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Identification of a Novel 81-kDa Component of the *Xenopus* Origin Recognition Complex*

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Phillip B. Carpenter‡ and William G. Dunphy§

From the Division of Biology, Howard Hughes Medical Institute, California Institute of Technology, Pasadena, California 91125

The *Xenopus* origin recognition complex is essential for chromosomal DNA replication in cell-free extracts. We have immunopurified the *Xenopus* origin recognition complex with anti-Xorc2 antibodies and analyzed its composition and properties. Xorc2 (p63) is specifically associated with Xorc1 (p115) and up to four additional polypeptides (p81, p78, p45, and p40). The cDNA encoding p81 is highly homologous to various expressed sequence tags from humans and mice encoding a protein of previously unknown function. Immunodepletion of p81 from *Xenopus* egg extracts, which also results in the removal of Xorc2, completely abolishes chromosomal DNA replication. Thus, p81 appears to play a crucial role at S phase in higher eukaryotes.

The regulation of DNA replication involves the coordinated action of various proteins that associate with chromosomes in a specific manner (reviewed in Refs. 1 and 2). In the budding yeast *Saccharomyces cerevisiae*, the origin recognition complex (ORC)[1] binds to origins of replication and is essential for the onset of DNA replication. ORC is bound to budding yeast origins throughout the cell cycle, implying that other factors dictate when initiation of replication will occur (3–8). Before S phase, ORC resides in a pre-replicative complex that contains Cdc6 and other key replication factors such as the Mcms (minichromosome maintenance proteins) (5–10). Upon the firing of origins, the footprint of ORC on the DNA switches to a post-replicative form. Because this pattern is similar to that which is observed for purified ORC bound to DNA, the post-replicative complex may consist only of ORC. The mechanisms that govern the switch from the pre-replicative complex to the post-replicative complex remain to be established fully. However, the cyclin-dependent kinases, including Clb-associated Cdc28 in budding yeast and Cdk2-cyclin E in higher eukaryotes such as *Xenopus*, are likely to play a key role (11, 12). Additionally, recent evidence suggests that the conserved kinase Cdc7 regulates the Mcms at S phase (8, 13, 14).

ORC components have been identified in a wide range of eukaryotes, including budding and fission yeast, flies, frogs, and humans, suggesting that the mechanism of initiation of eukaryotic DNA replication might be universally conserved (15–20). Despite the conservation of several ORC subunits from yeast to metazoans, the identification of replicator sequences in higher eukaryotes has proven elusive. The functional reconstitution of a metazoan ORC might provide an avenue for the identification of replication origins in more complex eukaryotes. Furthermore, this reconstituted complex would facilitate the elucidation of mechanisms governing initiation of DNA replication in higher eukaryotic chromosomes.

Xenopus egg extracts have proven highly valuable for the study of cell cycle events such as DNA replication *in vitro*. Previous studies have established an essential role for two *Xenopus* homologs of budding yeast ORC subunits (Xorc1 and Xorc2) in the initiation of DNA replication (18, 20). In this report , we present a further characterization of *Xenopus* ORC (XORC). In particular, we have focused on an 81-kDa protein (p81) that is tightly associated with a complex containing Xorc2. cDNA cloning studies indicate that p81 is highly homologous to putative human and mouse proteins of previously unknown function. Although p81 does not possess an obvious homolog in *S. cerevisiae*, it displays some limited sequence identity to the budding yeast Orc3 protein. Removal of p81 from *Xenopus* egg extracts completely abolishes the capacity of these extracts to carry out chromosomal DNA replication. Hence, p81 appears to be an essential replication factor in higher eukaryotes.

EXPERIMENTAL PROCEDURES

Xenopus Extracts—Cytostatic factor-arrested egg extracts were prepared from unactivated *Xenopus* eggs as described by Murray (21). Cytostatic factor-arrested extracts were induced to enter the interphase state by the addition of 0.4 mM $CaCl_2$. Interphase extracts were also supplemented with 100 μg/ml cycloheximide to prevent the entry into mitosis. S phase cytosol was prepared according to the method of Smythe and Newport (22). Extracts from developmentally staged *Xenopus* embryos were prepared as described (23).

Immunopurification and Isolation of Xorc2-associated Proteins—To isolate Xorc2-associated proteins, interphase egg extracts (3–5 ml) were treated with anti-Xorc2 antibodies (18) covalently coupled to the Affi-Prep protein A support (Bio-Rad) by the method of Schneider *et al.* (24). Typically, 100 μg of anti-Xorc2 antibodies was coupled to 50 μl of protein A beads and incubated with egg extract for 1 h at 4 °C. After incubation, the beads were collected and extensively washed three times with extract buffer (80 mM β-glycerophosphate, 20 mM EGTA, and 15 mM $MgCl_2$, pH 7.3) containing 1% Nonidet P-40, three times with extract buffer containing 1% Nonidet P-40 and 0.4 M sodium chloride, three times with extract buffer alone, and once with HEPES-buffered saline (10 mM HEPES-KOH and 150 mM NaCl, pH 7.4). Finally, the beads were resuspended in Tris-buffered saline (10 mM Tris-HCl and 150 mM NaCl, pH 7.4) containing 0.1% SDS and boiled for 5 min. A small aliquot of the eluted fraction was subjected to SDS gel electrophoresis and either silver-stained or processed for immunoblotting with either anti-Xorc1 (20) or anti-Xorc2 antibodies. The eluted proteins from several preparations were combined and concentrated with a Filtron microconcentrator. The concentrated eluate was treated with 15

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§ Supported by the Howard Hughes Medical Institute. To whom correspondence should be addressed. Tel.: 626-395-8433; Fax: 626-449-0679; E-mail: dunphy@cco.caltech.edu.

