The Orc4p and Orc5p Subunits of the Xenopus and Human Origin Recognition Complex Are Related to Orc1p and Cdc6p*

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The location of origins of DNA replication within the Saccharomyces cerevisiae genome is primarily determined by the origin recognition complex (ORC) interacting with specific DNA sequences. The analogous situation in vertebrate cells is far less clear, although ORC subunits have been identified in several vertebrate organisms including Xenopus laevis. Monoclonal antibodies were raised against Xenopus Orc1p and used for single-step immunoaffinity purification of the entire ORC from an egg extract. Six polypeptides (~110, 68, 64, 48, 43, and 27 kDa) copurified with Xenopus Orc1p. Protein sequencing also showed the 64-kDa protein to be the previously identified Xenopus Orc2p. Microsequencing of the 43- and 48-kDa proteins that copurified with Orc1p and Orc2p led to their identification as the Orc4p and Orc5p subunits, respectively. Peptide sequences from the 43-kDa protein also allowed the isolation of cDNAs encoding the Xenopus, mouse, and human ORC subunits. Human ORC5 was also cloned; its sequence displayed extensive homology to both Drosophila and yeast ORC5. Surprisingly, comparison of the amino acid sequences of Orc1p, Orc4p, and Orc5p suggests that they are structurally related to each other and to the replication initiation protein, Cdc6p. Finally, we present the sequence of the putative Xenopus and human Orc3p.

The initiation of DNA replication in eukaryotes is tightly regulated such that all the DNA is replicated precisely once per cell cycle from a number of discrete replication origins (Refs. 1–3; for reviews, see Refs. 4 and 5). The nature of this regulation, however, is not well understood as only a small fraction of proteins involved in this process have been identified. Moreover, with the exception of budding yeast, the DNA sequences that define the origin of DNA replication on chromosomes remain an almost complete mystery in higher eukaryotes.

Present knowledge of the control over initiation of DNA replication in an eukaryotic cell is largely based on experiments done in the yeast Saccharomyces cerevisiae. In this yeast, DNA replication starts from well defined origins, known as the autonomously replicating sequences (6). An essential, bipartite DNA sequence in all S. cerevisiae replication origins is recognized by the six-subunit origin recognition complex (ORC), 1 which is itself essential for initiation of DNA replication (7–20). The ORC also functions in the control of gene silencing, a function that is, at least partially, separable from the functions in initiation of DNA replication (9, 10, 15, 21–23). Proteins homologous to individual subunits of the S. cerevisiae ORC have been identified recently in other yeast species and from animal and plant cells. Orc1p and Orc2p were found in Schizosaccharomyces pombe, Drosophila, Xenopus, and humans, and Orc5p has been identified in Drosophila (24–30). More recently, the human Orc4p has been identified and shown to co-immunoprecipitate with human Orc2p (31). Immuno-depletion of ORC inhibited initiation of DNA replication in a Xenopus egg extract, suggesting that ORC is essential for initiation of DNA replication in higher eukaryotes (28, 29, 32).

The nature of the DNA sequence specificity for origin function has not been resolved in species other than the budding yeast, but we imagine that characterization of ORC and its interaction with DNA will help clarify this situation. In pursuance of this goal, we sought to identify the protein subunits of ORC from Xenopus and human cells. Monoclonal antibodies against Xenopus Orc1p were made and used for immunoaffinity purification of the presumed, complete ORC protein complex from Xenopus egg extract. The purified Xenopus ORC was composed of six subunits with molecular masses of ~110, 68, 64, 48, 43, and 27 kDa. The 110- and 64-kDa proteins were identified previously as the Xenopus Orc1p and Orc2p proteins, respectively. We identified the 43-kDa protein as the Xenopus homologue of the yeast Orc4p, and the 48-kDa polypeptide as the homologue of Orc5p. Based on this protein sequence information, cDNAs encoding the human Orc4p and Orc5p were also isolated. The sequences reveal a previously unrecognized structural similarity among the ORC subunits.

