that the remaining ice sheets (such as that of the West Antarctic) must be sensitive to minor climate warming. But the models for the glacio-hydro-isostatic adjustment of the Earth remove the principal argument for larger ocean volumes at that time, so that if the climate was indeed warmer during substage 5e then these residual ice sheets must be relatively stable in the presence of small temperature increases.

Another issue raised is the duration of the interglacial interval. The evidence from raised reefs is that sea levels were near their present level from at least 135 to ~120 kyr BP, an interval longer than the 7-12-kyr interval inferred from the oxygen isotope record from deep-sea cores and scaled by astronomical motions[4,5]. It has been usual to ascribe the discrepancy to inaccurate dating of the reefs, but even with recent improvements in uranium-series dating, ages as old as 130-132 kyr BP are still indicated for some Caribbean corals; because of the glacio-hydro-isostatic adjustments of the Earth at these localities, the ocean volumes would have reached their present value earlier than this, possibly by 135-137 kyr BP. Improved age constraints on interglacial reefs at or above present sea level, particularly from better sampling of the individual reefs, coupled to glacio-hydro-isostatic rebound models, should contribute considerably to resolving this apparent conflict of the evidence for the onset and duration of the last interglacial interval. □

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ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex

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A multiprotein complex that specifically recognizes cellular origins of DNA replication has been identified and purified from the yeast *Saccharomyces cerevisiae*. We observe a strong correlation between origin function and origin recognition by this activity. Interestingly, specific DNA binding by the origin recognition complex is dependent upon the addition of ATP. We propose that the origin recognition complex acts as the initiator protein for *S. cerevisiae* origins of DNA replication.

THE complex process of eukaryotic chromosomal replication must be carefully regulated throughout the cell cycle. It is likely that much of this regulation occurs at the level of the initiation of DNA synthesis. Studies of *Escherichia coli* chromosomal, bacteriophage, and eukaryotic viral DNA replication have resulted in a paradigm for the initiation of bidirectional DNA replication[1,2]. For each of these organisms, the first step is the sequence-specific recognition of the origin of DNA replication by an initiator protein. Binding of the initiator causes partial untwisting of the double helix adjacent to the recognition site and the subsequent action of a helicase leads to further unwinding of the DNA duplex. This unwound DNA structure serves as a template for the initiation of DNA synthesis. Although protein factors likely to be involved in the unwinding and elongation stages of eukaryotic chromosomal DNA replication have been described[3,4], proteins involved in the initial stage of eukaryotic origin recognition have not been identified.

The availability of short well characterized chromosomal origins of DNA replication derived from the yeast *S. cerevisiae* make it a particularly useful organism to study the earliest steps of eukaryotic DNA replication. Originally identified as chromosomal sequences able to confer high frequency of transformation on plasmid DNA[5], a subset of these autonomous replication sequences (ARS) were subsequently shown to act as true origins of replication in the chromosome[6-9]. Sequence comparison of numerous ARS elements led to the identification of an 11-base-pair sequence that is conserved across all ARS elements and is referred to as the ARS core consensus sequence (ACS)[10,11]. Studies of the sequences required for ARS function have found that although the ACS is necessary, sequences 3′ to the T-rich strand of the ACS also are required for ARS function[11]. A detailed analysis of *ARS1* identified four *cis*-acting elements that constitute this chromosomal origin of DNA replication[12]. In addition to an essential A element containing the ACS, three distinct elements within the 3′ region, B1, B2 and B3, are required for efficient *ARS1* function. One of these elements, B3, is a

binding site for the yeast DNA-binding protein ABF1 and can be substituted by the binding sites of other transcription factors. The remaining two elements, B1 and B2, have no known function, but seem to be functionally distinct from both each other and the B3 element.

Studies directed at identifying proteins that recognize *S. cerevisiae* origins of replication have identified several proteins that recognize sequences outside the ACS, including ABF1 (refs 11, 13–18). More recently, a single-stranded DNA-binding protein that recognizes the T-rich strand of the ACS has been described[19-21]. Despite these efforts, no protein has been identified that can recognize the double-stranded form of the ACS. We have used a DNase protection assay to identify a novel DNA-binding activity that specifically recognizes the double-stranded ACS in an ATP-dependent manner. Our findings suggest that this multiprotein complex acts as an initiator protein at chromosomal origins of DNA replication.

Identification and purification of ORC

Initial DNA binding studies using crude yeast nuclear extract and *ARS1* DNA detected only the abundant nuclear protein ABF1. Fractionation of this extract, however, revealed a distinct DNA binding activity. This activity protected both the A and B1 elements of *ARS1* from deoxyribonuclease I digestion. Moreover, this binding event showed a strong dependence on the addition of exogenous nucleoside triphosphates. Through the use of both conventional and DNA-affinity chromatography, this DNA-binding activity was purified to near homogeneity. Fractionation of the binding activity by glycerol gradient sedimentation indicated that it had a relative molecular mass of ~250,000 (M_r 250K). SDS–polyacrylamide gel analysis of fractions collected from the final glycerol gradient of the purified activity showed no proteins that could individually account for such a large size. Instead, eight polypeptides of M_r 120K, 116K, 97K, 72K, 62K, 56K, 53K and 50K co-sedimented with the DNA-binding activity (Fig. 1). Unless otherwise indicated, this material was used for all subsequent experiments. The abundance of the 120K, 116K and 97K polypeptides varied significantly between different preparations, suggesting that the 116K and 97K proteins are proteolytic products of the 120K polypeptide. Additional chromatographic steps were unable to separate this set of proteins. Together these findings suggest that a multiprotein complex is responsible for the origin binding activity. We will refer to this DNA-binding activity as the origin recognition complex (ORC).