[1] The abbreviations used are: ORC, origin recognition complex; XORC, *Xenopus* origin recognition complex; kb, kilobase pair; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

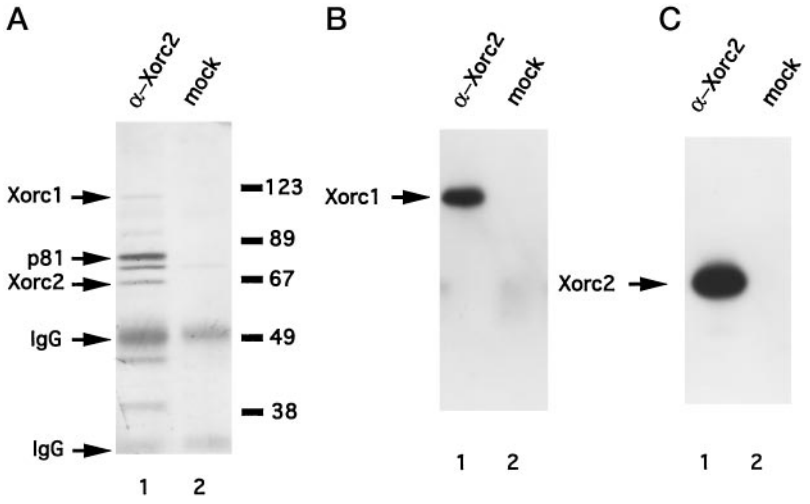

FIG. 1. **Characterization of Xorc2-associated proteins in interphase extracts.** *A,* immunoprecipitates prepared with anti-Xorc2 (*lane 1*) or control (*lane 2*) antibodies were silver-stained. *B* and *C,* anti-Xorc2 and mock immunoprecipitates were processed for immunoblotting with either anti-Xorc1 or anti-Xorc2 antibodies, respectively.

mM dithiothreitol and 50 mM iodoacetamide prior to electrophoresis on a preparative 8% polyacrylamide gel. Xorc2-associated bands were visualized by staining for 20 min with 0.2% Coomassie Brilliant Blue (dissolved in 20% methanol and 0.5% acetic acid) and destaining for 1 h with 30% methanol. One band, termed p81 due its apparent molecular mass of 81 kDa, was excised from the gel and processed for peptide sequencing by the California Institute of Technology Protein/Peptide Micro Analytical Laboratory. Amino acid sequences were obtained from three tryptic fragments as follows: peptide A, TSLTVADYFNEGLR; peptide B, *X*EFVFKPSAK; and peptide C, VALHTALNNPASYLK. *X* denotes an unreadable amino acid.

^{32}P Labeling of Xenopus Extracts—^{32}P-Labeled orthophosphate (500 μCi/μl) was added to cytostatic factor-arrested extracts containing 100 μg/ml cycloheximide and incubated for up to 1 h at 23 °C to allow incorporation into endogenous ATP. The extract was split in half. One aliquot was activated with calcium, and the other was kept arrested at metaphase. After an additional 30-min incubation, the extracts were immunoprecipitated with anti-Xorc2 antibodies bound to protein A beads. Immunoprecipitated proteins were washed as described above, except that the first three washes also contained 1 μM microcystin, resuspended in SDS gel sample buffer, and electrophoresed on 10% SDS gels. Radiolabeled bands were visualized by autoradiography or transferred to polyvinylidene difluoride membranes and processed for phosphoamino acid analysis (25).

Cloning of the cDNA Encoding p81—Six degenerate oligonucleotides were designed from the sequences of the p81 tryptic peptides. One combination of primers was found to amplify an internal 1.6-kb fragment of *Xenopus* p81 in a polymerase chain reaction (PCR). These two primers are as follows: B-forward, 5′-ACIGTIGCIGA(T/C)TA(T/C)TT(T/C)AA(T/C)GA(A/G)GG-3′; and C-reverse, 5′-(G/C)(T/A)IGCIGG(G/A)TT(G/A)TTIA(G/A)IGCIGT(G/A)TG-3′. The PCR conditions of Mueller *et al.* (26) were used. After electrophoresis of the PCR products, the 1.6-kb band was reamplified and subcloned into the pCRII cloning vector (Invitrogen). This fragment was radiolabeled by the random primer method using a kit from Life Technologies, Inc. and used to screen 10^6 clones from a *Xenopus* oocyte cDNA library (18, 26). Eighteen cDNA clones were isolated; the longest one (a 2.3-kb *Apa*I-*Xho*I fragment) was sequenced on both strands using an automated DNA sequencer. By conceptual translation, this cDNA was found to contain an 81-kDa open reading frame. Although the presumed initiation codon is not preceded by an in-frame termination codon, it does reside in a good Kozak consensus sequence (27). Furthermore, the apparent molecular mass of recombinant histidine-tagged p81 (84 kDa) is 3 kDa larger than that of endogenous p81 in *Xenopus* egg extracts detected with anti-p81 antibodies. Thus, the 2.3-kb cDNA appears to contain the full-length coding sequence of p81.

