is a matter of considerable debate (10). Furthermore, the association of Orc6 with Orc1–5 remains to be firmly established in mammalian cells (11).

The function of metazoan ORC in replication initiation is also dependent on ATP, best analyzed in Drosophila. There, ATP hydrolysis by Drosophila melanogaster (DmORC) and its ability to support in vitro DNA replication require an intact ATP binding and hydrolysis site in DmOrc1, but not in DmOrc4 or DmOrc5 (12). For recombinant human ORC, an ATP dependence for sequence-unspecific DNA binding has also been reported (8).

There are numerous examples of a role for Orc proteins in cellular processes other than the initiation of DNA replication, both in yeast (13, 14) and in metazoans (15–19). Thus, addressing the contribution of individual ATP binding events in the context of ORC might ultimately also require an experimental readout unrelated to ORC’s role as an initiator protein complex.

Here we report a detailed analysis of ORC subunit interactions. The stability of recombinant human ORC depended on the sustained presence of ATP. One subunit, HsOrc4, was critical for mediating these nucleotide-dependent protein interactions. Finally, we could show that the structural role of ATP is not limited to the maintenance of ORC’s integrity, but ATP also serves as a cofactor for complex formation.

Results

Purification of Functional Human ORC. Recombinant Homo sapiens ORC (HsORC) was purified from SP9 cells after baculovirus-mediated coexpression of individual ORC subunits. A modified tandem affinity purification (TAP) tag approach (20) was used for affinity purification of the complex, with the tag cassette comprised of a polyhistidine moiety, a triple hemagglutinin motif, and a tobacco etch virus (TEV) protease site allowing for the enzymatic removal of the tag cassette. This tag was fused either to the N-terminal end of HsOrc2 or the C-terminal end of HsOrc1, resulting in HsOrc2N and HsOrc1C, respectively (Fig. 7 and Supporting Materials and Methods, which are published as supporting information on the PNAS web site). HsORC was affinity-purified from the nuclear extract of infected SP9 cells by using anti-hemagglutinin beads recognizing the tagged HsOrc2 subunit. The protein complex was released from the matrix by TEV protease digestion. An example for such a HsORC preparation is shown in Fig. 1a. Using a Xenopus in vitro DNA replication assay (21) we showed that the recombinant HsORC

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: ORC, origin recognition complex; HsORC, Homo sapiens ORC; ScORC, Saccharomyces cerevisiae ORC; DmORC, Drosophila melanogaster ORC; TEV, tobacco etch virus.

*To whom correspondence should be addressed. E-mail: mgossen@mdc-berlin.de.

© 2006 by The National Academy of Sciences of the USA
purified according to our experimental procedures could efficiently restore DNA replication activity in egg extracts depleted of endogenous ORC (Fig. 1b). Thus, our recombinant HS Orc could bind to sperm chromatin and recruit other initiation factors present in Xenopus egg extracts to form a functional prereplicative complex.

The abundance of HS Orc1 and HS Orc6 subunits was mostly substoichiometric in these HS Orc preparations. Especially the amount of HS Orc6 copurifying with the HS Orc1–5 complex varied greatly between different preparations, without any indications to the experimental parameters causing these fluctuations. A one-step affinity-purified HS Orc from a preparation containing substantial quantities of HS Orc6 (Fig. 1a) was further resolved by centrifugation through a glycerol gradient. HS Orc1–5 moved as a single entity in the gradient, characterized by a sedimentation rate similar to catalase as found for ORC from other species (1, 6, 22) (Fig. 1c Upper). In contrast, HS Orc6 was exclusively detected in the low-molecular-weight fractions, separated from the other HS Orc subunits. The identity of HS Orc6 protein was confirmed by immunoblot analysis of the glycerol gradient fractions probed with an anti-HS Orc6 antiserum (Fig. 1c Lower).