ORC was biochemically unrelated to the previously identified factor(s) that recognize the single-stranded form of the ACS (refs 19–21). ORC binding was not reduced by the addition of up to 10,000-fold molar excess of the T-rich strand of the ACS. In addition, purified preparations of ORC were unable to alter the mobility of either single-stranded or double-stranded DNA fragments containing the ACS in non-denaturing polyacrylamide gels (data not shown).

Titration of ORC protein into the footprinting assay revealed

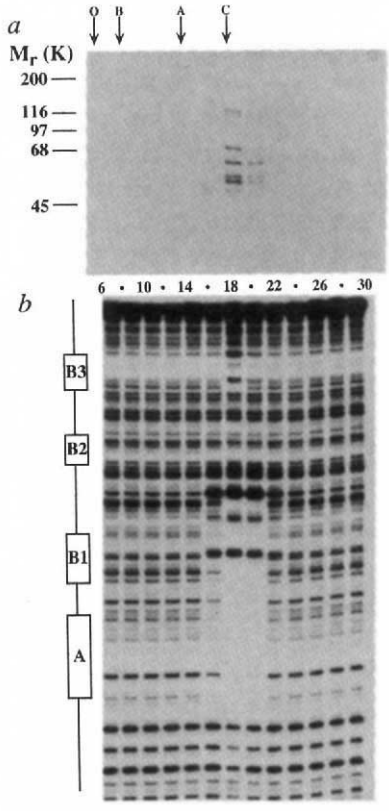

FIG. 1 Purification of ORC. *a*, Glycerol gradient sedimentation of ORC. Aliquots (40 μl) of every second fraction from the final glycerol gradient were precipated with trichloroacetic acid, dissolved in SDS sample buffer and analysed on a 10% SDS–polyacrylamide gel. Proteins on the gel were stained with silver. The fractions tested are indicated below. The position of the SDS–PAGE M_r size marker proteins are shown on the left. The position of protein markers sedimented in a parallel glycerol gradient are indicated above the gel: O, ovalbumin; B, bovine serum albumin; A, aldolase; C, catalase. *b*, Sedimentation of *ARS1* DNA-binding activity. DNase protection reactions were performed on the T-rich strand of the ACS of *ARS1* prepared as described in the legend to Fig. 2. Every second fraction (0.6 μl) collected from the final glycerol gradient was tested (indicated above each lane). The positions of the elements of *ARS1* are shown on the left[12]. No protein or DNA-binding activity was detected in fractions 1–5.

METHODS. Nuclear extract was prepared from 145 g (wet weight) of logarithmically growing BJ926 cells. All buffers contained 0.5mM PMSF, 1 mM benzamidine, 2 μM pepstatin A, 0.1 mg ml^{-1} bacitracin and 2 mM dithiothreitol. Nuclear extracts were prepared as described[43] with the following changes. Spheroplasts were washed by loading onto a 150-ml cushion of 0.8 M sucrose, 1.5% Ficoll and 20 mM Tris–HCl buffer, pH 7.5, in a 250-ml conical bottle. The spheroplasts were spun through the cushion at 3,000*g* for 10 min. Spheroplasts were lysed by a single pass through a Yamato LH-21 homogenizer. After dialysis against buffer H/0.1 (50 mM HEPES–KOH, pH 7.5, 0.1 M KCl, 1 mM EDTA, 1 mM EGTA, 5 mM magnesium acetate, 0.02% NP40, 10% glycerol) the nuclear extract was applied to an S-Sepharose column (Pharmacia-LKB) equilibrated with H/0.1 (2.5 × 9 cm, 15 mg protein per ml resin). The column was washed with buffer H/0.2 (the above buffer with 0.2 M KCl) and developed with a 500-ml linear gradient of KCl (0.2–0.6 M) in buffer H. The active fractions (0.38 M KCl) were pooled and dialysed against T/0.18 (25 mM Tris–HCl, pH 8.0, 0.18 M KCl, 1 mM EDTA, 1 mM EGTA, 5 mM magnesium acetate, 0.02% NP40, 1 mM DTT, 10% glycerol). Proteins were then loaded on to a Q-Sepharose column equilibrated in T/0.18 (1.5 × 3 cm, 6 mg protein per ml resin) and eluted with a 60-ml linear gradient of KCl (0.18–0.6 M) in buffer T. The active fractions (0.3 M KCl) were dialysed against buffer H/0.1. The dialysed pool was loaded onto a double-stranded DNA–cellulose column (Sigma) equilibrated in H/0.1 (0.3 × 3.5 cm, 5 mg protein per ml resin) and eluted by a linear gradient of KCl (0.1–0.6M) in buffer H. The active fractions (0.2M KCl) were dialysed against H/0.1. The dialysed pool (2.6 mg) was brought to 0.5 mM ATP, 5 mM $CaCl_2$ and 10 mM $MgCl_2$ and loaded onto a sequence-specific DNA affinity column equilibrated in buffer H^+/0.1 (H/0.1 containing 0.5 mM ATP, 5 mM $CaCl_2$, and 10 mM $MgCl_2$) and prepared as described[44] using the oligonucleotides 5′-GATCTAAACATAAAATCTGTAAAA-3′ and 5′-GATCTTTTACAGATTTTATGTTTA-3′. Bound proteins were eluted with a linear gradient of KCl (0.1–0.6M) in buffer H^+. The active fractions (0.2M KCl) were dialysed against H/0.1. Proteins were concentrated on a 200-μl double-stranded DNA–cellulose column equilibrated in H/0.1 followed by a step elution with H/0.5. The peak fractions were applied to a 5-ml, 15–35% glycerol gradient in buffer H/0.15 and spun at 225,000*g* for 12 h. Fractions (200 μl) were collected from the bottom of the gradient. The two peak fractions were concentrated on double-stranded DNA–cellulose and applied to a second glycerol gradient prepared and sedimented as before. Fractions (175 μl) were collected from the top of the gradient. This procedure yielded ~10 μg purified ORC protein. A footprinting assay was used to detect ORC activity throughout the preparation. Footprinting reactions are described in Fig. 2 legend.