Production of Recombinant p81 and anti-p81 Antibodies—We expressed *Xenopus* p81 using the BAC-to-BAC baculovirus expression system (Life Technologies, Inc.). The full-length coding sequence of p81 was subcloned into pFastBacHTa via a triple-ligation strategy. An *Nco*I restriction site was engineered at the initiation codon by PCR with *Pfu* polymerase. A 1.3-kb *Nco*I-*Xba*I fragment and a 1.0-kb *Xba*I-*Xho*I fragment, which together contained the entire coding sequence, were ligated to pFastBacHTa that had been digested with *Nco*I and *Xba*I. p81 protein was expressed in Sf9 insect cells as described previously (26, 28). The protein was isolated from the inclusion body fraction of solubilized Sf9 cells in essentially pure form and used to immunize rabbits for polyclonal antibody production. Antibodies directed against p81 were affinity-purified essentially as described (10), except that p81 was coupled to CNBr-Sepharose beads after it had been solubilized in 8 M urea.

Other Methods—GST-p21N (29) was prepared as described for p21 (11). Immunodepletions of *Xenopus* interphase extracts with anti-p81 antibodies were performed as described (18), except that three rounds of depletion were performed. For each round, 30 μg of anti-p81 antibodies was used per 50 μl of egg extract. For the rescue of p81-depleted extracts, demembranated sperm chromatin was incubated in S phase cytosol (22) for 5 min and then diluted with 2 volumes of extract buffer (10 mM HEPES-KOH, 0.1 M KCl, 0.1 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 50 mM sucrose, pH 7.7) supplemented with 1 mg/ml bovine serum albumin and 7 μM ATP. The "loaded" chromatin was then centrifuged in a Beckman Microfuge E containing a horizontal rotor for 1 min at 4 °C. Either p81-depleted or mock-depleted extracts were added to the pellet containing the loaded chromatin. DNA replication assays were performed as described (18). Immunoblotting with anti-Xorc2 antibodies was performed as described (18). Immunoblotting with anti-p81 antibodies was performed in BLOTTO (26), and proteins were detected with the ECL system (Amersham Pharmacia Biotech).

RESULTS

Identification of Xorc2-associated Proteins—We previously demonstrated that Xorc2 is essential for chromosomal DNA replication in *Xenopus* extracts (18). Xorc2 is found in a large multiprotein assembly, suggesting that it resides in XORC (18, 20, 30). By immunoprecipitation with anti-Xorc2 antibodies, we observed the presence of several Xorc2-associated proteins (Fig. 1*A*, *lane 1*). Immunoblotting of anti-Xorc2 immunoprecipitates confirmed that Xorc1 and Xorc2 reside in the same multiprotein complex (Fig. 1, *B* and *C*, *lanes 1*) (20, 30). Moreover, it was clear from silver staining of the immunoprecipitates that at least four or five proteins tightly associated with Xorc2 (63 kDa) even after stringent washes in 0.5 M NaCl and 0.1% CHAPS (Fig. 1*A*; see "Experimental Procedures"). In addition to Xorc1 (115 kDa), there were polypeptides with apparent molecular masses of 81, 78, 46, and 40 kDa. The 78-kDa band also appeared in immunoprecipitates with control antibodies (albeit at lower levels) and thus may represent a nonspecific binding protein. Typically, the 81-kDa band silver-stained more intensely than Xorc2 in these preparations, suggesting that it might be more abundant than Xorc2 in the complex. However, when large-scale anti-Xorc2 immunoprecipitates were stained with Coomassie Blue, the amounts of p81 and Xorc2 were found to be comparable (data not shown), suggesting that p81 may stain anomalously well with silver.

Xorc1 and Xorc2 Are Phosphorylated Differentially throughout the Cell Cycle—To characterize the Xorc2-containing complex in greater detail, we examined the phosphorylation of its