Architecture of Human ORC. A systematic approach was taken to identify the protein–protein interactions within the HS Orc complex. To analyze the contribution of individual ORC subunits to the formation and integrity of the holocomplex, in each of the missing-subunit assays shown in Fig. 2 one of the respective baculoviruses was left out during the infection of Sf9 cells as indicated. The proteins interacting with the tagged ORC subunit were affinity-purified from the infected Sf9 cells by means of tags on Orc1 or Orc2 and visualized by Coomassie staining of protein gels. Using HS Orc2N (Fig. 2a Left) we showed that dimeric HS Orc2,3 formed independent of, and was a prerequisite for, additional HS Orc subunit interactions (Fig. 2a, lanes 1 and 4–6; see also ref. 9). Note that in the absence of HS Orc3 even the tagged HS Orc2 was not recovered because of its poor solubility when not in complex with other proteins (data not shown). HS Orc2,3 formed a trimer with HS Orc5 but not HS Orc4 (Fig. 2a, compare lanes 4 and 5). However, HS Orc4 could interact with an HS Orc2,3,5 subcomplex (Fig. 2a), forming a “core complex” as discussed previously (9). The interaction of HS Orc1 with HS Orc2,3 is absolutely dependent on HS Orc4 and HS Orc5 (Fig. 2, lane 6; see also lanes 12 and 13). In an analysis based on HS Orc1C (Fig. 2a Right) we identified a prominent trimeric subcomplex. HS Orc1 interacted with HS Orc4,5 (Fig. 2a, lanes 8 and 9) but with neither of these proteins alone (Fig. 2a, lanes 10 and 11). The mutual dependence between HS Orc4 and HS Orc5 for this interaction to occur sets it mechanistically apart from

Fig. 1. Integrity and functionality of recombinant human ORC. Sf9 cells were coinfected with the six different baculoviruses encoding the human ORC subunits. (a) A silver-stained protein gel showing HS Orc affinity-purified from nuclear extracts of infected insect cells. In this experiment the hemagglutinin epitope tag was on the N terminus of HS Orc2. The complex was released from the affinity matrix by TEV protease digestion. In this particular preparation both HS Orc1 and HS Orc6 appear to be present in roughly stoichiometric amounts when judged by the intensity of the stained protein bands. The TEV protease bands are marked by asterisks. (b) Xenopus egg extracts either mock-depleted or depleted of Xenopus laevis ORC were used to verify the activity of the recombinant HS Orc in DNA replication. Quantification in different experiments shown was by measuring the incorporation of radiolabeled dNTP into sperm chromatin from three independent reactions done in parallel, with error bars indicating the standard deviation. Purified Xenopus laevis ORC as well as recombinant HS Orc support DNA replication as indicated. (c Upper) A silver-stained protein gel showing the comigration of HS Orc subunits 1–5 when affinity-purified HS Orc (a) was further resolved by glycerol gradient centrifugation. Gradient fraction numbers and the peak positions of size markers (lgG, 150 kDa; C, catalase, 232 kDa; A, apoferritin, 440 kDa) resolved on a parallel glycerol gradient are indicated at the top. (c Lower) An immunoblot for HS Orc6, which migrated in the low-molecular-weight fractions.

Fig. 2. Subunit interactions within the human ORC. (a) ORC subcomplex formation as assayed by a missing-subunit assay. Sf9 cells were infected with a mixture of Orc-encoding baculoviruses except those indicated above the lanes. Affinity purification was by tags fused to Orc2 (Left) or Orc1 (Right). Proteins retained on the affinity matrix were eluted by TEV protease digestion. The eluate was electrophoresed on a protein gel and stained with Coomassie. The positions of the individual ORC subunits as well as of marker proteins are indicated. The TEV protease bands are marked by asterisks. (b) A model of the interaction map emerging from the missing-subunit assay shown in a.
their interaction with HsOrc2,3. It should be noted that the interaction of HsOrc1 with HsOrc4 and HsOrc5 is weaker than the interaction of HsOrc1 with the HsOrc2–5 complex. Interaction of HsOrc2,3 with HsOrc1 appeared to be indirect, requiring HsOrc4,5, in agreement with the reciprocal experiment using tagged HsOrc2 (Fig. 2a, compare lanes 8–11 with lanes 12 and 13). When detectable, HsOrc6 in the complex was underrepresented, and interpreting its presence in some of the subcomplexes under the chosen conditions requires further experimental confirmation. The map of subunit interactions emerging from these experiments is depicted in Fig. 2b.