several interesting features of this DNA-binding event (Fig. 2, lanes 1–7). At low concentrations of ORC protein, three sites of enhanced DNase I cleavage were observed, one within element B1 and two between elements B1 and B2 of *ARS1* (lane 2). Increasing amounts of ORC protein protected a 50-base-pair region that included elements A and B1 and enhanced DNase cleavage at two additional sites between elements B1 and B2 (lanes 3 and 4). Binding of ORC to the A-rich strand of *ARS1* showed a similar DNase footprint, but the sites of enhanced cleavage were offset two to three base pairs towards the ACS (Fig. 2, lanes 8–10). The observed 10-base-pair periodicity of DNase cleavage on both strands of *ARS1* suggests that the DNA may wrap around ORC during binding[22]. At higher concentrations there were new regions of protection, including weak protection of the B2 element and a second region of alternating protection and enhanced cleavage with a 10-base-pair periodicity encompassing the B3 region of *ARS1* (Fig. 2, Lanes 5 and 6).

Specificity of ORC DNA binding

The DNase protection pattern produced by ORC binding encompassed two DNA sequence elements that are critical for *ARS1* function. To address whether either of these elements are recognized by ORC, several linker scanning mutants in *ARS1* (ref. 12) were tested directly for ORC binding (Fig. 3*a*). An *ARS1* mutant simultaneously defective in B1, B2 and B3 function (B123-KO) showed essentially the same DNase protection pattern as wild-type *ARS1*, indicating that the B elements were not required for specific recognition (lanes 9–11). Similarly, a linker scanning mutant located between elements A and B1 that did not affect *ARS1* function[12], had no effect on ORC binding (lanes 3 and 4). In contrast, two mutants that disrupt the A element and prevent *ARS1* function (ARS1/858–865 and ARS1/865–872)[12] both dramatically altered the DNase protection pattern observed in the presence of ORC protein (lanes 5–8). Unlike the wild-type situation, no protection was observed over the A or B1 elements. Instead, there was a new region of protection encompassing the B2 element. The B2 element of *ARS1* is composed of a 9 out of 11 match to the ACS in the opposite orientation to the exact match located within the A element. The observed protection pattern, which initiates at B2 and extends towards the B1 element, suggested that in the absence of an exact match to the ARS consensus the ORC protein bound to the imperfect ACS at B2. Interestingly, in the absence of a functional A element the protection pattern at B3 is unchanged, suggesting additional interactions with these sequences independent of the A element. These findings, however, suggest that the ACS is the predominant element responsible for ORC DNA binding.

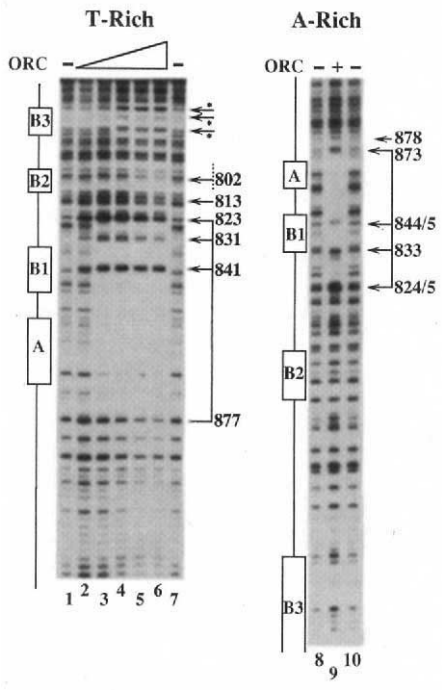

FIG. 2 ORC binds multiple regions of the *ARS1* origin of DNA replication. DNase footprints were done with DNA fragments containing *ARS1* sequences between 734 and 926 (ref. 5) labelled with ^{32}P at the 5′ end of either strand (lanes 1–7, the T-rich strand of the A element labelled; lanes 8–10, the A-rich strand of the A element labelled). Lanes 1, 7, 8 and 10 contain no protein. Lanes 2 to 6 contain 4, 8, 16, 32 and 64 ng ORC respectively. Lane 9, 16 ng ORC. The positions of the elements of *ARS1* are indicated to the left of each footprint. The positions of regions of enhanced or reduced DNase cleavage are indicated to the right of each panel. Brackets and asterisks indicate regions of DNase protection. Arrows indicate sites of enhanced DNase cleavage and the numbers to the right indicate their position.

METHODS. Binding reactions (25 μl) contained 37.5 mM HEPES–KOH, pH 7.5, 10 mM magnesium acetate, 3 mM EGTA, 0.5 mM EDTA, 200 μM each of CTP, GTP and UTP, 4 mM ATP, 125 μM each of dCTP, dGTP and TTP, 25 μM dATP, 2% polyvinyl alcohol, 5% glycerol, 200 ng poly d(A-C), 40 mM creatine phosphate and 0.06 units of creatine phosphokinase. Binding was at 30 °C for 10 min, followed by standard DNase digestion[45].