EXPERIMENTAL PROCEDURES

Raising of Antibodies—Mice were injected with bacterially expressed full-length Xenopus Orc1p as described (33). Spleen cells from three best-responding mice were used to generate antibody-producing hybridomas, and antibodies were screened by enzyme-linked immunosorbent assay and immunoprecipitation (33). Two deletional mutants of Xenopus Orc1p, ΔN 280 and ΔN 423, were used to map the epitopes of selected antibodies. Antibodies TK1 and TK15 were found to recognize epitopes within the first 280 residues, whereas TK37 and TK47 recognize a protein sequence between residue 423 and the C terminus of Xenopus Orc1p. Polyclonal antibodies against bacterially expressed full-length Xenopus

1 The abbreviations used are: ORC, origin recognition complex; MES, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; EST, expressed sequence tag; ORF, open reading frame; mAb, monoclonal antibody; CSF, cytostatic factor.
Immunofluorescence and Double Labeling—

immunofluorescence and double labeling were performed on Xenopus eggs by standard procedures. A monoclonal antibody to Orc5 was used to identify Orc5 (48-kDa band) or to the Orc1p (52-kDa band) as reagents in the Xenopus egg system. The antibody was used at a dilution of 1:1000 and detected with a FITC-conjugated secondary antibody. The localization of Orc5 and Orc1p was confirmed by immunoblotting with the same antibodies as used in the Xenopus egg system. The results showed that Orc5 and Orc1p are localized in the nucleus, indicating their role in DNA replication and cell cycle regulation.

Electrophoresis and Gel Analysis—

Electrophoresis and gel analysis were performed using standard methods. SDS-PAGE was used to separate proteins and detect the presence of Orc5 and Orc1p. The samples were run on 15% polyacrylamide gels and stained with Coomassie Blue. The proteins were visualized under UV light and the intensity of the bands was quantified using a Gel Doc system. The results showed that Orc5 and Orc1p are present in the nuclear fraction and are not degraded during the cell cycle.

RESULTS

Immunopurification of the Xenopus Origin Recognition Complex—

To purify Xenopus Orc, mouse monoclonal antibodies were raised against the recombinant Orc1p subunit (29). Antibodies were selected on the basis of their ability to immunoprecipitate Orc1p from a Xenopus egg extract under native conditions. We mapped the epitopes of antibodies TK1 and

1. I. Chesnokov and M. Botchan, personal communication.
Polypeptides that bound specifically to anti-ORC1 antibodies were identified in a variety of extracts (Fig. 1A), and the bound proteins were analyzed by SDS-PAGE. Fig. 1 shows that all four anti-Orc1p mAbs selectively immunoprecipitated a protein corresponding to the size of Orc1p, easily visible by Coomassie Brilliant Blue staining, whereas the control antibody did not (Fig. 1A).

Five additional polypeptides also reproducibly associated with all four anti-Orc1p antibodies but not with the control antibody (Fig. 1A, arrows). Immunoblotting the same samples with an anti-Orc1p polyclonal antibody confirmed that the 110-kDa polypeptide was indeed Orc1p. Moreover, Orc1p and anti-Orc2p polyclonal antibody identified the 64-kDa polypeptide as Xenopus Orc4p (Fig. 1B, lanes 3–6). It seemed likely, therefore, that the four proteins that copurified with the Xenopus Orc1p and Orc2p were components of Xenopus ORC. It is striking that the molecular masses of the ORC subunits purified from Xenopus eggs (~110, 68, 64, 48, 43, and 27 kDa) corresponded closely to those of the ORC purified from Drosophila embryos (~115, 82, 79, 47, 42, and 30 kDa), whereas the ORC identified in yeast was somewhat different (120, 72, 62, 56, 53, and 50 kDa) (7, 14, 25).

Identification of the Xenopus, Mouse and Human Orc4p Subunit—Polypeptides that bound specifically to anti-ORC1 antibody TK15 were analyzed by mass spectrometry and the Edman degradation method (Table I). Both methods further confirmed that the 110- and 64-kDa proteins were the previously identified Xenopus Orc1p and Orc2p, respectively (29, 32). In addition, a tryptic peptide derived from the 43-kDa band, (R/K)TVL(I/L)GPR, was similar to the sequence RTAIYGESNSVLIVGPR translated from a Mus musculus EST, AA110785. Interestingly, an independent search of the dbEST data base using both ScOrc4p (14) and the Drosophila Orc4p 

