Participation of ATP in the binding of a yeast replicative complex to DNA

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The activity that replicates yeast DNA *in vitro* can be isolated from cells of the budding yeast *Saccharomyces* in a high- M_r ($\sim 2 \times 10^6$) form. Several lines of evidence indicate that this fraction contains a multiprotein replicative complex. A functional assay has been developed for the analysis of the interaction of the replicating activity with DNA. Binding of the activity required Mg^{2+} , but did not require the addition of ATP or the other ribo- or deoxynucleoside triphosphates. However, the ATP analogues adenosine 5'-[γ -thio]triphosphate and adenosine 5'-[$\beta\gamma$ -imido]triphosphate blocked the binding, suggesting that ATP participates in the interaction at some stage. The binding was template (origin)-specific in either the presence or the absence of ATP and the other nucleoside triphosphates; however, ATP stabilized the replicating activity. The preferential inhibition of binding that was observed in the presence of the DNA topoisomerase II inhibitor coumermycin suggests that the requirement for ATP may be at least partially accounted for by the involvement of this enzyme in the initial interaction of the replicating activity with DNA. Finally, the binding was rapid. In contrast, DNA synthesis displayed a lag when assayed directly without first allowing a period for the replicating activity to bind to the DNA. In addition, binding was 'tight', as judged by the resistance of the protein–DNA complexes to salt in comparison with the relative sensitivity of binding. The replicating activity was not readily displaced from the complexes by exogenous DNAs, either possessing or lacking yeast origins of replication. The results suggest that the interaction of the replicating activity with the DNA occurs in more than one stage.

INTRODUCTION

The control of initiation of DNA replication plays a central role in the regulation of the eukaryotic cell cycle [1]. There are several levels at which this control may be mediated: synthesis and activation of the proteins involved in replication, assembly of a multiprotein replicative complex and its transport to its site of action, and the intracellular/intranuclear locus of a given replicon and its association with supramolecular structures in the cell. With regard to the latter two, recognition of origin-specific nucleotide sequences may or may not be important. One means of approaching the question of the relative importance of these putative control mechanisms involves the development of cell-free systems for DNA replication and the analysis of the replication process *in vitro* [2].

The budding yeast *Saccharomyces* is an attractive organism for the examination of replication control in the cell cycle [3]. A considerable body of information concerning certain yeast replication proteins has been acquired recently [e.g. 4–12]. Similarly, DNA elements possessing the characteristics of replication origins have been identified in yeast [13,14]. Some of these autonomously replicating sequences (*ARS*) have been examined in much detail, both in a structural and in a functional context [for review see 3,15]. In addition,

the organism possesses an extrachromosomal DNA element, called the 2- μ m yeast plasmid, which can be used as a model of a nuclear replicon [16]. Finally, the genetic analysis of the cell cycle is well advanced in yeast [17].

I have been studying DNA replication in yeast with a view to its control in the cell cycle for several years [7, 18–25]. These studies have depended in a large part on the development of a system *in vitro* for yeast DNA replication [19]. Similar systems have been described by others [26,27]. With the use of the system *in vitro*, a high- M_r ($\sim 2 \times 10^6$) form of yeast DNA-replicating activity has been identified [22,23]. Analysis of this activity has provided evidence for the participation of a multiprotein complex in yeast DNA replication *in vitro* [24,25]. In certain respects, this multiprotein complex resembles the replicative complex (replisome) isolated from mammalian cells [28]. Both complexes may be assembled and disassembled during the cell cycle.

To gain a better understanding of the properties of the yeast replicative complex and of its interaction with DNA, a rapid assay for binding is required. Such an assay has been developed. Using this assay, some of the requirements for complex binding to DNA have been examined and are reported here. Also, some of the features of the protein–DNA complexes that are formed have been determined.

Abbreviations used: ATP[S], adenosine 5'-[γ -thio]triphosphate; p[NH]ppA, adenosine 5'-[$\beta\gamma$ -imido]triphosphate; R complex, DNA replicating activity in the high- M_r replicative fraction from yeast.