To address more precisely the role of the ACS in ORC DNA binding, seven point mutations were constructed in this sequence at the A element of *ARS1*. All of the mutants showed reduced DNase protection by ORC relative to wild-type *ARS1* DNA, although the extent of this reduction varied (Fig. 3*b*). Only one mutant, 860T→A, retained significant, albeit reduced, protection of the A element of *ARS1* in the presence of ORC protein. Four other mutants, 859T→A, 859T→C, 860T→C, and 863A→G, all showed minimal, if any, protection of the ARS consensus sequence, but retained the strong enhanced DNase cleavages characteristic of ORC binding and significant protection of a portion of the B1 element (lanes 4–7, 10–11, and 16–17). The most deleterious of the point mutations tested were 860T→G and 863A→C (lanes 12–15). These mutants showed little or no protection over either the A or B1 elements of *ARS1* and exhibited a significant reduction in the extent of enhanced DNase cleavage. In addition the point mutants also show weak protection of the B2 element, consistent with the binding observed with the linker scanning mutants within the A element of *ARS1*. Taken together, the results of the mutant DNA binding studies confirm that the ACS is required for the recognition of *ARS1* by ORC.

To correlate the effect of these *ARS1* point mutations on ORC binding with the function of *ARS1* as an autonomous replicating sequence, these same point mutants were tested for their function in a plasmid stability assay. The results of these studies show a strong correlation between ORC binding and plasmid stability. Thus, the mutation that reduced ORC DNA binding the least, 860T→A, retained the ability to transform yeast at high frequency. The plasmid stability of these transformants was about five-fold less than wild-type *ARS1*, consistent with the reduced binding of ORC to this mutant. Of the remaining mutants, only 859T→C transformed yeast at high frequency, and the plasmid stability of these transformants was reduced by at least 50-fold relative to 860T→A, as expected, given the reduced binding of ORC to this mutant. The close correspondence between ORC binding and ARS function exhibited by these mutants supports the hypothesis that ORC both recognizes and functions at the ACS.

ORC binding at other ARS elements

To address whether ORC binds other yeast origins of replication or exclusively *ARS1*, we tested ORC binding to three additional well characterized ARS elements, *ARS 307*, *H4 ARS* and *ARS 121*. Like *ARS1*, *ARS 307* and *ARS 121* act as chromosomal origins of DNA replication (C. Newlon, personal communication)[23]. The *H4 ARS* has not been tested for origin function. Strong ORC-dependent DNase protection was observed over the minimal sequences required for the function of the

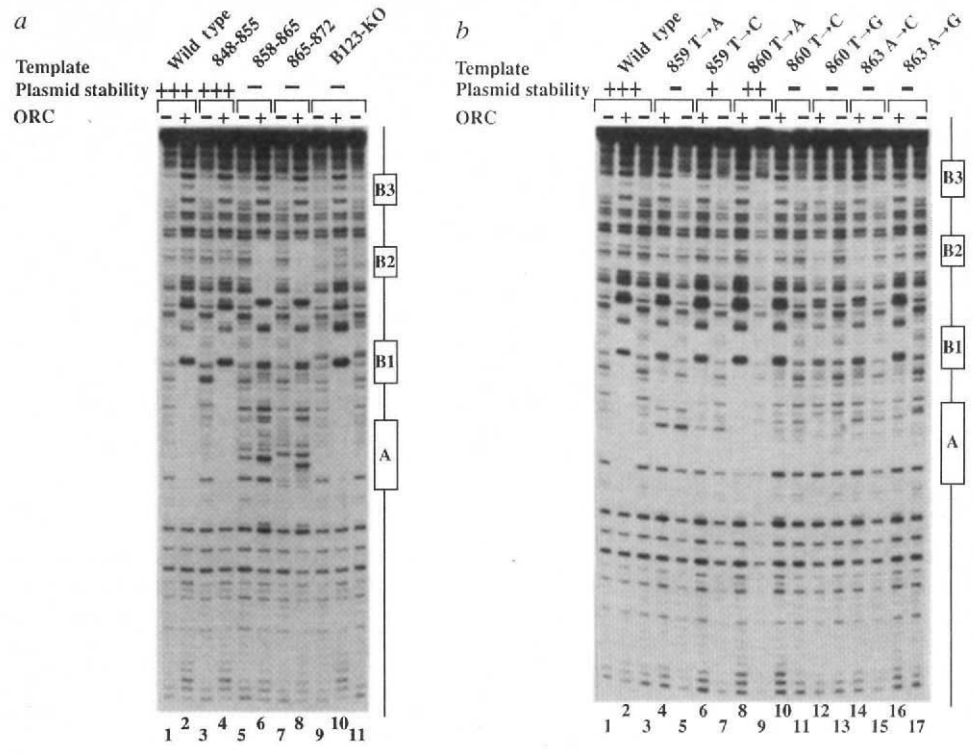

FIG. 3 DNA-binding specificity of ORC. *a*, ORC binding to *ARS1* is disrupted by mutants in the A element. Wild-type or mutant *ARS1* DNA fragments labelled at position 926 and extending to position 734 were used for DNase footprinting (T-rich strand of the A element is labelled). Templates used are indicated above each set of reactions (B123-KO is ARS1/756, 758, 802-808, 835-842)[12]. Lanes 1, 3, 5, 7, 9 and 11 contain no protein. Lanes 2, 4, 6, 8 and 10 contain 32 ng ORC. The elements of *ARS1* are indicated to the right. The plasmid stability of each construct is indicated above each set of reactions[12]. *b*, Point mutants in the *ARS1* ACS reduce ORC binding and *ARS1* function. Wild-type *ARS1* or point mutants in the 11 out of 11 match to the ARS consensus sequence at *ARS1* labelled at position 943 and extending to position 734 were used for DNase footprinting (T-rich strand of the A element is labelled). Templates used are indicated above each set of reactions. Lanes 1, 3, 5, 7, 9, 11, 13, 15 and 17 contain no protein. Lanes 2, 4, 6, 8, 10, 12, 14 and 16 contain 24 ng ORC. The elements of *ARS1* are indicated to the right. The plasmid stability of each construct is indicated above each set of reactions: +++, 30–40%; ++, 6–7%; +, high frequency of transformation-positive, plasmid stability below detection (<0.05%); −, minus for high-frequency of transformation. METHODS. DNase footprinting was performed as for Fig. 2. High frequency of transformation and plasmid stability assays were as described[12]. Point mutants in the *ARS1* ACS were constructed by site-directed mutagenesis.