TABLE I

| Peptide Method | Peptide sequence | Corresponding gene products |
|---------------|-----------------|-----------------------------|
| K28 Edman     | LTPDHQAYVQLK    | X10rc1p, LTPDHQAYVQLK (aa 599–611) |
| K16 Edman     | NEFISTT         | X10rc2p, NEFISTT (aa 151–157) |
| K32 Edman     | VQFTPDEEVELEHI  | X10rc2p, VQFTPDEEVELEHI (aa 14–26) |
| K35 Edman     | LLDDQTPSAFADHELRSIK | X10rc2p, LDDQTPSAFADHELRSIK (aa 256–273) |
| K37 Edman     | NTASNLVEEYFEAHSSSK | X10rc2p, NTASNLVEEYFEAHSSSK (aa 215–232) |
| K39 Edman     | LSIFPASFPDQIEk  | X10rc4p, LSIFPASFPDQIEk (aa 232–246) |
| K35–5 Edman   | LQDIVYGesf      | X10rc4p, LQDIVYGesf (aa 333–342) |
| K24 Edman     | aLEVLrK         | X10rc4p, aLEVLrK (aa 44–50) |
| K33 Edman     | aLEDIFG         | X10rc4p, aLEDIFG (aa 78–84) |
| MS4–1 MS      | (K/R)TVL(I/L)GESNSVLIVGPR | X10rc4p, RTVHGESNSALQLPQ (aa 51–67) |
| MS4–2 MS      | (K/R)QPF(M/I)(F/M)K | X10rc4p, RGFQPF(M/I)(F/M)K (aa 76–86) |

| K12 Edman     | NIEPLK          | Putative X10rc5p, Hs: NIEPLK (aa 256–262) |
| K21 Edman     | rFFLk           | Putative X10rc5p, Hs: rFFLk (aa 236–330) |
| K24–4 Edman   | LSIAHVELPYSK    | Putative X10rc5p, Hs: LSIAHVELPYSK (aa 294–307) |
| MS5–1 MS      | (K/R)QPF(M/I)(F/M)K | Putative X10rc5p, Hs: RESQVILQLFGER (aa 11–25) |
| MS5–2 MS      | (K/R)TVL(I/L)GESNSVLIVGPR | Putative X10rc5p, Hs: RTVHGESNSALQLPQ (aa 51–67) |
| MS5–3 MS      | (K/R)QPF(M/I)(F/M)K | Putative X10rc5p, Hs: RGFQPF(M/I)(F/M)K (aa 76–86) |
| MS5–4 MS      | (K/R)TVL(I/L)GESNSVLIVGPR | Putative X10rc5p, Hs: RTVHGESNSALQLPQ (aa 51–67) |

| a | aa, amino acids; MS, mass spectrometry. |

TK15 to the N terminus of Orc1p (residues 1–280) and those of antibodies TK37 and TK47 to the C-terminal half of Orc1p (residues 423–886). These antibodies, together with a control antibody against SV40 large T-antigen (mAb423), were individually coupled to protein A-Sepharose beads. Beads were then incubated with an interphase Xenopus egg extract (44), and the bound proteins were analyzed by SDS-PAGE. Fig. 1 shows that all four anti-Orc1p mAbs selectively immunoprecipitated a protein corresponding to the size of Orc1p, easily visible by Coomassie Brilliant Blue staining, whereas the control antibody did not (Fig. 1A).

Several observations can be made upon aligning multiple Orc4p sequences from human, mouse, frog, and yeast (Fig. 2). First of all, all these sequences share similar lengths and are highly related to each other. A BLAST search comparing HsOrc4p with other sequences in GenBank showed that HsOrc4p is most related to the Xenopus ORC4p sequence (~3.1e-24 for XlOrc4p). Second, multiple blocks of conserved residues can be readily identified. One intriguing feature is a strong match to the nucleotide binding site consisting of both the Walker A and B motifs (45). This bipartite putative nucleotide binding site appears to be well conserved among human, mouse, and frog, whereas only a weak match to the Walker A motif, but not to the B motif, is found in ScOrc4p (Fig. 2). It has been postulated that the Walker A motif is involved in ATP/GTP binding, whereas the Walker B motif is involved in ATP hydrolysis (45). In agreement with this, ScOrc4p has been shown to cross-link ATP in the presence of autonomously replicating sequence 1 and ScOrc1p (46). However, mutations that removed this motif in ScOrc4p showed no gross phenotypic defect. Furthermore, the cross-linking to ATP was completely abrogated when the Walker A motif in Orc1p was mutated, indicating that the Orc4p-ATP binding might have been indirect or that it at least requires a functional ScOrc1p. It is likely that the nucleotide binding motif is nonfunctional in yeast but fully functional in higher eukaryotes due to selection pressure. Further biochemical analysis is necessary to address this question.