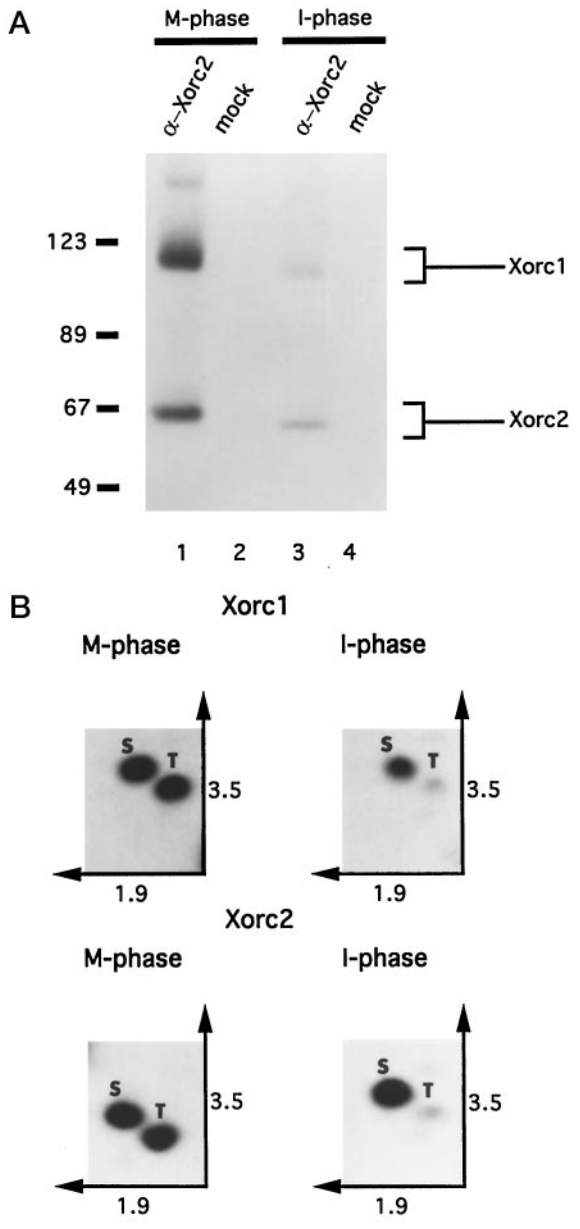

FIG. 2. **Phosphorylation of XORC constituents during the cell cycle.** *A,* M phase and interphase (*I-phase*) extracts were labeled with [^{32}P]orthophosphate and immunoprecipitated with either anti-Xorc2 (*lanes 1* and *3*) or control (*lanes 2* and *4*) antibodies. *B,* shown are the results from the phosphoamino acid analysis of Xorc1 and Xorc2. *S*, phosphoserine; *T*, phosphothreonine.

various constituent proteins during the cell cycle. For this purpose, M phase and interphase egg extracts were radiolabeled with ^{32}P and subjected to immunoprecipitation with anti-Xorc2 antibodies (Fig. 2*A*). Only Xorc1 and Xorc2 became detectably phosphorylated under these experimental conditions. The labeling of both proteins was stronger at M phase (Fig. 2*A*, *lane 1*), but significant phosphorylation of both Xorc1 and Xorc2 could also be detected in interphase extracts (*lane 3*). Similar amounts of both Xorc1 and Xorc2 were immunoprecipitated from M phase and interphase extracts under these conditions (data not shown). The functional significance of the M phase phosphorylation is not known, but it does correlate with the dissociation of XORC from the condensed chromosomes at M phase (10). Furthermore, recent studies with both human and frog Mcm (mini-chromosome maintenance) replication proteins have also revealed a similar correlation between protein phosphorylation and chromatin association (reviewed in Ref. 2). Thus, it is possible that extensive phosphorylation of some replication proteins serves to remove them from the chromatin during M phase. Significantly, the interphase phosphorylation of Xorc1 and Xorc2 was unaffected by 15 μM GST-p21N, a Cdk (cyclin-dependent kinase) inhibitor that completely blocks both Cdk2-cyclin E activity and DNA synthesis in egg extracts (data not shown). By phosphoamino acid analysis, we found that the M phase forms of Xorc1 and Xorc2 were phosphorylated on both serine and threonine, whereas the interphase forms appeared to contain mostly phosphoserine. In conclusion, Xorc1 and Xorc2 appear to be the most heavily phosphorylated subunits of XORC, and their phosphorylation increases greatly at M phase.

Cloning of the 81-kDa Xorc2-associated Protein—To isolate enough of the Xorc2-containing complex for peptide sequencing, we carried out multiple large-scale immunoprecipitations. From ~20 ml of egg extract, we obtained approximately 10 μg of each Xorc2-associated protein. Our interest first centered on p81, an 81-kDa polypeptide that migrates between Xorc1 and Xorc2 (Fig. 1*A*, *lane 1*). The sequences of three tryptic peptides from p81 were used to design degenerate oligonucleotides for PCR. One combination of primers yielded a 1.6-kb PCR fragment, which was used to screen a *Xenopus* oocyte cDNA library (18, 26). Eighteen positive clones were isolated, and the largest one, pC84-1A, was found to contain ~2300 nucleotide base pairs and to encode a predicted protein of 81 kDa (Fig. 3).

We used the sequence of *Xenopus* p81 to search against current data bases using the BLAST and PSI-BLAST programs (31). p81 possesses no obvious structural motifs that might lend a clue to its biochemical function, although it does have a potential bipartite nuclear localization signal at residues 533–546 (KKLLEMKETRRTKK). p81 was found to be highly homologous to several human expressed sequence tags of unknown function (GenBank™ accession numbers of a few examples are U50950, AA317247, and H94935). The homologous expressed sequence tags were derived from a variety of tissues including retina, heart, colon, and brain. In addition, p81 was found to be strongly homologous to several expressed sequence tags from embryonic mice (*e.g.* GenBank™ accession numbers AA073564 and W98988). We found no significant sequence similarities between *Xenopus* p81 and any proteins from *Caenorhabditis elegans*, *Drosophila*, or *Arabidopsis.* A directed search with *Xenopus* p81 against the *S. cerevisiae* genome yielded no significant sequence homologies with an *E* value of <0.01. A subthreshold score was detected between p81 and budding yeast Orc3 (GenBank™ Z73109; *E* value of 3.4 with the PSI-BLAST program). Three other budding yeast proteins scored higher than Orc3 in this search. These include a protein of unknown function (GenBank™ D50617), a putative transcriptional regulatory protein (GenBank™ Z35827), and a subunit of the proteasome (GenBank™ Z75169). Overall, p81 shares 21% identical residues with budding yeast Orc3. An internal region of p81 (residues 215–406) is ~25% identical to residues 201–400 of budding yeast Orc3. Thus, it is possible that p81 represents a novel, highly diverged form of Orc3 present in higher eukaryotes.