three ARS elements (Fig. 4). In each case, the region of protection included the essential match to the ACS as determined by previous mutagenesis studies[24–26]. As with *ARS1*, these protected regions were ~50 base pairs in length and were located asymmetrically with respect to the ACS, extending significantly further in the direction 3′ to the T-rich strand of the consensus sequence. The *ARS 307* and *H4 ARS* footprints also showed similar ORC induced sites of enhanced DNase cleavage as seen in *ARS1*. At each ARS these sites initiate 12–16 base pairs 3′ to the T-rich strand of the ACS and are spaced by ~10 base pairs (lanes 1–6), with the exception of one slightly offset site in *ARS 307*. The absence of enhanced bands at *ARS 121* may be due to the lack of an exact match to the ACS at this origin of DNA replication. Although there are a number of other partial matches to the ARS consensus sequence found in all of these ARS elements, only the essential match is fully protected from DNase digestion. The selective protection of the essential match to the ACS at these three distinct ARS element indicates that ORC recognizes other origins of replication in addition to *ARS1* and strongly suggests that ORC mediates the function of the ACS at these sequences as well.

Identification of DNA proximal subunits of ORC

To identify the subunits of ORC close to the DNA at the time of binding we crosslinked the complex to radiolabelled DNA. To prepare the DNA for crosslinking, ^{32}P-labelled dCTP and a photocrosslinking nucleotide (N_3RdUTP; ref. 27) were incorporated at specific sites within the region of *ARS1* protected from DNase digestion by ORC. When these modified *ARS1* DNA fragments were exposed to ultraviolet light in the presence of purified or partially purified ORC, polypeptides within reach of the photoreactive nucleotide were covalently linked to the ^{32}P-labelled DNA (Fig. 5). Using partially purified preparations of ORC, multiple polypeptides were crosslinked to DNA in this manner (Fig. 5, lane 2). DNA competition experiments using *ARS1* DNA fragments that contained either a wild-type or mutant ACS identified three polypeptides of M_r 72K, 62K and 53K that competed specifically with wild-type but not mutant DNA (compare lanes 3 and 4). Crosslinking of the same set of three polypeptides was reduced significantly when nucleoside triphosphate (ATP) was omitted from the reaction mixture. The remaining polypeptides exhibited enhanced crosslinking, presumably because of increased accessibility to the modified nucleotide (lane 6). When the same crosslinking reaction was done using purified ORC preparations, only four polypeptides were crosslinked, including the three polypeptides identified as sequence-specific by DNA competition experiments (lane 1). These same four polypeptides are abundant in purified ORC preparations and comigrated exactly with the crosslinked adducts when run side-by-side on the same SDS gel (data not shown). The finding that the 120K subunit of ORC is non-specifically crosslinked suggests that either it is a nonspecific DNA-binding protein associated with ORC or a contaminant of the ORC preparation. Although our experiment used a unique site of modification (position 853 in *ARS1*; between B1 and A), similar results were obtained when other sites of modification were used (data not shown). These findings indicate that the 72K, 62K and 53K polypeptides constitute part of the origin recognition complex and are closely juxtaposed to the DNA during binding. Nevertheless, owing to the long reach of the photocrosslinking nucleotide used (9–10 Å)[27], it is possible that either one or some combination of the crosslinked polypeptides is responsible for specific recognition of the ACS.

Nucleotide requirement for ORC DNA binding

The unusual requirement for nucleoside triphosphates to allow specific DNA binding by ORC was maintained throughout its purification. To address the specificity of this requirement, footprinting experiments were done in the absence or presence of a number of different nucleotides (Fig. 6). In the absence of added nucleotides, the DNase cleavage pattern was nearly identical to that observed in the absence of added protein (compare

lanes 6 and 7). Addition of ATP alone resulted in a protection pattern that was indistinguishable from that when all eight nucleoside triphosphates were added to ORC footprinting reactions (compare Fig. 6, lane 2 with Fig. 2). None of the remaining three ribonucleoside triphosphates stimulated DNA binding at a level comparable to ATP, although the addition of CTP allowed a low but significant level of ORC binding. At higher concentrations (1mM), all four ribonucleoside triphosphates stimulated ORC DNA binding (data not shown) but the effect of ATP remained significantly stronger than CTP, GTP and UTP. To determine the role of the triphosphates during ORC binding, two ATP analogues were tested for their ability to function in the DNA binding assay. ATP-γ-S stimulated ORC DNA binding at a level comparable to ATP (lane 10). In contrast, AMP-PNP (β-γ-imidoadenosine 5′-phosphate), which unlike ATP-γ-S has a non-hydrolysable β-γ bond, was unable to stimulate ORC DNA binding (lane 11).