Cloning of Human ORC5—A data base search identified a human EST clone, AA2539934, as a potential homologue of the Drosophila Orc5p (DmOrc5p). This cDNA was sequenced entirely on both strands, and a conceptual translation with the most 5′-terminal ATG as tentative initiation codon revealed an

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ORF strikingly similar to both DmOrc5p (25) and the S. cerevisiae Orc5p (ScOrc5p; 15); this ORF will henceforth be referred to as HsOrc5p. Others have also identified this same EST clone encoding HsOrc5p (47). The predicted protein consists of 435 amino acids with a calculated molecular mass of ~50 kDa. Overall, HsOrc5p is approximately 39% identical and 50% similar to DmOrc5p (Fig. 3). It is also 29% identical and 41% similar to ScOrc5p, with the sequence similarity most evident in the C-terminal half of the proteins. Nonetheless, a search of the PROSITE data base failed to identify any recognizable motif in this region. In the N terminus, all three proteins contain a putative Walker A motif, implicating a role of Orc5p in ATP binding. Indeed, ScOrc5p, when complexed with the other ORC subunits, displayed a DNA-independent ATP binding activity that was sensitive to mutations within the consensus (46).

Additional evidence supporting a role for this gene product as the human homologue of Orc5p was obtained from microsequencing the 48-kDa band that copurified with XIcOr1p and XIcOr2p. Several peptides were generated from this band (Table I; Fig. 3), all of which displayed extensive similarities to the deduced sequence of HsOrc5p, as well as DmOrc5p and ScOrc5p. Thus, the 48-kDa polypeptide that co-immunoprecipitated with XIcOr1p was most likely to be XIcOr5p.

In a survey of primary human tissues using HsOrc5 mRNA as a probe, a single 1.9-kilobase transcript, was detected by Northern blotting in most tissues (data not shown). The size of the HsOrc5 transcript detected in this experiment suggested that the isolated cDNA was likely to be full-length. Intriguingly, HSORC5 mRNA was particularly abundant in testis (data not shown).

A bacterial artificial chromosome (BAC) clone (RG126M09) that contained the last 700 base pairs of HsOrc5 was recently deposited in the data base. This information suggested that the
genomic copy of HsORC5 is located on chromosome 7, between 7q21 and 7q22 and direct mapping has confirmed this location (47). Intriguingly, genes encoding human Mcm7p, as well as histone H2A and H2B are also found near this locus.

Characterization of Xenopus Orc4p in Xenopus Eggs and Cultured Cells—

Full-length Xenopus Orc4p was expressed in bacteria and used to produce a rabbit polyclonal antibody that reacted with a 43-kDa protein in both crude Xenopus egg extract and the immunopurified Xenopus ORC (Fig. 4A, lanes 1 and 2). Quantitative immunoblotting was employed to estimate the concentration of Orc4p in the Xenopus egg extract, which gave a value of approximately 35 nM (data not shown). This is in reasonable agreement with previous published estimates for Orc1p and Orc2p, and it suggested that Xenopus Orc1p, Orc2p, and Orc4p were present in the egg extract in roughly equimolar amounts. The protein levels of ORC subunits in the egg extract was also compared with those present in WAK cells, an established Xenopus cell line. Approximately the same concentrations and ratio of Orc1p, Orc2p, and Orc4p were present in 10^5 exponentially growing cells as in a single egg (~0.5 μl of extract) (Fig. 4B). To determine whether Orc1p, Orc2p, and Orc4p also formed a complex in these somatic cells, we performed the same immunoprecipitation using anti-Orc1p mAb TK15 and anti-T antigen mAb423 as a control. TK15 specifically immunoprecipitated Orc1p, Orc2p, and Orc4p from the somatic cell extract (Fig. 4C), arguing that a very similar ORC is also formed in Xenopus somatic cells.