Characterization of Xenopus p81—To characterize the functional properties of p81, we first expressed p81 with a six-histidine tag at its N terminus in baculovirus-infected Sf9 insect cells. Recombinant His_6-p81 (Fig. 4*A*), which migrated as an 84-kDa band on SDS gels due to the addition of the histidine tag, was used to generate polyclonal antibodies in rabbits. The anti-p81 antibodies, which reacted strongly with His_6-p81, also recognized an endogenous 81-kDa polypeptide in *Xenopus* egg extracts (Fig. 4*A*), which migrated at the same position as immunopurified p81 (data not shown).

Since p81 had been isolated originally by immunoprecipita-

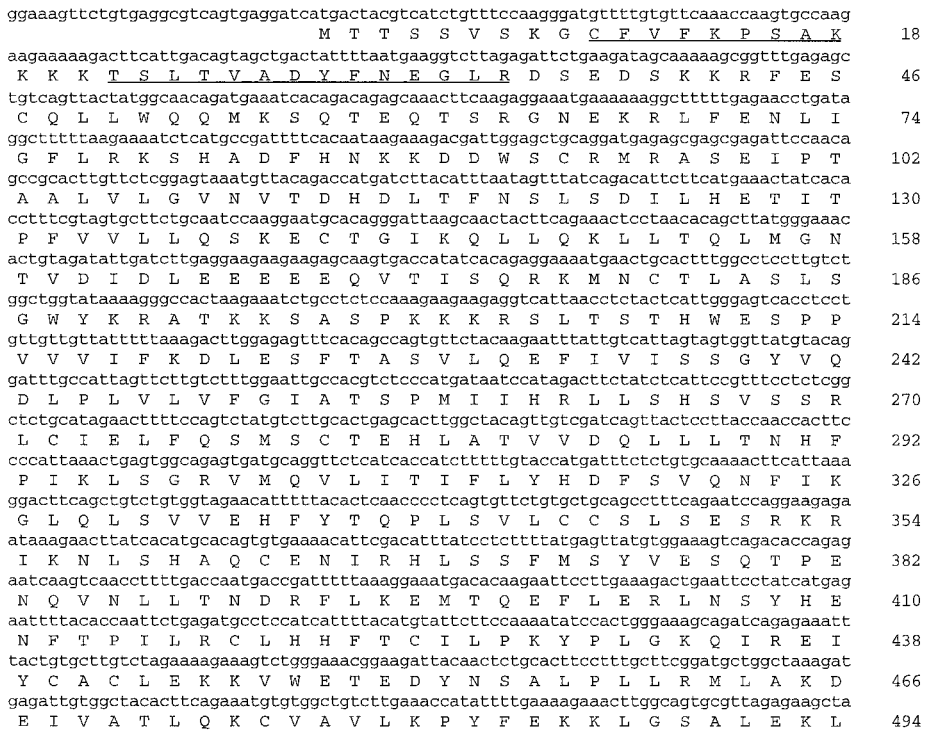

FIG. 3. **Nucleotide and predicted amino acid sequences of the p81 cDNA.** The sequences corresponding to the three tryptic fragments from immunopurified p81 are *underlined*.

tion with anti-Xorc2 antibodies, we asked whether the anti-p81 antibodies could be used to immunoprecipitate Xorc2 from egg extracts. Co-immunoprecipitation experiments were performed with anti-p81, anti-Xorc2, or control antibodies that had previously been coupled to protein A. After a 1-h incubation at 4 °C, the beads were washed extensively and eluted with SDS, and the SDS-eluted proteins were analyzed by immunoblotting with either anti-Xorc2 (Fig. 4*B*) or anti-p81 (Fig. 4*C*) antibodies. The results clearly demonstrate that Xorc2 could be efficiently immunoprecipitated with anti-p81 antibodies and that these antibodies could immunoblot the 81-kDa protein that had been immunoprecipitated with anti-Xorc2 antibodies. p81 and Xorc2 appeared to be tightly associated in the same complex since these two proteins could still be co-immunoprecipitated after washing in solutions containing 2 M NaCl or 1 M urea or in radioimmune precipitation assay buffer containing 1% Nonidet P-40 and 0.1% SDS (data not shown).

To characterize the expression of p81, we examined the levels of p81 as well as those of Xorc2 in developmentally staged *Xenopus* embryos. Embryos from a variety of stages, including ones before and after the mid-blastula transition (stage 8.5), were prepared and processed for immunoblotting with anti-p81 and anti-Xorc2 antibodies (Fig. 4*D*). The results indicate that p81 and Xorc2 are present through at least stage 28 of development (tail bud stage). Additionally, we found that p81 is present in *Xenopus* tissue culture cells and could be co-immunoprecipitated with Xorc2 in these cells (data not shown). Thus, p81 does not appear to be restricted to early embryonic cells with a very rapid S phase.