The exact role of ATP in ORC DNA binding remains unclear. The inability of AMP-PNP to substitute for ATP during footprinting suggests that hydrolysis of the ATP β-γ bond is required for sequence-specific DNA binding by ORC. These findings suggest that ORC undergoes a significant change in conformation upon binding, and possibly hydrolysis, of ATP. Such a change could involve an alteration in the ORC DNA binding domain or alternatively a rearrangement of one or more of the ORC subunits. Despite the apparent requirement for ATP hydrolysis, we have yet to detect an ATPase activity in purified ORC preparations. It is also possible that the ATP requirement reflects a protein phosphorylation event, although autophosphorylation has not been detected in purified preparations of ORC either in the presence or absence of DNA.

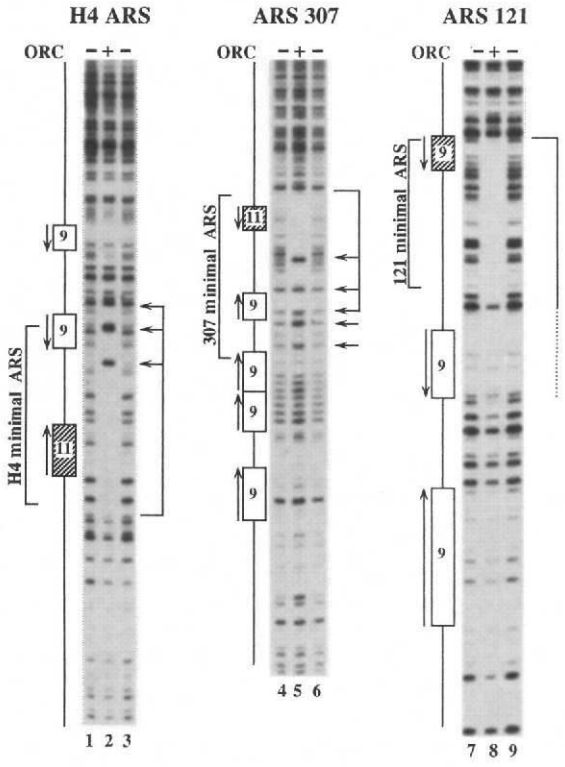

FIG. 4 ORC recognizes essential sequences in three other ARS elements. In lanes 1–3 an *H4 ARS*-containing DNA fragment labelled at position 1 and extending to position 174 was used for DNase footprinting (the T-rich strand of the ACS match is labelled; numbering according to ref. 24). In lanes 4–6 an *ARS 307*-containing DNA fragment labelled at position 1 and extending to position 522 was used for DNase footprinting (the A-rich strand of the ACS match was labelled; numbering according to ref. 25). In lanes 7–9 an *ARS 121*-containing DNA fragment labelled at position 486 and extending to position 1 was used for DNase footprinting (the A-rich strand of the essential match to the ACS is labelled, numbering according to ref. 26). Lanes 1, 3, 4, 6, 7 and 9 contain no protein. Lanes 2, 5 and 8 contain 16 ng ORC. Matches to the ACS are shown in boxes to the left of each panel (9, 9 out of 11 match; 11, exact match). The essential match to the ACS for each ARS is shaded[24–26]. Vertical arrows indicate the 5′ to 3′ direction of the T-rich strand of the ACS. The horizontal arrows indicate the DNase hypersensitive sites are at positions 126, 116/117 and 107 from top to bottom at *H4 ARS*. For *ARS 307* hypersensitive sites are at positions 170, 158, 149, 146 and 139 from top to bottom. Bracketed regions to the left of each panel indicate the minimal regions of each ARS required for high frequency of transformation[23–25]. Bracketed regions to the right of each panel indicate regions of DNase protection.

Discussion

The replicon model[28] hypothesized that DNA replication would be controlled by a positive regulator (the initiator) acting on a *cis*-sequence (the replicator). Studies of *E. coli*, bacteriophage and eukaryotic viral DNA replication have largely upheld these predictions. Unlike these simple replication units in which a single bidirectional origin of DNA replication is sufficient to direct all DNA synthesis, eukaryotic chromosomal DNA replication involves coordinated initiation from multiple origins. In yeast, a subset of ARS elements act as origins of replication and the ARS consensus sequence very likely fulfills the role of a replicator. The corresponding initiator protein, however, has

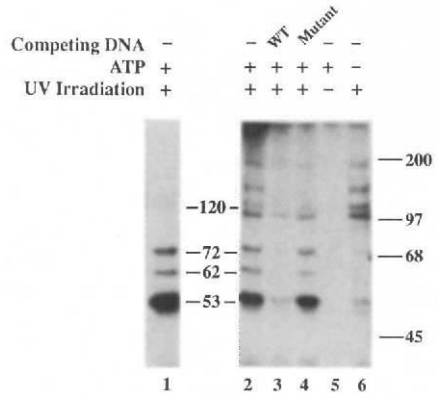

FIG. 5 Ultraviolet (UV) photocrosslinking of ORC polypeptides to *ARS1*. A DNA fragment of ARS1/848–855 (wild type for ARS function[12] and ORC DNA binding; Fig. 3) extending from position 734 to position 926 was modified with N_3RdUMP at position 853 and with [^{32}P]dCMP at positions 852, 854 and 855 and used for ultraviolet photocrosslinking (4 ng per reaction). Lane 1 contains 16 ng glycerol-gradient-purified ORC; lanes 2–6 contain 80 ng partially purified ORC preparation following sequence-specific DNA affinity chromatography. All reactions contained 200 μM ATP except 6. Reactions 3 and 4 contained 40 ng of an *ARS1* DNA fragment extending from position 852 to 926 and containing 35 base pairs of linker DNA 3′ to the T-rich strand of the ACS. The fragment added to reaction 3 contained a wild-type (WT) ACS, whereas the fragment added to reaction 4 was derived from the ACS mutant ARS1/858-865 (ref. 12). All reactions were irradiated with UV light except 5. The positions of the M_r ($\times 10^{-3}$) size marker proteins are shown on the right. The migration of the corresponding unmodified ORC polypeptides is shown between the two panels.