**Fig. 2.** ATP dependence of HsORC integrity. (a) SF9 cells were coinfectd with the six ORC-encoding baculoviruses. The subsequent preparation of nuclear extracts, affinity purification, and washing steps were done in parallel with or without 1 mM ATP. A Coomassie-stained protein gel with these two HsORC preparations is shown. Note that HsOrc2N and HsOrc3 do not resolve because purified proteins were not proteolytically removed from the affinity matrix. (b and c) After proteolytic cleavage by TEV, the different HsORC preparations shown in a were subjected to glycerol gradient centrifugation as described for Fig. 1c. Although the recombinant complex purified in the presence of ATP migrates as a single entity (b), the respective complex purified in the absence of ATP disintegrates (c). ATP was omitted from the glycerol gradient medium itself, because the presence or absence of ATP during the affinity purification was the decisive factor for complex stability.

**Effect of ATP on the Stability of ORC.** With the notable exception of *Schizosaccharomyces pombe* (23), biochemical analysis of ORC from different species revealed an increasing affinity of ORC to DNA in the presence of ATP, dependent on ATP binding sites in some, but not all, of the subunits (4, 12). However, there appear to be species-specific differences in the nucleotide-dependent increment of this gain in affinity, which might be related to different modes of origin selection mediated by ORC. To analyze in more detail possible mechanisms behind the influence of nucleotide binding on ORC function, HsORC was affinity-purified in parallel in the absence and presence of 1 mM ATP. Note that in the HsORC purification scheme described above 1 mM ATP was added during these steps. As shown in Fig. 3a, ATP did not affect the appearance of HsORC when judged by protein gel analysis. However, when these different preparations of recombinant protein were resolved by glycerol gradient centrifugation (Fig. 3b and c), HsORC affinity-purified in the absence of ATP disintegrated. The HsOrc1 and HsOrc4 subunits were lost from the HsOrc2,3,5 subcomplex during sedimentation through the glycerol gradient. The HsOrc4 subunit was detected in the low-molecular-weight fractions, whereas the majority of HsOrc1 could not be recovered from the gradient. Because HsOrc1 was not found in the pellet fraction (data not shown) we suspect it to be degraded during sedimentation upon loss of subunit contacts within HsORC purified in the absence of ATP. A fraction of HsOrc4 was still associated with HsOrc2,3,5. Inclusion of ADP during purification did not stabilize the HsORC, whereas its integrity upon inclusion of ATP was indistinguishable from the results obtained with hydrolyzable ATP (data not shown). These results indicate that the continuous presence of ATP is critical for the stability of the multi-protein complex HsORC.

**ATP-Dependent Protein–Protein Interactions in ORC Subcomplexes.** Based on the identification of critical HsORC subunit interactions resulting in the formation of stable subcomplexes we were able to directly address whether the observed dependence of HsORC integrity on ATP could be attributed to the formation or stability of individual subunit interactions. As in the missing subunit assay, affinity purification was through the HsOrc1C or HsOrc2N subunit. The experimental scheme was modified by performing the matrix binding, washing, and elution steps either in the absence or the presence of ATP (Fig. 4). The formation of two subcomplexes was strictly ATP-dependent. The interaction of HsOrc4 with HsOrc2,3,5 and the interaction of HsOrc1 with HsOrc4,5 were found to be ATP-dependent. For the interaction of HsOrc2,3,5 with HsOrc4 a titration experiment showed that ATP concentrations <100 μM (i.e., well below the intracellular concentration) destabilized the complex (Fig. 8, which is published as supporting information on the PNAS web site). The same concentration dependence was found with ATP$\gamma$S. Thus, nucleotide hydrolysis is not required for the ATP-dependent subunit interactions.