Depletion of p81 from Egg Extracts—To assess the role of p81 in DNA replication, we depleted endogenous p81 from interphase egg extracts by treatment with anti-p81 antibodies. Because of the high abundance of p81 in egg extracts (~5 ng/μl; data not shown) and the relatively low affinity of the anti-p81 antibodies, we found it necessary to use three rounds of depletion. After this procedure, we could no longer detect any p81 in the depleted extracts relative to extracts that had been treated with a control antibody (Fig. 5*A*, *lanes 1* and *2*). Significantly, we also could not detect any Xorc2 protein in p81-depleted extracts (Fig. 5*B*). Because Xorc2 could not be detected in p81-depleted extracts, we asked if p81 could be found in extracts that had been depleted of Xorc2. Interphase extracts were treated with anti-Xorc2 or control antibodies as described previously (18). Essentially all of the Xorc2 was removed (Fig. 5*C*), and DNA replication was reduced to 5% of the control level in this extract (data not shown). We then determined the concentration of p81 in this Xorc2-depleted extract. By immunoblotting, there was no detectable p81 protein in Xorc2-depleted extracts (Fig. 5*D*). Collectively, these results indicate that Xorc2 and p81 are associated quantitatively in the same

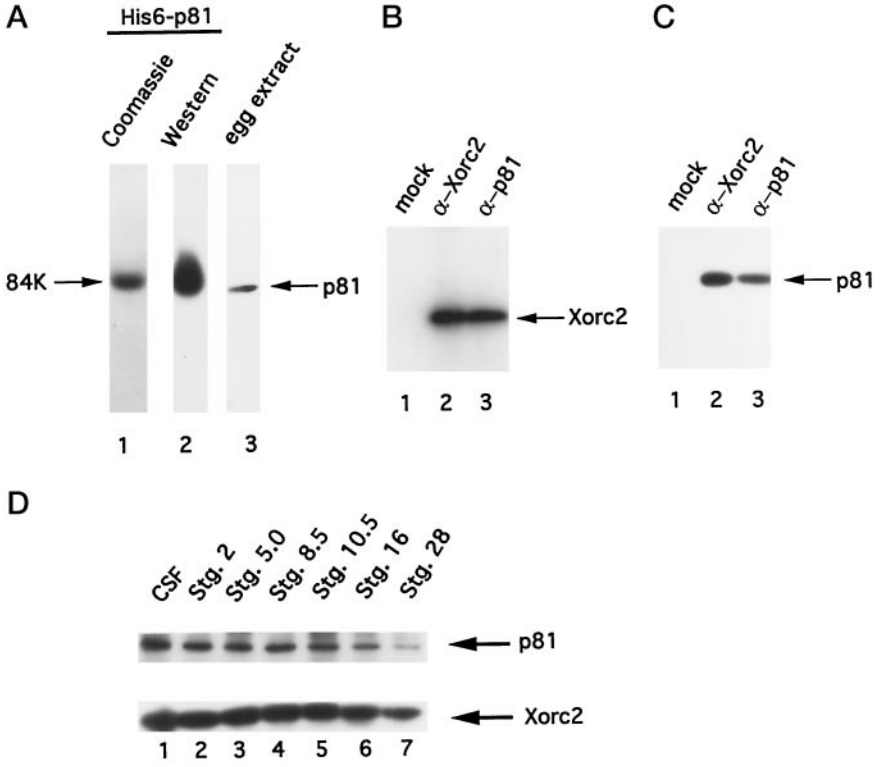

FIG. 4. **Characterization of *Xenopus* p81.** *A,* recombinant histidine-tagged p81 was stained with Coomassie Blue (3 μg of protein; *lane 1*) or immunoblotted with anti-p81 antibodies (50 ng of protein; *lane 2*). Interphase egg extract was immunoblotted with anti-p81 antibodies (*lane 3*). *B* and *C*, Xorc2 and p81 were co-immunoprecipitated. Interphase extracts (50 μl) were treated with control (*lane 1*), anti-Xorc2 (*lane 2*), or anti-p81 (*lane 3*) antibodies bound to protein A beads. The washed beads were processed for immunoblotting with either anti-Xorc2 (*B*) or anti-p81 (*C*) antibodies. *D,* p81 and Xorc2 were detected during *Xenopus* development. Extracts from *Xenopus* eggs (cytostatic factor (*CSF*) or staged (*Stg.*) embryos were immunoprecipitated with anti-p81 antibodies and immunoblotted with either anti-p81 (*upper panel*) or anti-Xorc2 (*lower panel*) antibodies.

complex.

Next, we measured the abilities of the p81-depleted and mock-depleted extracts to carry out the replication of exogenously added sperm chromatin. We performed a continuous labeling in the presence of [α-^{32}P]dCTP for 2 h at 23 °C and assessed the extent of DNA synthesis by agarose gel electrophoresis (Fig. 5*E*, *lanes 1* and *2*). We observed that incorporation of ^{32}P into chromosomal DNA was virtually abolished in interphase extracts that had been depleted of p81. This inhibition of replication was not a consequence of the inability of the extract to form intact nuclei around sperm chromatin, a requirement for DNA replication in the *Xenopus* cell-free system, since nuclear assembly occurred at similar rates in the p81-depleted and control extracts (data not shown).