METHODS. The substituted N_3RdUMP probe was prepared as described[27] with the following changes: the *Hin*dIII to *Eco*RI fragment of pARS1/WTA (ref. 12) was subcloned into *Hin*dIII- and *Eco*RI- cut M13mp19 replicative form DNA and the single-stranded DNA produced (the A-rich strand of the ACS) by the resulting phage was used as template. The annealed oligonucleotides used were the −40 universal sequencing primer and a second oligonucleotide hybridizing to the A-rich strand of the *ARS*1 between positions 871 and 856. DNA binding as described in Fig. 2 legend, except the only nucleoside triphosphate added was ATP (200 μM) and polyvinylalcohol was not added to the reactions. After binding, reactions were transferred to a microtitre plate and irradiated for 4 min with a short-wave UV light at a distance of 0.75 cm. Crosslinked proteins were treated with DNase (1 μg) and micrococcal nuclease (7.5 U) and separated on a 10% SDS–polyacrylamide gel. Competitor DNA was prepared by cutting the *Hin*dIII–*Kpn*I fragment from the plasmids pABox/WT and pABox/858–865. These plasmids were prepared by cloning the *Bgl*II to *Kpn*I fragments of either pARS1/WTA or pARS1/858–865 (ref. 12) into pUC119 cut with the same enzymes.

been elusive. Our results make ORC a strong candidate initiator protein in yeast cells.

The DNA-binding specificity of ORC strongly supports this hypothesis. Point mutants within the ACS of *ARS1* that reduced ORC DNA binding in each case resulted in an equivalent reduction of *ARS1* function. At three other ARS elements the essential match to the ACS is also protected from DNase by ORC. Moreover, at all the ARS elements tested for ORC binding, the boundaries of the observed footprint correlate with the minimal sequences required for ARS function. Despite recognizing the ACS, ORC protects a much larger region of DNA extending predominantly 3′ to the T-rich strand of the ACS. This asymmetric pattern of protection suggests that one or more of the ORC subunits are in an ideal position to act upon these essential 3′ sequences. Although ORC does not specifically recognize the DNA 3′ to the T-rich strand of the ACS, both DNA competition experiments (data not shown) and the alternating pattern of enhanced and protected DNase cleavage observed at these sequences suggest that important nonspecific contacts are made with this region of ARS elements. Consistent with this hypothesis, mutations that would be expected to disrupt the interaction of ORC with the 3′ region, such as the precise inversion of the ACS, result in a loss of ARS function[23,29]. Furthermore, the enhanced DNase cleavages observed at the 3′ sequences suggests that the DNA is wrapped around ORC[22] and is reminiscent of the pattern observed when the *E. coli* initiator protein dnaA binds to its cognate origin, *oriC*[30]. Protein–DNA complexes involving wrapping have been implicated in the early events of DNA replication in several organisms[31,32].

The intriguing finding that ATP is required for specific DNA binding by ORC distinguishes it from most DNA-binding proteins. Of the sequence specific DNA-binding proteins known to bind ATP, many but not all (for example, see refs 33 and 34) are involved in the recognition of origins of replication, including SV40 and polyoma T-antigen, dnaA protein, bovine papillomavirus E1 protein, and herpes simplex virus UL9 protein (OBP)[2,35]. Of these initiator proteins, only the DNA recognition properties of T-antigen are directly affected by ATP[36–38]. Studies of these intiator proteins suggest that ATP may have other roles in ORC function in addition to facilitating sequence-specific DNA binding. For example, ATP is required for the untwisting of the DNA helix by T-antigen and dnaA protein[1,39]. ATP hydrolysis could also be important in regulation. Studies of *oriC* replication indicate that hydrolysis of ATP by dnaA protein results in the inactivation of its replicative properties[40,41]. A similar mechanism involving eukaryotic initiator proteins could help prevent reinitiation during the S phase of the cell cycle. Further studies of the ATP requirement of ORC will be necessary to address these possibilities.

In addition to the results of the purification, the multiprotein nature of ORC is supported by the large size predicted by glycerol gradient sedimentation and the multiple proteins identified by ultraviolet photocrosslinking experiments (Fig. 5). Despite these findings, the exact composition of ORC remains to be confirmed. The development of new reagents, such as antibodies against the individual subunits, will help us understand the subunit structure of ORC. There is precedent for the use of multiple proteins to recognize an origin of DNA replication. The bovine papilloma virus initiator proteins E1 and E2 recognize their cognate origin of replication cooperatively[35]. Mutiprotein complexes are frequently assembled after origin recognition by an initiator protein[2]. In yeast, ORC may obviate a subset of these assembly steps by maintaining a complex of proteins associated at all times.

The properies of ORC and the recently identified single-stranded DNA-binding protein(s) that recognize the ACS (refs 19–21) are distinct. While the single-stranded binding proteins recognize only the T-rich strand of the ACS, ORC recognizes the consensus sequence in its duplex form. In addition, the multiprotein nature of ORC, the ATP dependence of ORC DNA