MATERIALS AND METHODS

Strains, plasmids and replicative fraction

Saccharomyces cerevisiae A364A (a *ade1 ade2 ura1 his7 lys2 tyr1 gal1*) was from the Yeast Genetic Stock Center (Berkeley, CA, U.S.A.). This strain was cultured as described previously [18]. Supercoiled YEp3 [22] and YEp3ori⁻ [24] plasmids were prepared [22] and used as the 2- μ m yeast plasmid templates. YEp3ori⁻ was derived from YEp3 by deletion of the 2- μ m origins of replication. U.v.-irradiated YEp3 DNA was prepared by illumination of a 4 μ l drop of DNA solution at 254 nm for 20 min at 9.6 μ W/cm². The high- M_r replicative fraction was prepared as before [24], except that Zymolyase 60000 (0.5 mg/ml) was used for cell lysis. DNA replication was assayed essentially as described before [24] by incorporation of ³²P from [α -³²P]dATP into acid-precipitable material.

Binding assay

Binding of replicating activity to DNA [24] was determined as follows. The DNA was incubated briefly at 30 °C with the high- M_r replicative fraction in a complete replication reaction mixture that included ATP, the three remaining ribonucleoside triphosphates, the four deoxynucleoside triphosphates, and MgCl₂. Alternatively, one or more of these components was absent during binding. After chilling on ice, DNA and protein-DNA complexes were isolated from the reaction mixture by sedimentation through a bed of Sepharose CL-4B equilibrated in 20 mM-Tris/HCl/2 mM-EDTA (pH 7.5 at 20 °C) in a 1.5 ml Microfuge tube at room temperature in an IEC Clinical centrifuge at 400 g for 1 min. In some instances, various additions were made to the samples either before or after the isolation of the protein-DNA complexes, and the samples were

either incubated or processed further immediately. The isolated protein-DNA complexes were then assayed for DNA replication under standard replication assay conditions at 30 °C. No replicating activity other than that isolated in the protein-DNA complexes was present; however, in all cases, except in the experiment shown in Table 3 (expt. 3), an aliquot of YEp3 DNA (0.3 μ g) was added. Radioactive label was present only during this final incubation for determination of DNA synthesis.

Restriction analysis of newly synthesized DNA

Replication reactions were stopped on ice, and the samples were dialysed rapidly against 20 mM-Tris/HCl/8.5 mM-MgCl₂/45 mM-NaCl (pH 7.4 at 20 °C), first in the presence of AG1-X8 ion exchange resin (BioRad) and then alone. The DNA was digested to completion with restriction endonucleases *Hind*III and *Ava*I. The digests were electrophoresed in 1.4% agarose gels containing 90 mM-Tris/90 mM-borate/1.25 mM-EDTA (pH 8.35 at 20 °C). DNA bands were visualized by fluorescence after staining with ethidium bromide (0.5 μ g/ml). The DNA bands were excised, and the gel slices were minced. After incubation of the minced gel slices in 0.5 ml of water at 37 °C for 15 min on a shaker, radioactivity was determined by liquid-scintillation spectrometry.

RESULTS

Requirements for binding

Incubation of DNA with a fraction containing the high- M_r ($\sim 2 \times 10^6$) form of yeast DNA-replicating activity results in the appearance of protein knobs on the DNA, as determined by electron microscopy [22,23]. Several lines of evidence suggest that the presence of these knobs on the DNA is associated with replicating activity [22–25]. Most importantly, these knobs have been mapped at or near the origins of replication on both the extrachromosomal 2- μ m yeast plasmid [22] and the chromosomal replicator *ARS1* [23]. Using electron microscopy as an assay, it has been determined that the recovery of protein knobs on the DNA requires ATP and the deoxynucleoside triphosphates [24].

Further evidence for the participation of a multiprotein complex in yeast DNA replication was obtained by the examination of individual enzymic activities in the high- M_r fraction [24]. In particular, the binding of these activities to DNA was analysed. This formed the basis for a rapid biochemical assay for the binding of the replicating activity (R complex). Briefly, the binding assay consists of two consecutive incubations. First, the DNA is incubated with the high- M_r replicative fraction under any one of a variety of conditions. The DNA and any protein-DNA complexes are separated from the reaction mixture by sedimentation through a bed of Sepharose CL-4B. This excluded material can be subjected to various treatments before it is analysed further. Finally, as exemplified in this study, the complexes are incubated under conditions allowing replication to proceed.