To rule out the possibility that the immunodepletion procedure might irreversibly inactivate the extracts, we attempted to restore the replicative capacity to the p81-depleted extracts. Because recombinant p81 from Sf9 insect cells is completely insoluble and because endogenous p81 is present in a multiprotein complex in egg extracts, we did not attempt to rescue the p81-depleted extracts with exogenously added, recombinant p81. Instead, we asked whether chromatin that had been preloaded with XORC in S phase cytosol (22) could be replicated upon subsequent transfer to p81-depleted extracts. Indeed, we found that loaded chromatin underwent replication at an efficiency of ~80% in p81-depleted extracts relative to control depleted extracts (Fig. 5, *E* and *F*). The loaded chromatin by itself did not undergo replication unless incubated in a complete, membrane-containing extract (data not shown). Taken together, these results indicate that removal of p81 and its associated proteins (including Xorc2) from egg extracts completely abolishes the replication of chromosomal DNA. However, this defect can be bypassed if chromatin already containing XORC is added to the p81-depleted extract.

DISCUSSION

In this report, we have analyzed the composition of XORC from *Xenopus* egg extracts. We have used highly specific antibodies against Xorc2 to immunopurify a multiprotein complex consisting of Xorc2 and up to five additional polypeptides. Similar preparations of anti-Xorc2 antibodies, which recognize a single 63-kDa protein in egg extracts by immunoblotting, were used previously to immunodeplete Xorc2 and its associated proteins quantitatively from *Xenopus* egg extracts (18). These Xorc2-depleted extracts are completely defective in chromosomal DNA replication, indicating that the Xorc2-containing complex has an essential role in this process.

In addition to Xorc2 (p63), the immunoisolated complex consists of Xorc1 (p115), p81, p78, p45, and p40. Although a protein with the same mobility as p78 can be immunoprecipitated to some extent with control antibodies, the remaining five polypeptides (p115/Xorc1, p81, p63/Xorc2, p45, and p40) bind specifically to beads containing anti-Xorc2 antibodies. Among the unidentified proteins, we first focused our attention on p81. By cloning the cDNA encoding p81 and expressing recombinant His_6-p81, we were able to prepare antibodies that could immunodeplete this protein from extracts of *Xenopus* eggs. Immunodepletion with anti-p81 antibodies also resulted in the quantitative removal of Xorc2. Similarly, by immunodepleting with anti-Xorc2 antibodies, we were able to completely remove p81 from egg extracts. These experiments establish that both p81 and Xorc2 are quantitatively associated in the same complex. Furthermore, both p81 and Xorc2 appear to be tightly associated with this complex since both polypeptides can be co-immunoprecipitated with one another after washes in buffers containing 2 M NaCl, 1 M urea, or relatively harsh detergents (*e.g.* 1% CHAPS or a mixture of 1% Nonidet P-40 and 0.1% SDS). Significantly, p81-depleted extracts are also completely defective in chromosomal DNA replication.

In previous studies, two groups have also observed multiple proteins within putative XORC. Rowles *et al.* (20) isolated a complex of up to nine polypeptides using anti-Xorc1 antibodies to follow its purification through a number of chromatographic steps. These polypeptides include p168, p115/Xorc1, p110, p86, p74, p63/Xorc2, p50, p46, and p40. Of these, p110 and p50 were