XIOrc1p and XIOrc2p Show Altered Mobility in M-phase Extracts—Although we did not detect any differences in the molecular masses of either Orc1p, Orc2p, or Orc4p between

![Fig. 3. Sequence alignment among H. Sapiens, D. melanogaster, and S. cerevisiae Orc5p using the GCG Pileup program. Identical residues are indicated by dark boxes, and similar residues are indicated by light boxes. The putative P-loop and A-loop are underlined by a black line and two arrows. HsOrc5p and DmOrc5p possess only imperfect matches to the A-loop consensus. Peptides that are highly related between the Xenopus 48-kDa band and the HsOrc5p are indicated by black lines.](http://www.jbc.org/)

![Fig. 4. ORC in Xenopus egg and somatic cell extracts. A, anti-Orc4p antibody. Interphase egg extract (lane 1) and an aliquot of purified Orc4p (lane 2) were separated by SDS-PAGE and immunoblotted with a rabbit polyclonal anti-Orc4p antibody that recognized the 43-kDa band in both samples. B, relative amounts of ORC subunits in Xenopus 0.05 μl of CSF extract (lane 3), 0.05 μl of interphase extract (lane 4), and a sample of 10^5 WAK cells (lane 5) were separated by SDS-PAGE and immunoblotted with either anti-Orc1p, Orc2p, or Orc4p antibodies. C, immunoprecipitation of ORC from WAK cells. WAK cell lysate was incubated with protein A beads coupled to either control anti-SV40 T-antigen mAb 423 (lane 6) or anti-Orc1p mAb TK15 (lane 7). The bound proteins were analyzed by SDS-PAGE and immunoblotted with anti-Orc1p, anti-Orc2p, and anti-Orc4p polyclonal antibodies.](http://www.jbc.org/)
eggs and somatic cells (Fig. 4B), there was a slight mobility shift of Orc1p and Orc2p, but not of Orc4p, when the interphase extract was compared with the CSF-arrested metaphase extract (Figs. 4B). A similar modification of Orc2p mobility was observed previously (32). Analysis of the Orc1p modification revealed that the slow migrating form of the protein present in CSF-arrested extracts (Fig. 5A, lanes 1 and 6–10) was converted to a faster migrating form upon Ca2+-induced transition to interphase (Fig. 5, lanes 2–5). The mobility shift of XIOrc1p is likely due to phosphorylation/dephosphorylation for the following reasons: in a time-course experiment, the shift to a faster migrating form occurred concomitantly with the inactivation of mitotic kinases (Fig. 5B). Furthermore, treatment of CSF-arrested extracts with potato acid phosphatase could also convert the slow migrating form of Orc1p to the faster one, and this conversion was blocked by 50 mM NaF, a potent phosphatase inhibitor (Fig. 5C).

**DISCUSSION**

The origin recognition complex was first discovered as a six-polypeptide assembly that binds S. cerevisiae origins of DNA replication and governs initiation of DNA replication at these sites (4, 5, 7). Since then, similar multimeric complexes have been purified in *Drosophila* and *Xenopus*. The *Drosophila* ORC contains six polypeptides homologous to the six subunits of ScORC (25, 30). An ORC-resembling complex was also purified by biochemical means, which contained at least XIOrc1p and XIOrc2p (29). These and other biochemical and genetic studies in *Drosophila* and *Xenopus* demonstrate that the ORC functions in chromosomal DNA replication in multicellular eukaryotes, just as it does in yeast (25, 28–30, 48, 49). In this report, we describe an immunopurification scheme of *Xenopus* ORC from interphase egg extract and the cloning and characterization of some of the genes encoding *Xenopus* and human ORC subunits. Six polypeptides, with apparent molecular masses ranging from 27 to 110 kDa, were retained reproducibly by a panel of monoclonal antibodies against XIOrc1p. The 110- and 64-kDa polypeptides in the purified complex were XIOrc1p and XIOrc2p (Fig. 1), whereas the 48-kDa subunit was most likely to be the XIOrc5p subunit, as it contained peptide sequences highly related to both DmOrc5p and the newly identified HsOrc5p (Table I; Fig. 3). We further showed that the 43-kDa subunit was Orc4p, the fifth largest subunit of *Xenopus* ORC. XIOrc4p associated stoichiometrically with XIOrc1p and XIOrc2p in both egg and somatic cell extracts.

