**Communication**

*In Vitro* Association of a Replication Complex with a Yeast Chromosomal Replicator*

(Received for publication, November 24, 1982)

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The activity that replicates the 2-μm yeast DNA plasmid *in vitro* can be isolated in a high molecular weight form (M₀ ≈ 2 × 10⁹) from cells of the budding yeast *Saccharomyces*. Protein complexes from fractions containing this activity associate with the replication origins of this extrachromosomal DNA element. In order to investigate the possibility that partially purified preparations of this high molecular weight material also contain a "replication complex" capable of initiation of DNA replication from a yeast chromosomal origin, the binding of protein complexes from these preparations to a yeast autonomously replicating sequence (ars 1) was analyzed by electron microscopy. Incubation of the complex with a plasmid carrying *ars* 1 resulted in the appearance of a protein "knob" on the DNA. This association was limited to a unique site on the *ars* 1, as determined after restriction endonuclease digestions. Incubation of the complex with the *ars* 1-carrying plasmid also resulted in the appearance of replication intermediates of the θ-form in the reaction mixtures. The origin of replication was mapped on the plasmid, using the same procedures, and it was localized at a single position on the *ars* 1 corresponding to the site at which protein knobs associated with the DNA. This result, coupled with the observation that no complexes were detected on replication intermediates, raises the possibility that the protein complex may be involved in initiation of replication. Although this replication complex resembled the one utilized by 2-μm DNA, it is not clear at present whether they are identical. A comparison of the known nucleotide sequences at the 2-μm and *ars* 1 origins of replication, however, reveals a limited homology.

Recent studies in prokaryotic systems have revealed extensive interactions among the various proteins involved in DNA replication (1, 2). Indications that complexes of replication proteins may exist in eukaryotic systems have also been obtained. Some of these complexes are small, composed of a DNA polymerase and a primase (3, 4). Others are large and appear to contain many proteins (5, 6); thus, they may represent an intact "replisome" (7).

We have been studying the replication of the yeast 2-μm DNA plasmid *in vitro* (8, 9). The replication of this double-stranded, closed-circular DNA molecule provides a model for the initiation of chromosomal replication (10) and a means for approaching the problem of control of nuclear DNA replication in the cell division cycle (11).

During the course of fractionation of the replication proteins in yeast extracts, we identified and partially purified a high molecular weight (~2 × 10⁹) form of 2-μm DNA replicating activity (6, 9). When fractions containing this putative replication complex were incubated with DNA, protein knobs were found associated nonrandomly with the 2-μm plasmids origins of replication (6). Comparison of the replication complex from early and late logarithmic phase yeast cells (6, 9) suggested that it may be assembled and disassembled during the cell cycle, prompting the hypothesis that control of assembly of the complex during the G₁ phase of the cell cycle may provide one means of exerting control over entry into, or orderly temporal progression through, the S phase (9).

In view of these findings, it was of interest to determine whether protein complexes from our preparations associate with yeast chromosomal replicators. Several yeast DNA fragments containing putative chromosomal origins of replication have been isolated by high frequency transformation of yeast cells (12-15). For a few of these, called *ars*, the portion of the DNA responsible for high frequency transformation and the nucleotide sequence have been determined (12, 13). We have chosen one of these, *ars* 1, for our studies. This sequence was isolated as a 1,453-base pair EcoRI fragment of yeast DNA inserted into the EcoRI site of the *Escherichia coli* plasmid pBR322 and carries, in addition, the yeast *TRP* 1 gene (16); this hybrid plasmid is known as YRp7.

In this communication, we show that protein complexes from active 2-μm DNA replicating fractions associated nonrandomly with *ars* 1 DNA *in vitro*. The site at which the complex was bound coincided with the origin of replication utilized by *ars* 1 in our *in vitro* system. The nucleotide sequences in the regions of the replication origins of *ars* 1 (12, 13) and 2-μm DNA (17) possess a limited homology.

**EXPERIMENTAL PROCEDURES**

Supercoiled YRp7 plasmid DNA was prepared as described (6) under P1 + EK1 containment. Replication complex was prepared from crude extracts of the yeast *Saccharomyces cerevisiae* C-105 by gel filtration and rate-zonal sedimentation, and fractions were assayed for DNA replication as before (6).

DNA was prepared for electron microscopy as described previously (6). Briefly, replication reaction mixtures were chilled and centrifuged through Sepharose CL-6B. The excluded material was fixed with glutaraldehyde and this reaction was quenched with ethanolamine. Ethanol-precipitated DNA was either spread for electron microscopy or digested with restriction endonuclease EcoRI or with EcoRI and HindIII. These enzymes were obtained from Bethesda Research Laboratories. EcoRI digestion was carried out according to the instructions of the supplier. Double digestion with EcoRI and HindIII was performed in 150 mM Tris-HCl, 6 mM MgCl₂, 50 mM NaCl, 2 mM 2-mercaptoethanol (pH 7.3). After digestion was completed, the reaction mixtures were centrifuged through Sepharose CL-6B, and the excluded material was prepared for electron microscopy by aqueous spreading.