In order to gain further insight into the interaction of the replicating activity with DNA, the requirements for R complex binding to the DNA were examined in some detail (Table 1). Only an absolute requirement for Mg²⁺

Table 1. Requirements for R complex binding

Binding of R complex from the high- M_r replicative fraction to YEp3 DNA was compared under a variety of conditions during a 10 min incubation. In the complete reaction, 10 mM-MgCl₂, 1 mM-ATP, 100 μ M each of CTP, UTP, GTP, dCTP, dGTP, dTTP, and 10 μ M-dATP were added prior to incubation. In other cases, only the components of the complete reaction that are indicated were present. Where indicated, ATP[S] and p[NH]ppA were 2 mM during binding. Hexokinase (1 unit) and 5 mM-glucose were present during binding as indicated. Isolated protein-DNA complexes were assayed for replicating activity in a 60 min incubation to estimate the extent of binding. In controls in which DNA was omitted during the binding phase of the assay, no DNA synthesis above the background was detected.

Conditions	Nucleotide incorporation (pmol)	Binding (%)
Complete reaction	1.56	(100)
Mg ²⁺ , ATP	1.43	92
Mg ²⁺	1.28	82
No additions	0	0
Mg ²⁺ , ATP[S]	0.22	14
Mg ²⁺ , p[NH]ppA	0.14	9
Mg ²⁺ , hexokinase, glucose	1.09	70

Fig. 1. Time course of R complex binding to DNA and time course of DNA synthesis directed by the high- M_r replicative fraction

(a) Binding to YEp3 DNA was carried out for various lengths of time in the presence of 10 mM-MgCl₂ with 5 μ l of the high- M_r replicative fraction. The extent of binding was then determined in a second incubation by assaying the isolated protein-DNA complexes for replicating activity in a 60 min incubation. There was no DNA synthesis observed above the background in controls in which no DNA was present during the binding reaction (first incubation). (b) DNA synthesis with a YEp3 template was determined after incubation for various lengths of time in a replication reaction containing 5 μ l of the high- M_r replicative fraction. In this case, preformed protein-DNA complexes were not isolated prior to the assay of replicating activity.

was detected. This result was in striking contrast with the apparent requirement for both ATP and the deoxynucleoside triphosphates for protein complex binding that was determined by electron microscopy [24]. In the presence of these nucleotides, DNA replication could take place. One explanation for the difference may reside in the fact that the samples for electron microscopy were subjected to fixation. Some nucleotide polymerization may have simply been necessary to provide a protein-DNA complex that was resistant to this treatment. We suggested that this polymerization involved the synthesis of a primer by DNA primase, because the detection of the protein-DNA complexes was not prevented by aphidicolin, a DNA polymerase inhibitor that blocks replication in this system. Another possibility may be that the protein-DNA complexes identified in the previous study [24] and the ones formed in the presence of Mg²⁺ alone differ. This latter possibility will be explored below.

Despite the fact that the presence of ATP had only a slight stimulatory effect on binding, the addition of ATP[S] or p[NH]ppA, non-hydrolysable analogues of the nucleotide, virtually abolished binding (Table 1). These effects of ATP[S] and p[NH]ppA were exerted during the binding of R complex itself rather than during subsequent DNA synthesis, as evidenced by control experiments. In these controls, the binding reaction mixtures were sedimented through Sephadex G-25 rather than Sepharose CL-4B, and the excluded material was assayed for replication. Gel filtration through the Sephadex was sufficient to reverse completely inhibition by the nucleotide analogues; however, this matrix

Fig. 2. Dependence of R complex binding on the amount of replicative fraction added and template specificity of binding

Binding to YEp3 and YEp3ori⁻ DNAs was compared in 10 min incubations in the presence of 10 mM-MgCl₂ with various amounts of high- M_r replicative fraction in parallel reactions. Isolated protein-DNA complexes were assayed for replicating activity for 60 min to determine the extent of binding. No DNA synthesis was detected above the background when DNA was omitted from the binding reactions.

excluded the free R complex, in contrast with the Sepharose. The results with the ATP analogues imply that ATP participates at some point in the initial interaction of the R complex with DNA. Whether ATP induces a conformational change in the complex or whether ATP hydrolysis itself is important is not clear at present. It is also not known at what stage of binding ATP is required. In any case, the presence of hexokinase (and glucose) during binding appeared to have only a small inhibitory effect (Table 1), indicating that either ATP was largely absent from the R complex preparation or that it was not accessible to hexokinase. The former alternative is favoured because the inhibition of binding by ATP[S] and p[NH]ppA suggests that if ATP was present that it readily exchanged with its analogues.