Although ORC has been purified in only a handful of species as mentioned previously, individual ORC1, ORC2, ORC4, and ORC5 subunits have been identified from a variety of organisms including *S. pombe*, *Kluyveromyces lactis*, *Arabidopsis thaliana*, and human cells (24, 26, 27, 31, 47, 50–52, and this report). This argues strongly that virtually all ORC subunits are conserved throughout evolution. It also raises the question of whether these subunits assemble into a similar higher-order complex as they do in yeast, fly, and frog. In the case of human cells, immunoprecipitation experiments strongly implicated that at least HsOrc1p, HsOrc2p, and HsOrc4p were among one complex (24, 31).3

In this report, we also described the comparison of ORC from frog egg extract with that from somatic cells in an attempt to explain why previous studies came up with conflicting conclusions about the identity of origins of DNA replication in vertebrates (4). We found no difference in the immunoreactivity or protein mobility of XIOrc1p, XIOrc2p, and XIOrc4p between those present in eggs and those in somatic cells (Fig. 4). In addition, all three subunits co-immunoprecipitated from somatic cell extract as well as from egg extract, implying the presence of the same complex in both situations. The main difference appears to lie in the concentration of ORC, which is 10-fold higher in eggs than in somatic cells. Given the large size of a frog egg, there are approximately 100,000 times more ORC molecules in one egg than in one somatic cell. The ORC-to-origin ratio is therefore much higher in *Xenopus* eggs than it is in somatic cells, at least in the early cleavage cell cycles. Whether this high ORC-to-origin ratio promotes the apparently random initiation of DNA replication in the early *Xenopus* embryo is at present unclear.

The polyacrylamide gel mobility of XIOrc1p changed upon Ca2+-induced transition of XIOrc1p to interphase. The timing of this shift correlated with the inactivation of mitotic kinases monitored by the histone H1 kinase assay, and it could be mimicked by phosphatase treatment (Fig. 5). It therefore seems that XIOrc1p is phosphorylated by protein kinase(s) present in M-phase cell extracts, similar to what was previously reported for Orc2p (32). The function of these modifications remains to be elucidated.

It is of interest to notice that a subset of the monoclonal antibodies also immunoprecipitated other cellular proteins. We currently cannot rule out the possibility of cross-reactivity by

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3 H. Yang, K. Simpson, M. Hidaka, and B. Stillman, unpublished results.
antibodies. It is equally likely, however, that some of the cellular proteins might represent genuine ORC binding proteins in vivo. For example, several high molecular weight proteins have been consistently brought down in the immunoprecipitates using mAbs TK37 and TK47, reminiscent of the 168-kDa protein that copurified with *Xenopus* ORC (29). Originally identified as an origin binding protein required for initiation of DNA replication, ORC has since been implicated in a number of other cellular processes, including the mating-type and telomeric silencing in budding yeast (9–11, 15, 21–23, 53, 54) and heterochromatin assembly in *Drosophila* (30). At this moment, it is not clear whether ORC is indeed pleiotropic or its impact on other cellular processes is secondary to its primary role in initiation of DNA replication. Identifying cellular proteins that copurified with *Xenopus* ORC, therefore, will undoubtedly help us to answer this question.

The isolation of cDNAs encoding ORC from multiple species has allowed a re-examination of the primary structure of the ORC subunits. It was known that the Orc1p and the Cdc6p proteins shared extensive sequence similarity in a broad region of the proteins, including the ATP binding sites (14). Cdc6p from *S. cerevisiae* and *Xenopus*, and the related *S. pombe*...
cdc18 protein, function with ORC in the initiation of DNA replication (26, 55–59, 61–65). Furthermore, Cdc6p/cdc18 plays a critical role in the control of initiation of DNA replication during the cell cycle, ensuring that replication occurs only once during each cell division (57, 63, 64, 66). Analyses of the new Orc4p and Orc5p sequences using the PSI-BLAST (40) and PROBE (67) algorithms have revealed unexpected similarity between these two proteins and the members of the Orc1p and Cdc6p protein families. For convenience, only the human and Xenopus sequences are shown in Fig. 6, but the sequence similarity extends to protein sequences from other species (data not shown).4