**Fig. 4.** Nucleotide effects on ORC subcomplexes. Nuclear extracts of SF9 cells coinfectd with baculoviruses coding for HsOrc2, HsOrc3, HsOrc4, and HsOrc5 (a) or HsOrc1, HsOrc4, and HsOrc5 (b) were purified in parallel in the absence and presence of ATP. Material bound to the affinity matrix was either directly resolved by SDS/PAGE (b) or eluted by TEV protease digestion before SDS/PAGE analysis (a), with subsequent Coomassie staining.
interaction between HsOrc5 and HsOrc1 was detected. Thus, the
as the cause of this effect. In line with earlier results no binary
pressed in the Sf9 cells, ruling out differences in expression levels
lanes clearly show that HsOrc4A protein was efficiently ex-
complete than that of wild-type HsORC in the absence of ATP
predicted from the previous purifications with or without ATP,
purified from infected Sf9 cells in the presence of ATP. As
in HsOrc4A. The respective complex (HsORC4A) was affinity-
the lysine to alanine in the conserved GKT sequence, resulting
primary structure HsOrc4 is one of the three human ORC
subunits showing a strongly conserved ATP binding Walker A
motif (24). We reasoned that ATP binding by this protein motif
is responsible for the observed nucleotide dependence of HsOrc4 integration into the holocomplex and the subcomplexes.
The ATP binding Walker A motif was disrupted by substituting
the lysine to alanine in the conserved GKT sequence, resulting
in HsOrc4A. The respective complex (HsORC4A) was affinity-
purified from infected Sf9 cells in the presence of ATP. As
predicted from the previous purifications with or without ATP,
the TEV eluate was indistinguishable from wild-type HsORC
purified in parallel (data not shown). Upon further resolution on
a glycerol gradient the mutant complex fell apart, resulting in a
pattern of disintegration highly reminiscent but even more
complete than that of wild-type HsORC in the absence of ATP
(Fig. 5a; see Fig. 2 for comparison). The effect of the HsOrc4A
mutation on HsORC subunit interactions was further analyzed
by subcomplex analysis. Of the two HsORC subcomplex
interactions we identified before to be ATP-dependent, both involved
HsOrc4. The interaction experiments were repeated with the
mutated HsOrc4A subunit. ATP (1 mM) was added to all of the
buffers. Unlike the wild-type HsOrc4, HsOrc4A interacted with
neither HsOrc2,3,5 (Fig. 5b) nor HsOrc1 (Fig. 5c). The input
lanes clearly show that HsOrc4A protein was efficiently ex-
pressed in the Sf9 cells, ruling out differences in expression levels
as the cause of this effect. In line with earlier results no binary
interaction between HsOrc5 and HsOrc1 was detected. Thus, the

ATP is Required for the Formation of the ORC. Next we tried to
address the question of whether the observed ATP requirement
is limited to the stability of HsORC once the complex is formed
or whether there is also a nucleotide requirement for complex
formation itself. In the absence of a true in vitro reconstitution
system, ORC and its subcomplexes are formed in a cellular
environment, i.e., in the presence of ATP. To perform this
analysis we affinity-purified HsOrc2,3,5 in the absence of ATP,
which is not required to maintain stability of this subcomplex.
HsOrc4/-A turned out to be largely soluble in the cytoplasmic
fraction of infected Sf9 cells (data not shown). Without further
purification these cytoplasmic fractions were dialyzed to remove
ATP from the extract. Combining the HsOrc2,3,5 and the HsOrc4
fraction with the HsOrc2,3,5 subcomplex, without proteolytic removal from the matrix, was
incubated with the HsOr4A-containing cytoplasmic extract for 1 h at 4°C in the
absence or presence of ATP. The pull-down lanes show matrix-bound proteins
after combining the HsOr2,3,5 and the HsOr4A fractions as indicated
(lanes 4–7). Proteins were resolved on an 8% polyacrylamide protein gel and
stained with Coomassie.

Discussion

HsORC Subunit Interactions. Among the different species where it
has been identified, the eukaryotic initiator protein ORC is best
characterized in S. cerevisiae, in both genetic and molecular terms.
One of the hallmarks of ScORC is its binding to specific DNA
sequences located within origins of bidirectional DNA replication
(1). These DNA binding characteristics are shared by the hetero-
hexameric ScOrcl–6 as biochemically purified from the yeast as well
as by a recombinant ScOrcl–5 (25). Otherwise, there are few
indications for a further variety of ScORC subassemblies participat-
ing in replication initiation. In contrast, for metazoan organisms
with their diverging modes of origin selection there is mounting
evidence for an increased heterogeneity of ORC composition. One
of these additional features is the dynamic behavior of the HsOrcl
subunit, which is widely seen as one of the mechanisms preventing
unscheduled replication of DNA (10). The transitory in vivo
association of Orc1 with the other ORC subunits (and thus with

Fig. 5. Instability of HsORC4A in the presence of ATP. (a) HsORC4A was
prepared in the presence of ATP as described before for HsORC. The complex
disintegrated upon glycerol gradient centrifugation, with HsOr4A4A moving
exclusively in the low-molecular-weight fractions. (b and c) Subcomplex for-
formation was analyzed in the presence of ATP. The left part of each panel shows
the input lanes as indicated, and the right lanes show the respective TEV
eluates after affinity purification. Baculoviruses encoding either HsOr4 or
HsOr4A were used in parallel. The previously defined subcomplexes HsOr2–5
(b) and HsOr1,4,5 (c) are shown.