Properties of the binding reaction

The binding of R complex to DNA was rapid, and it was essentially complete within 10 min (Fig. 1a). Interestingly, a lag of approx. 10 min was routinely observed in the onset of DNA replication carried out by the high- M_r replicative fraction (Fig. 1b) when protein-DNA complexes were not first allowed to preform and the assay for DNA synthesis was carried out without prior isolation of these complexes. This lag may well be accounted for by the period of R complex binding to DNA, because no lag in DNA synthesis was detected with isolated protein-DNA complexes whether binding was carried out in the presence of ribo- and deoxynucleotides [24] or in the presence of Mg²⁺ alone (results not shown).

Binding in the presence of Mg²⁺ alone was dependent upon the amount of replicative fraction present during incubation with DNA (Fig. 2). In this regard, the

Table 2. Site-specificity of DNA initiation

(a) YEp3 DNA was incubated with the high- M_r replicative fraction for 10 min in a standard replication reaction. The reaction was stopped, and nucleotide incorporation into various portions of the DNA molecule was determined as described in the Materials and methods section. The data have not been normalized with regard to fragment size and base composition. Such normalization further amplifies the difference between fragments F and G and the remaining portions of the DNA molecule. Fragments F and G contain the 599 base pair inverted repeats of 2- μ m DNA. Fragment D is composed entirely of pBR322 DNA, while A and C contain the junctions between 2- μ m and pBR322 DNAs in the YEp3 hybrid. The order of the fragments is F-A-D-C-E-G-B on the circular map of YEp3, starting from one of the repeats and continuing through the larger unique region of 2- μ m DNA [22]. Fragment F contains the 2- μ m *ARS* [3,15]. The fragment sizes range from 2940 to 1133 base pairs in A through G, respectively. (b) DNA synthesis directed by equal amounts of either YEp3, YEp3ori⁻ or pBR322 DNA was compared in a 60 min replication reaction with the high- M_r replicative fraction without prior isolation of protein-DNA complexes.

(a)		
Restriction fragment	Nucleotide incorporation (c.p.m.)	Fraction of newly synthesized DNA (%)
A	6882	12.3
B	5752	10.3
C	7035	12.5
D	6053	10.8
E	7114	12.7
F	10841	19.3
G	12371	22.1
Total	56048	(100)

(b)		
DNA	Nucleotide incorporation (pmol)	Relative activity (%)
YEp3	25.2	(100)
YEp3ori ⁻	4.6	18
pBR322	2.9	12

reaction was indistinguishable from that found in the presence of the nucleoside triphosphates. Importantly, the binding was template-specific both under conditions in which DNA replication can proceed [24], as well as in the presence of Mg^{2+} alone (Fig. 2). This conclusion was drawn from the fact that binding to the plasmid YEp3ori⁻ was drastically reduced as compared to the binding obtained with YEp3 (Fig. 2). The former plasmid was derived from YEp3 by deletion of the 2- μ m yeast plasmid origins of replication [24]. Thus, the R complex-DNA interactions that occur in the presence or in the absence of ATP and the deoxynucleoside triphosphates and that are measured in this binding assay involve the origin-region of the DNA.

The origin-specific nature of the interaction of R complex with YEp3 DNA was further examined by localizing the sites on the template where nascent DNA appeared in a brief pulse (Table 2a). A 10 min pulse was

chosen to take advantage of the lag in DNA synthesis (Fig. 2b). Because initiation of replication is rate-limiting in this system [19,22], it was reasoned that this would optimize for detection of replicating molecules over the background of labelled molecules that had completed replication. Furthermore, the majority of the replicative intermediates *in vitro* are ones in which less than 5% of the molecule has replicated [22], suggesting that this type of analysis can point to actual DNA initiation events in portions of the molecule [29]. (In fact, many of the replicative intermediates may be molecules in which less than 200 base pairs are replicated; such small replication 'eyes' lie below the limits of resolution of the electron microscopic techniques that were employed [22]). After digestion of plasmid DNA with the enzymes *Hind*III and *Ava*I, it was found that at least 41% of the nascent DNA originated from two restriction fragments (F and G) in about equal amounts (Table 2a). The remaining fragments were labelled almost equally at approx. 2-fold lower levels. This result indicates that initiation took place, preferentially at least, in fragments F and G, and that indeed the bulk of the replicative intermediates were molecules in which less than 5% of the DNA had replicated.