* This work was supported by Grants GM-28099 and AI-11378 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviation used is: *ars*, autonomously replicating sequence.
RESULTS AND DISCUSSION

A restriction endonuclease fragment containing \textit{ars} 1 has been shown to be utilized in preference to adjacent DNA as a template for DNA synthesis by yeast cell extracts (18). We have found that the \textit{ars} 1-carrying plasmid YRp7 directed DNA synthesis \textit{in vitro} as efficiently as 2-\mu DNA.\footnote{S. M. Jazwinski, A. Niedzwiecka, and G. M. Edelman, unpublished results.} In view of the possible involvement of a replication complex in 2-\mu DNA replication (6, 9), the question arose whether the same or similar complex was involved in the replication of YRp7 DNA.

In order to determine whether a protein complex binds to YRp7 DNA, fractions containing the partially purified replication complex were incubated briefly with YRp7 DNA under replication reaction conditions. The DNA was separated from the reaction mixture, fixed, and prepared for electron microscopy. Among the supercoiled and open-circular plasmid molecules expected in the reaction mixture, molecules possessing what appeared to be protein knobs were found (Fig. 1A). These knobs resembled closely those found previously on 2-\mu DNA (6) and were of the same size (~40 nm, diameter), consistent with a complex of \textit{M}, $\approx 2 \times 10^6$. These knobs were only found when the DNA was incubated in the presence of replication complex (Table I, Experiment I) and were present on supercoiled and open-circular molecules with equal frequency. No molecules with more than one knob were found. Open-circular molecules with double-stranded “eyes” (theta forms) were also found (Table I), but none of these presumptive replication intermediates possessed protein knobs.

The knobs visualized on the plasmid DNA appeared to represent material from the replication complex (Table I, Experiment II). First, when DNA replicating activity was heat-inactivated prior to incubation with the plasmid, knobs were not found associated with the DNA. Second, when the replication reaction mixture was treated with proteinase K prior to the separation and fixation of the DNA, no knobs were detected. These results suggested that the presence of knobs on the DNA was dependent on protein contained in the plasmid replicating activity and that the knobs were not simply derived from the cytochrome \textit{c} added during DNA spreading for electron microscopy. Inasmuch as it was previously observed that knobs were not found associated with pBR322 DNA (6), their association must be limited to the yeast chromosomal DNA insert in YRp7. This conclusion will be substantiated further below.

In all their features, the protein knobs on YRp7 DNA resembled those found previously on 2-\mu DNA (6). Because protein knobs were not found associated with replicative intermediates, we surmise that, as with 2-\mu DNA, the replication complex as a unit may function in initiation rather than elongation, although this does not preclude a role of particular components of the complex in the latter process.

To determine the localization of the protein knobs on YRp7 DNA, their position was measured with respect to the nearest end of DNA fragments obtained by digestion of fixed plasmid DNA with the restriction endonuclease EcoRI. This enzyme excises precisely the yeast chromosomal DNA insert in YRp7 (16) resulting in two fragments, yeast DNA (1,453 base pairs) and pBR322 (4,362 base pairs). Protein knobs were found exclusively on the smaller, yeast DNA fragment (Fig. 1B) at a mean distance from the nearest end of 42%. In order to orient this nearest end with respect to the restriction map of the yeast DNA fragment, a double digestion of the YRp7-protein complex was performed with EcoRI and HindIII. This digestion results in the cleavage of the yeast DNA into two fragments (12, 16), one containing full \textit{ars} 1 activity (638 base pairs) and the other one the bulk of the TRP 1 gene (615 base pairs). Protein knobs were found exclusively on the larger of these fragments localizing the protein knobs in the \textit{ars} 1-containing portion of the yeast DNA in YRp7. The position of the knobs on the \textit{ars} 1 fragment corresponded to the position of the knobs mapped on the yeast DNA fragment obtained by digestion with EcoRI alone. For this reason, the results of the two experiments were combined and are shown in Fig. 2A. The knobs were located predominantly at ~42% coinciding with the position of the known \textit{Bgl}II site on the restriction map of \textit{ars} 1 (Fig. 2C). It should be noted that the size of the pBR322 portion of YRp7 does not change in the electron microscope after EcoRI and HindIII cleavage, be-
cause the unique EcoRI and HindIII sites in pBR322 are separated by only 25 base pairs.

In the same series of experiments, the positions of the centers of replication eyes in the plasmid DNA was determined using the same mapping procedure. They were found exclusively on the yeast DNA insert of YRp7 (Fig. 1C) and were localized to the ars 1-containing portion. No molecules with more than one eye were found. The position of the eyes found on the DNA after double digestion with EcoRI and HindIII corresponded to that obtained with EcoRI digestion alone. The data were combined and are shown in Fig. 2B. The eyes mapped predominantly at -42% on the ars 1 DNA (Fig. 2C) and coincided with the sites of binding of the protein knobs. Thus, the putative replication complex appears to bind to the DNA at the in vitro origin of ars 1 replication. The origin is located in the vicinity of the BglII site in ars 1, although some skewing to the left appears to exist (Fig. 2). No DNA fragments with both eyes and protein knobs were found. It was not possible to determine whether replication was bidirectional or unidirectional in vitro, because the size distribution of the eyes on the DNA fragments was too narrow.