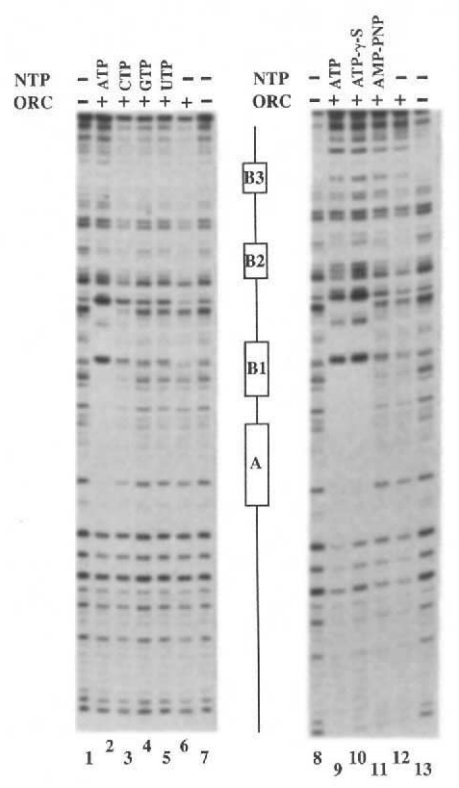

FIG. 6 ATP dependence of ORC DNA binding. *ARS1* DNA fragments labelled at position 926 and extending to position 734 were used for DNase footprinting (T-rich strand of the A element was labelled). Binding conditions were as described for Fig. 2 except that only a single indicated nucleoside triphosphate was added (at 80 μM) and creatine phosphate and creatine phosphokinase were omitted from the reactions. The NTP added is shown above each lane. Reactions 2–6 and 9–12 contained 16 and 24 ng of ORC respectively. Reactions 1, 7, 8 and 13 contained no protein. The position of the elements of *ARS1* are indicated between the two panels.

binding, and the inability of ORC to alter the mobility of ACS-containing DNA fragments in non-denaturing polyacrylamide gels, all set it apart from the single-stranded DNA-binding protein(s). Although it remains possible that these single-stranded DNA-binding proteins function at *S. cerevisiae* origins of replication, on the basis of studies of bidirectional DNA replication in other organisms[2], it is unlikely that they function in the initial recognition of the origin. Instead, we propose that ORC recognizes the origin in its double-stranded form and that if the single-stranded DNA-binding proteins play any part, they are involved in events that occur after the DNA duplex is opened. Recent high-resolution *in vivo* footprinting experiments support the hypothesis that ORC mediates ACS function[46]. These studies show a pattern of DNase protection at *ARS1 in vivo* that is remarkably similar to the pattern induced by ORC *in vitro*, including the characteristically enhanced cleavages at B1 and B2.

If ORC is the *S. cerevisiae* initiator protein, its characterization will allow significant insights into eukaryotic DNA replication and its regulation. In addition to the information gained from understanding the properties of ORC itself, the availability of a eukaryotic initiator protein complex would provide a powerful tool for the development of a *in vitro* replication system directed by a chromosomal origin of DNA replication. ORC also would represent a prime target for proteins that regulate the initiation of S phase. Finally, if ORC does represent the yeast initiator protein, it is likely that related proteins will function at the origins of replication in metazoa. The identification and characterization of these related initiator protein complexes could, in turn, facilitate identification of chromosomal replicator

sequences in multicellular organisms, which remain poorly understood[42]. □

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Glutamate-induced long-term potentiation of the frequency of miniature synaptic currents in cultured hippocampal neurons

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Glutamate application at synapses between hippocampal neurons in culture produces long-term potentiation of the frequency of spontaneous miniature synaptic currents, together with long-term potentiation of evoked synaptic currents. The mini frequency potentiation is initiated postsynaptically and requires activity of NMDA receptors. Although the frequency of unitary quantal responses increases strongly, their amplitude remains little changed with potentiation. Tests of postsynaptic responsiveness rule out recruitment of latent glutamate receptor clusters. Thus, postsynaptic induction can lead to enhancement of presynaptic transmitter release. The sustained potentiation of mini frequency is expressed even in the absence of Ca^{2+} entry into presynaptic terminals.

LONG-TERM potentiation (LTP), an activity-dependent change in synaptic efficacy, may be essential to learning and memory in the mammalian brain[1–5]. At hippocampal CA3–CA1 synapses, LTP is initiated by postsynaptic events that are relatively well understood[5–14]. By contrast, the mechanism of expression of increased synaptic strength is controversial[15–26]. Is presynaptic transmitter release enhanced during LTP? This possibility has been intensely debated since its first proposal by Bliss and colleagues[15]. A presynaptic component of LTP expression would require retrograde signalling from postsynaptic spines to presynaptic terminals[2–5,15–19], with important functional and developmental implications[27]. In studies of evoked synaptic events, some laboratories reported large increases in quantal content, consistent with presynaptic enhancement[16–19], whereas another group found only increased quantal size[20]. Rigorous quantal analysis is more difficult for excitatory transmission in the hippocampus[15–26] than in other systems[21,26,28–30].

Miniature synaptic events (minis) are synaptic responses to single packets of transmitter[28–30]. Minis are generally seen as isolated events, aiding distinctions between quantal size and release probability, and have thus provided insights into synaptic plasticity[31–38]. Changes in mini frequency and evoked responses occur in parallel during post-tetanic potentiation[31] and perturbations of presynaptic signalling[34–38]. Minis can be easily recorded in hippocampal neurons[37,39–41] but their usefulness in understanding mechanisms of LTP has been hampered by uncertainty about whether minis arise from potentiated or non-potentiated synapses[17,41]. Furthermore, findings of apparent changes in mini amplitude or frequency are by themselves ambiguous[20,24,41] without direct tests of postsynaptic responsiveness. Increased quantal size might signify enhanced postsynaptic responsiveness or elevated presynaptic vesicular content; elevated quantal frequency might reflect enhanced vesicular release or recruitment of latent postsynaptic receptor clusters[24,42].

To overcome these problems, we used minis in combination with direct measurements of postsynaptic responsiveness. We obtained clear evidence that postsynaptic mechanisms of LTP induction can lead to sustained enhancement of presynaptic

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