The location of the origins determined here corresponds closely to that found previously by electron microscopy in the case of fragment F [22]. For fragment G, the determination does not coincide exactly. The position of the centres of replication 'eyes' at this origin maps adjacent to the *Ava*I site that joins fragments G and B and resides in fragment B. These findings can be reconciled by postulating that initiation occurs in fragment G and is initially unidirectional towards fragment B. This is similar to the situation that pertains at the SV40 origin [29]. Interestingly, sequences within fragment B appear to be essential for origin function. Removal of fragments B and F from YEp3 drastically reduced DNA synthesis, as seen in the comparison of YEp3 and YEp3ori⁻ templates (Table 2b). Some specific synthesis still occurred as compared to pBR322, however (Table 2b).

Further characterization of the interaction of R complex with DNA was obtained by estimating how 'tight' the binding actually was. This was achieved by determining the effect of various agents that disrupt the interaction of protein and DNA. First, the effect of various concentrations of KCl on binding was determined (Table 3, expt. 1). In the presence of 100 mM-KCl, binding was reduced to one-fourth of the maximum level. However, the same salt concentration had a much smaller effect when KCl was added after binding was essentially complete. Thus, the protein-DNA complex, once formed, was relatively resistant to salt. Next, the effect of heparin on the protein-DNA complex was examined (Table 3, expt. 2). The efficiency with which this negatively-charged polysaccharide 'stripped' the R complex from the DNA (Table 3) was very similar to its inhibitory effect on DNA synthesis in a reaction containing the high- M_r replicative fraction and DNA but without prior isolation of preformed protein-DNA complexes (results not shown). Finally, competition by added DNA for the R complex present in a protein-DNA complex was determined (Table 3, expt. 3). It was found that addition of an equal amount of u.v.-irradiated YEp3 DNA to a protein-YEp3 DNA complex inhibited DNA synthesis by about one-third. This suggests that binding

Table 3. Effect of various agents on the association of R complex with DNA

Binding of R complex from the high- M_r replicative fraction to YEp3 DNA was carried out in 10 min incubations in the presence of 10 mM-MgCl₂. KCl was present during binding or it was added immediately after, and the samples were sedimented through Sepharose CL-4B. Heparin (1 µg/ml) was added after binding, and the samples were incubated on ice for 5 min prior to isolation of protein-DNA complexes. Competing DNAs were added to isolated protein-DNA complexes in the presence of 10 mM-MgCl₂, and samples were incubated for 5 min at 30 °C before placing them in replication reactions. U.v.-irradiated YEp3 DNA and YEp3ori⁻ DNA were added in equal amount or in 2-fold excess, respectively, to the amount of YEp3 DNA present in the original binding reaction. Determination of replicating activity in isolated protein-DNA complexes was carried out in a 60 min incubation to quantify binding. DNA synthesis above the background was not observed when DNA was absent during binding.

Conditions	Nucleotide incorporation (pmol)	Binding (%)
Expt. 1		
+ KCl during binding		
20 mM	3.45	(100)
50 mM	1.82	53
100 mM	0.82	24
+ KCl after binding		
100 mM	2.24	65
Expt. 2		
Control	5.30	(100)
+ Heparin	2.78	52
Expt. 3		
Control	0.78	(100)
+ U.v.-irradiated YEp3	0.52	67
+ YEp3ori ⁻	0.83	106

of R complex to the DNA was tight, and that this complex did not dissociate freely from the DNA to bind to the exogenous u.v.-irradiated YEp3 DNA. If this were not the case, it would be expected that the exogenous DNA would inhibit binding by approx. 50%. (It was determined that the induction of pyrimidine dimers in the DNA by the u.v. irradiation inhibited DNA synthesis on the irradiated template by 95% but did not block R complex binding, as determined in competition experiments between u.v.-irradiated DNA and unirradiated template for binding of R complex from the high- M_r replicative fraction.) In contrast with the effect of yeast origin-containing DNA, the addition of YEp3ori⁻ DNA, even in 2-fold excess, to the protein-DNA complex had no significant effect on DNA synthesis (Table 3, expt. 3), supporting the conclusion that the binding was tight. The lack of competition by YEp3ori⁻ provides further evidence that the binding of R complex to DNA is template (origin)-specific.