The nucleotide sequences in the region of one of the in vivo 2-μm yeast DNA plasmid origins and of ars 1 have been compared (13) revealing a limited homology. In Fig. 3, the published nucleotide sequences of regions of the DNA lying in the vicinity of both of the 2-μm origins (6) and in the vicinity of the ars 1 in vitro origin found here are shown. In all three cases, a homologous element having the consensus sequence TAAAPyrPurPurAAPur, similar to that pointed out previously (13), can be found. Within the limits of resolution, this sequence is located at the sites of binding of the putative replication complex to 2-μm and ars 1 DNA, which coincide with the origins of replication. This sequence is also found in ars 2 (12) and ars 3 (13). It is not found in pBR322 DNA. However, features of the DNA other than the consensus sequence alone appear to be important in defining the origin, because 2-μm plasmid possesses another copy of this sequence starting at nucleotide 5,806 (17) and ars 1 at nucleotide 1,139 (19) on the strand opposite the one shown in Fig. 3C. These sequences do not appear to lie in the vicinity of replication origins. The region of DNA depicted in Fig. 3A lies in the larger unique portion of the 2-μm plasmid adjacent to the inverted repeat, an extended region of symmetry elements (17). Similarly, several symmetry elements are adjacent to the ars 1 sequence shown in Fig. 3C (12). Subcloning experiments have suggested that this region of ars 1 DNA, adjacent to the BglII site, is essential for ars 1 activity (19). It will be of interest to determine more precisely the relationship between replication complex binding and initiation of replication at the various origins, particularly when pure complex becomes available.

The results presented here suggest that our preparations contain replication complexes capable of binding to three origins (two in 2-μm plasmid, one in ars 1) and also of initiating replication from these origins. Although a limited sequence homology exists between these origins, it does not appear sufficient to explain their function in replication. The ars 1 sequence belongs to a class of unique yeast genomic sequences (12), while the 2-μm origins reside on an extrachromosomal element. These studies can be extended to ars 2, one of a family of dispersed, repetitive chromosomal sequences (12, 14). At present, the ars sequences are defined only by their capacity to confer high frequency transformation on colinear DNA, and a distinct origin of replication has not been shown to reside in these sequences in vivo (12, 13). The in vivo 

![Fig. 2. Electron microscopic localization of protein knobs and replication origins on YRp7 plasmid DNA. Plasmid DNA was incubated with replication complex, prepared, and digested with EcoRI or EcoRI and HindIII and visualized in the electron microscope as for Fig. 1. Protein knobs and replication eyes were found only on the yeast DNA portion of YRp7. Micrographs were projected and traced with a planimeter. The positions of knobs and of the centers of replication eyes were determined as the fractional distance from the nearest end of the linear DNA. The maps obtained for the single- and double-digested molecules were aligned, taking into account the known relationship of the EcoRI and HindIII sites and the fact that knobs and eyes were found only on the larger of the EcoRI-HindIII fragments of yeast DNA. These maps were coincident; the data were combined and are depicted as length histograms of protein knob location (A) and replication eye location (B) on the EcoRI yeast DNA fragment of YRp7 plasmid. In C, the corresponding restriction map of this EcoRI fragment, verified by us, is depicted with the ars 1-containing portion of the DNA demarcated. The BglII site is indicated to define further the orientation of this EcoRI fragment.](http://www.jbc.org/)

![Fig. 3. Nucleotide sequences of three yeast origins of DNA replication. Only one strand is shown for each DNA, S' to S from left to right. A, the 2-μm origin located in the larger unique region of the plasmid adjacent to an inverted repeat at position 3,692 to 3,718 in the nucleotide sequence (17); B, the 2-μm origin located in the smaller unique region of the plasmid on the opposite strand at position 5,086 to 5,060 (17); C, the ars 1 chromosomal origin located at position 847 to 873 (12, 13, 19). The homologous consensuses sequence (13) TAAAPyrPyrPyrAAPur (where Pyr represents a pyrimidine and Pur represents a purine) is underlined.](http://www.jbc.org/)
studies described here demonstrate the presence of a unique origin in \textit{ars} 1 that is utilized by an established \textit{in vitro} replication system. If the \textit{in vivo} origin in \textit{ars} 1 corresponds to the one used \textit{in vitro}, binding of the replication complex to yeast DNA might serve more generally to identify replication origins.

The question arises whether one or a few species of replication complex are present that are capable of initiation from many or all origins, or whether there are many different complexes, one for each origin. The resolution of this problem has important implications for cell cycle control. If only a few replication complexes exist inside the cell, factors other than origin specificity may play a more dominant role in control of entry and progression through the S phase of the cell cycle. These other factors could include the intranuclear location of replicons (9, 20).

Acknowledgments—We wish to thank A. Wolff for excellent technical assistance. We are grateful to Dr. B. K. Tye for providing the YRp7 plasmid.

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