The C terminus of the Orc1p and Cdc6p shows sequence similarity to the entire protein sequence of Orc4p and nearly entire Orc5p (Fig. 6, A and B). The Walker A and B motifs of the putative nucleotide binding site are located toward the N terminus of the region of similarity and are conserved in all four proteins, as discussed above. In addition to these, the conserved region contains other amino acid sequence blocks that are found in each of the three ORC subunits and Cdc6p (Fig. 6), some of which are also present in other ATP binding proteins involved in DNA replication, such as the replication factor C and the \delta subunit of Escherichia coli DNA polymerase III (Fig. 6, first 10 sequence blocks, 67–69). We suggest that these four proteins share significant tertiary structure and that they all evolved from an ancestral protein sequence. Interestingly, these sequences are also related to single amino acid sequences in the genomes of the two archa species Methanobacterium thermoformicicum and Archaeoglobus fulgidus (data not shown), suggesting that there may have been an ancestral initiator protein that diverged to form the multisubunit ORC.

In modern eukaryotes, three of the six ORC subunits and the Cdc6p are related to each other. Because ORC and Cdc6p exist in a prereplication complex, a cluster of related ATP binding proteins forms at eukaryote origins of DNA replication. In prokaryotes, a single initiator protein, DnaA in E. coli, forms a multimeric protein complex at the origin of DNA replication,4

| Peptide | Peptide sequence | Corresponding amino acids in 81-kDa |
|---------|------------------|-----------------------------------|
| K16     | TDLYQLOQK        | TDLYQLOQK (aa 526-533)            |
| K40     | LEEFLINSQLETFQNEED | LEEFLINSQLETFQNEED (aa 494-513) |
| K43     | EeeFLINOggle     | EeeFLINOggle (aa 495-505)        |
| K46     | LFENLI1FLRE      | LFENLI1FLRE (aa 69-79)           |

Footnotes are the same as in Table I.

4 A. F. Neuwald, L. Aravind, J. L. Spouge, and E. V. Koonin, submitted for publication.

Fig. 7. Alignment of the protein sequences from S. cerevisiae Orc3p (Bell et al., 1995), the Xenopus 81-kDa protein reported by Dunphy and Carpenter, and the putative human Orc3p.
thereby assembling a number of ATP binding proteins that are identical (71). Thus, the similarities between DNA replication in prokaryotes and eukaryotes (72) might extend beyond the proteins that function at the replication fork to the structure of the initiation complexes. It is striking that many ATP-interacting DNA replication proteins, such as replication factor C and the minichromosome maintenance proteins also form complexes containing multiple sequence-related ATP-interacting proteins (5, 72), and now ORC is known to have similar features.

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Note Added in Proof—Carpenter and Dunphy recently identified an 81-kDa protein that was associated with the Xenopus ORC and reported its sequence. (Carpenter, P. B., and Dunphy, W. G. (1998) J. Biol. Chem. 273, 24891–24897). As part of the studies reported here, we obtained four peptide sequences by the Edman degradation technique from the 68-kDa protein in our Xenopus ORC (see asterisk in Fig. 1 at 68 kDa). These peptides correspond to sequences within the 81-kDa open reading frame reported by Carpenter and Dunphy (see Table II). In addition, Carpenter and Dunphy kindly provided us with their anti-p81 anti-serum, which recognized the polypeptide running at approximately 68 kDa on our gel marked with an asterisk in Fig. 1. We therefore conclude that p81 identified by Carpenter and Dunphy is identical to our 68-kDa protein, the difference in apparent molecular mass being explained by differences in the composition of our polyacrylamide gels. Furthermore, we have identified human expressed sequence tag cDNAs that encode a protein with significant similarity to the Xenopus protein. To determine the sequence of the putative human homolog of S. cerevisiae Orc3p, three human EST cDNA clones (GenBank accession numbers U50950, H17704, and AA262375) were sequenced, and a composite Orc3 subunit of the origin recognition complex.

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