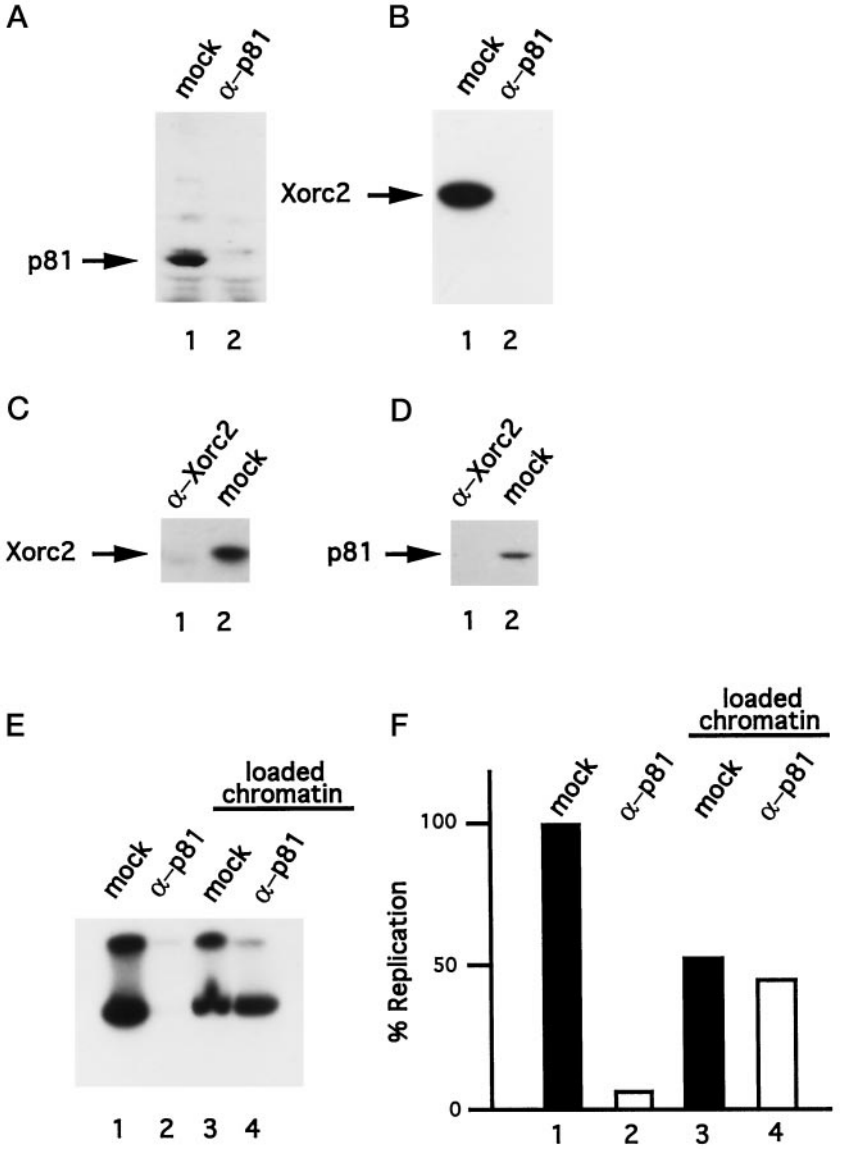

FIG. 5. **Immunodepletion of p81 from *Xenopus* egg extracts abolishes DNA replication.** *A* and *B,* immunoprecipitation with anti-p81 antibodies quantitatively removes both p81 and Xorc2 from egg extracts. Interphase egg extract (50 μl) was treated with either control (*lane 1*) or anti-p81 (*lane 2*) antibodies bound to protein A beads. Following removal of the beads by centrifugation, the supernatants were subjected to immunoblotting with either anti-p81 (*A*) or anti-Xorc2 (*B*) antibodies. *C* and *D,* immunoprecipitation with anti-Xorc2 antibodies quantitatively removes both Xorc2 and p81. Interphase egg extracts were treated with either anti-Xorc2 (*lane 1*) or control (*lane 2*) antibodies bound to protein A beads. Following removal of the beads, the supernatants were immunoblotted with either anti-Xorc2 (*C*) or anti-p81 (*D*) antibodies. In this experiment, DNA replication in the Xorc2-depleted extract was reduced to 5% of the control value. *E* and *F,* measurement of DNA replication in p81-depleted extracts. Control depleted (*mock*; *lanes 1* and *3*) or p81-depleted (*lanes 2* and *4*) extracts were supplemented with [α-^{32}P]dCTP and sperm chromatin to measure DNA replication. The same extracts were also assayed for their ability to replicate chromatin that had been preloaded with XORC by incubation in S phase cytosol (*lanes 3* and *4*). *F* is a quantitation of *E*.

relatively minor components. Furthermore, p86 did not cofractionate precisely with p115/Xorc1 and p63/Xorc2 during MonoS chromatography. In other studies with anti-Xorc1 antibodies, Romanowski *et al.* (30) were able to immunoprecipitate a complex of five proteins, including Xorc1, Xorc2, a protein that migrated between Xorc1 and Xorc2 on SDS gels, and two proteins that migrated between Xorc2 and a 45-kDa marker protein. Although the composition of XORC could not be established conclusively in these two studies, it appears that this complex consists of five to nine polypeptides, including at least one unknown protein with a molecular mass of 74–85 kDa that migrates between Xorc1 and Xorc2 on SDS gels. A generally similar polypeptide composition has been observed for ORC from *Drosophila* (17). Based upon our studies, it appears that the p81 described in this paper is also present in the XORC preparations of both Rowles *et al.* (20) and Romanowski *et al.* (30).

Interestingly, although p81 possesses close homologs of previously undetermined function in humans and mice, there appears not to be an obvious homolog of this protein in budding yeast ($E < 0.01$). This conclusion is based on a search of the *S. cerevisiae* genome with the BLAST and PSI-BLAST programs. Among the budding yeast ORC subunits, p81 scored highest with Orc3, raising the possibility that p81 might represent a highly diverged form of Orc3 found in higher eukaryotes. Although homologs of *S. cerevisiae* Orc1, Orc2, Orc4, and Orc5 have been readily identified in metazoans (2), homologs of Orc3 and Orc6 have not been described thus far in other organisms. This might suggest that these particular subunits have not been conserved as stringently during evolution.

In principle, p81 could be an integral component of XORC that is required for initiation of replication on DNA templates, and indeed, this is the simplest and most obvious possibility. The limited similarity between p81 and budding yeast Orc3 would be consistent with this interpretation. The lack of close similarity between p81 and the budding yeast ORC subunits might be explained by a difference between origins of replication in higher eukaryotes and yeast. Alternatively, it is formally possible that p81 could represent either a tightly associated regulator of XORC or a factor that collaborates with XORC in the replication process. The functional reconstitution of XORC with recombinant proteins will be necessary to elucidate the precise function of p81 in the control of DNA replication.

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