Fig. 6. ATP and the formation of HsORC. The input lanes show a lysate of Sf9
cells infected with HsOr2,3,5 (lane 1) and the cytoplasmic extracts containing
HsOr4 (lane 2) or HsOr4A (lane 3). The cytoplasmic fractions were dialyzed
to remove ATP. The HsORC subcomplex in the nuclear fraction was bound to
the affinity matrix and purified under ATP-free conditions as described. This
HsOr2,3,5 subcomplex, without proteolytic removal from the matrix, was
incubated with the HsOr4A-containing cytoplasmic extract for 1 h at 4°C in the
absence or presence of ATP. The pull-down lanes show matrix-bound proteins
after combining the HsOr2,3,5 and the HsOr4A fractions as indicated
(lanes 4–7). Proteins were resolved on an 8% polyacrylamide protein gel and
stained with Coomassie.
growth retardation (13, 28), which might be explained by an AAA subunits encoding consensus ATP binding and ATP hydrolysis allowed us to define a HsOrc1,4,5 complex, i.e., the human ORC to consensus ATP binding maintenance proteins (5). ScOrc4 itself shows only a weak match with DNA (4). ScOrc4-assisted hydrolysis of this ScOrc1-bound function in replication initiation by facilitating the interaction binding to the ScOrc1 subunit has been correlated with a distinct of how individual ATP binding of them contacting ATP (27), providing a plausible explanation is often located at the interface between two subunits, with both binary or ternary interaction studies vs. the missing-subunit interactions caused by the high expression levels of the subunits. To determine whether under physiological conditions the human Orc6 protein is an integral part of HsORC, either on or off chromatin, will require further experimental scrutiny. It is well possible that the conditions in our experiments using recombinant HsORC are not compatible with HsOrc6’s association in the holocomplex, despite occurring in vivo. Alternatively, some functions of human ORC, like its eponymous activity of origin recognition, may be executed by a heterotetramer.

Based on the results of a missing-subunit assay (25) we present a model for interactions between individual ORC subunits. Similar attempts have been made to analyze the subunit interactions of human ORC by coexpression of various subunit combinations (7, 9). These studies place HsOrc6 and HsOrc1 outside the central interaction map, in agreement with the mostly substoichiometric presence of these subunits in our subcomplex analysis. There is also an agreement about the prominent HsOrc2,3 interaction. Different conclusions about individual subunit interactions could be because of the different tags and/or antibody reagents used and, in particular, the mainly binary or ternary interaction studies vs. the missing-subunit assay.

A Structural Role of ATP for HsORC. Our experimental approach allowed us to define a HsOrc1,4,5 complex, i.e., the human ORC subunits encoding consensus ATP binding and ATP hydrolysis motifs, which, among other structural features, place them in the AAA+ family of proteins (3). However, two recent in silico analyses using different structure prediction algorithms suggest that also Orc2 and Orc3 share these structural features (M. Botchan, personal communication, and ref. 26). In this scenario Orc1–5 would form a heterooligomeric assembly of all AAG+ or AAG+-like proteins commonly found in other multisubunit protein complexes involved in replication initiation (27). One hallmark of this protein family are the conformational changes triggered by ATP binding and/or hydrolysis. Structural analysis revealed that in AAG+ oligomers the nucleotide binding site is often located at the interface between two subunits, with both of them contacting ATP (27), providing a plausible explanation of how individual ATP binding/hydrolysis events result in structural alteration of a holocomplex. For ScORC only the ATP binding to the ScOrc1 subunit has been correlated with a distinct function in replication initiation by facilitating the interaction with DNA (4). ScOrc4-assisted hydrolysis of this ScOrc1-bound ATP is required for the reiterated loading of minichromosome maintenance proteins (5). ScOrc4 itself shows only a weak match to consensus ATP binding/hydrolysis motifs, and respective mutations do not result in discernable phenotypes either in vitro or in vivo (4). In budding yeast a mutation of the consensus Walker A motif of ScOrc5 resulted in a temperature-dependent growth retardation (13, 28), which might be explained by an increased genomic instability in such mutants (14). Biochemical analysis of this mutant complex, however, did not show a major effect on ScORC/DNA interactions. Thus, the picture emerging from the analysis of ScORC is largely in line with that of Drosophila, where only ATP-defective DmOrc1, but not DmOrc4 or DmOrc5, showed an effect on ORC’s ATPase activity and its function in an in vitro replication system (12). In contrast, all human Walker A box mutant ORCs fail to stably associate with chromatin (22). Thus, in the various organisms analyzed specific ATP interactions of ORC appear to have different functional consequences, possibly contributing to the species-specific differences in its part in origin selection and replication control.

To gain a better understanding of ORC’s functional dependence on nucleotide binding we extended our initial subunit interaction studies to a comparative analysis with or without ATP. Complex formation in our experiments is intracellular, i.e., in the presence of ATP. We therefore decided to isolate the recombinant HsORC in the absence and presence of ATP during the affinity purification and washing steps. In this situation the procedure we followed did not result in any appreciable difference in the integrity of the holocomplex upon SDS/PAGE analysis. Upon glycerol gradient centrifugation, however, ORC purified in the absence of ATP partially disintegrated, pointing to structural differences caused by the removal of ATP. Most prominently, the HsOrc4 subunit dissociated from the core complex. The effect is not quantitative, which might be because of persistent nucleotide binding as prolonged washing aggravated the loss of HsOrc4 (data not shown). Results obtained after the inclusion of ATP-binding-deficient recombinant HsOrc4A in the protocol point in the same direction. The mutant subunit participates in initial complex formation when all subunits are overexpressed (see also ref. 22), showing that the alterations in the amino acid sequence of the Walker A motif did not cause gross changes in protein conformation. With respect to the ATP dependence of subunit interactions shown here a perspicuous explanation for ORC formation under these conditions is the masking of such defects upon overexpression, as previously discussed for the formation of ScORC5-A (29). However, HsOrc4A lost the ATP-binding-deficient subunit completely upon further purification; this result is similar to but more quantitative than our results obtained by purification of wild-type HsORC in the absence of ATP. All of these experiments indicated structural changes in the HsORC in the absence of ATP binding, which we tried to detail in subcomplex analyses. Indeed, by assaying the previously defined HsOrc1,4,5 and HsOrc2,3,4,5 subcomplexes we were able to demonstrate a striking effect of ATP on specific subunit interactions. ATP was required to maintain the interaction of HsOrc4 with the stable HsOrc2,3,5 subcomplex and that of HsOrc4,5 with HsOrc1. The emerging picture in which the HsOrc4 subunit plays a critical role in mediating the ATP effect on subunit interactions was confirmed by using the ATP binding mutant of HsOrc4.

Structural Dynamics of HsORC. Mechanistically, the structural changes of HsORC upon loss of ATP as assayed by the stability of the complex could reflect steps involved in replication initiation downstream of ATP-dependent DNA binding and subsequent nucleotide hydrolysis (5). The interpretation of such an event completely upon further purification; this result is similar to but depend on whether ADP dissociates rapidly from an initiator protein (28) or whether it is stably bound (30), potentially requiring the assistance of exchange factors for reloading of ATP. Another conceivable nucleotide-dependent control mechanism over ORC function is the formation of the protein complex itself. Bipartite ATP interaction domains constitute the interface between AAA+-type oligomeric initiator proteins (3), compatible with a role for the nucleotide in the oligomer-
incubated for 5 min. The nuclei were pelleted by centrifugation at 20,000 \( \times \) g for 20 min. The nuclear salt extract was used for HsORC affinity purification experiments. A typical experiment involved infection of 50 ml of SF9 cells. The cell pellet was lysed in 8 ml of lysis buffer. Isolated nuclei were extracted in 2 ml of lysis buffer with 300 mM KCl.

A total of 25 \( \mu \)l of 50% anti-hemagglutinin affinity matrix (rat monoclonal 3F10; Roche) was added to 1 ml of nuclear extract and incubated overnight at 4°C with overhead rotation. The antibody binding was done in siliconized 1.5-ml Eppendorf tubes. The beads were washed five times with a total of 5 ml of ice-cold lysis buffer. The proteins bound to the affinity matrix were eluted by proteolytically cleaving the tag on HsORC using TEV protease enzyme (Invitrogen). For TEV digestions, 1X TEV protease enzyme buffer and 10 units of TEV protease enzyme were added to the washed beads in a total volume of 100 \( \mu \)l. The beads were incubated at 16°C for 2 h for TEV digestion. For SDS/PAGE analysis 12% polyacrylamide protein gels were used unless indicated otherwise. Either the eluate was collected after centrifugation or the proteolysis reaction was loaded directly on the gel. Where indicated, the affinity-purified recombinant HsORC was subjected to a 15–28% glycerol gradient centrifugation in 20 mM Hepes, pH 7.6/150 mM NaCl/1 mM MgCl\(_2\)/0.5 mM EDTA/1 mM DTT. Ultra centrifugation was carried out on a SW 60 Ti rotor at 42,500 rpm for 16 h at 2°C by using a L-70 Ultracentrifuge from Beckman. Fractions (200 \( \mu \)l) were collected from the top, and aliquots were resolved by SDS/PAGE.

**Materials and Methods**

**Purification of Recombinant HsORC**

SF9 cells were infected simultaneously with the six baculoviruses coding for the different ORC subunits. One of the ORC subunits, either Orc1 or Orc2, was always tagged to facilitate affinity purification of the complex. The baculovirus-infected SF9 cells were kept at 27°C, and the cells were harvested 60 h after infection. Harvested cells were washed twice in ice-cold PBS. Cells were then either lysed immediately or snap-frozen in freezing buffer (PBS/2 mM MgCl\(_2\)/10% glycerol). For lysis, ice-cold lysis buffer (PBS/2 mM MgCl\(_2\)/0.1% Nonidet P-40/10% glycerol/1 mM PMSF) was added to the cells. This lysis buffer was supplemented with 1 mM ATP in all experiments unless stated otherwise in the text. All further steps were done at 4°C. Cells were resuspended and incubated for 5 min. The nuclei were pelleted by centrifugation at 1,500 \( \times \) g for 4 min. The nuclei pellet was washed once in lysis buffer and salt-extracted in lysis buffer supplemented with 300 mM KCl for 1 h. The soluble proteins were collected by centrifugation at 20,000 \( \times \) g for 20 min. The nuclear salt extract was used for HsORC affinity purification experiments. A typical experiment involved infection of 50 ml of SF9 cells. The cell pellet was lysed in 8 ml of lysis buffer. Isolated nuclei were extracted in 2 ml of lysis buffer with 300 mM KCl.

A total of 25 \( \mu \)l of 50% anti-hemagglutinin affinity matrix (rat monoclonal 3F10; Roche) was added to 1 ml of nuclear extract and incubated overnight at 4°C with overhead rotation. The antibody binding was done in siliconized 1.5-ml Eppendorf tubes. The beads were washed five times with a total of 5 ml of ice-cold lysis buffer. The proteins bound to the affinity matrix were eluted by proteolytically cleaving the tag on HsORC using TEV protease enzyme (Invitrogen). For TEV digestions, 1X TEV protease enzyme buffer and 10 units of TEV protease enzyme were added to the washed beads in a total volume of 100 \( \mu \)l. The beads were incubated at 16°C for 2 h for TEV digestion. For SDS/PAGE analysis 12% polyacrylamide protein gels were used unless indicated otherwise. Either the eluate was collected after centrifugation or the proteolysis reaction was loaded directly on the gel. Where indicated, the affinity-purified recombinant HsORC was subjected to a 15–28% glycerol gradient centrifugation in 20 mM Hepes, pH 7.6/150 mM NaCl/1 mM MgCl\(_2\)/0.5 mM EDTA/1 mM DTT. Ultra centrifugation was carried out on a SW 60 Ti rotor at 42,500 rpm for 16 h at 2°C by using a L-70 Ultracentrifuge from Beckman. Fractions (200 \( \mu \)l) were collected from the top, and aliquots were resolved by SDS/PAGE.

**Missing-Subunit Assay.** To study the contribution of individual HsORC subunits to the architecture of the complex, an assay was devised where one subunit at a time was left out during the infection of SF9 cells. A comparable approach was previously used to analyze the DNA binding properties of ScORC subassemblies (25). Affinity purification was by means of HisOrc1C or HisOrc2N. Complex purification was done as described above.

We thank Anna Woodward and Julian Blow for their generous help with the *Xenopus* experiments, which were also facilitated by a European Molecular Biology Organization Short-Term Fellowship (to A.R.). Mike Botchan is acknowledged for sharing unpublished observations with us. This work was supported by Deutsche Forschungsgemeinschaft Grant Go 628/3 (to M.G.).