Role of ATP in binding

In order to shed light on the mechanism of binding of R complex to DNA, the apparent involvement of ATP in the binding reaction was examined further. Although there was no obvious difference in binding of R complex

Fig. 3. Effect of ATP on R complex stability

Binding of R complex to YEp3 DNA was carried out in a 10 min incubation with 10 mM-MgCl₂ in the presence (+ ATP) and in the absence (- ATP) of 1 mM-ATP. The protein-DNA complexes isolated by sedimentation through Sepharose CL-4B were preincubated at 30 °C for various lengths of time prior to placing them into a replication reaction for 60 min. Results are expressed as a percentage of the DNA synthesis obtained without the preincubation.

to the DNA in the presence or absence of this nucleotide, the protein-DNA complexes that were formed were distinguishable. A comparison of the isolated protein-DNA complexes revealed a significant effect of ATP on stability (Fig. 3). Stability here denotes the maintenance of the capacity to support DNA replication. Binding in the presence of ATP rendered the complexes resistant to preincubation at 30 °C prior to the assay of DNA-synthetic ability. This stabilizing effect did not appear to reside simply in the prevention by ATP of the dissociation of R complex from the DNA, because exogenous origin-containing DNA was equally effective in displacing R complex from isolated protein-DNA complexes that were formed in the presence or the absence of the nucleotide (results not shown).

One of the possible modes in which ATP might play a role in the initial interactions of R complex with DNA involves the activity of DNA topoisomerase II, for which the nucleotide is a cofactor [9]. This enzyme appears to be a component of R complex [24]. The inhibition by coumermycin, a topoisomerase II inhibitor, of DNA replication supported by the high- M_r replicative fraction implicates this enzyme in the overall replication process carried out by R complex [24]. In view of these considerations, the effect of coumermycin on R complex binding to DNA was determined. The inhibitor preferentially blocked binding of R complex to DNA. When added solely during the first incubation (binding phase) of the binding assay at 100 µg/ml, it blocked subsequent DNA synthesis in the second incubation by 70%. This was nearly as effective as in a replication reaction containing the high- M_r fraction and DNA, in which the inhibition was by 74% with the same drug concentration. Nevertheless, an exclusive effect of the drug in these experiments on the binding of R complex cannot be

concluded because the controls to establish the reversal of inhibition, identical to those described above for ATP[S] and p[NH]ppA, showed a reduction of the inhibition to 47% rather than complete reversal (0% inhibition). This may have been due to tenacious binding of coumermycin to R complex. The preferential effect of the drug on binding does, however, suggest the involvement of DNA topoisomerase II in the initial interaction of R complex with DNA.

DISCUSSION

The use of a functional assay for the analysis of the interaction of R complex with DNA possesses both advantages and disadvantages as compared with physical assays such as DNAase footprinting. Although the assay cannot provide the resolution of the latter method, it does provide some assurance that most, if not all, of the functional components of the replicating activity present in an enriched (100–200-fold) fraction do bind to the test DNA. The fact that this functional binding (Fig. 2) coincides with the binding of protein complexes to DNA at replication origins, as determined by electron microscopy [24], suggests that the functional assay measures the association of a replicative complex with the DNA, rather than the random binding of individual activities. This conclusion is strengthened by the fact that DNA polymerase and DNA primase activities bind to the DNA along with total replicating activity in a template (origin)-specific manner [24]. It should be stressed that the interactions described in this report also involve the origin region of the DNA (Fig. 2). It is not known what role interactions outside this region play in the binding observed at the origin. However, it is reasonable to assume that such interactions take place, especially in view of the kinetics of binding [30] (Fig. 1a).

The binding assay used in this study requires the separation of protein–DNA complexes from the unbound R complex. As shown previously [24], this is readily achieved by the methodology employed. This is also demonstrated here in both controls in which no DNA was present during the binding reaction, as well as in experiments in which origin-minus DNA replaced the 2- μ m origin-containing template (Fig. 2). In that experiment, as well as in the controls containing no DNA during binding, YEp3 DNA was added during the assay for DNA synthesis to detect the possible presence of any R complex. For consistency, this additional YEp3 DNA was included during the DNA synthesis step of the binding assay throughout this study, where feasible. The results in Table 3 (expt. 3) indicate the adequacy of this approach for detection of even DNA-bound R complex. It should be pointed out that no claims are made as to whether DNA synthesis occurs on this added YEp3 DNA or on the YEp3 DNA associated with R complex or both. This question does not bear directly on the examination of actual R complex binding to DNA that is the subject of this report. In fact, the presence of the added YEp3 DNA referred to above does not alter significantly the amount of DNA synthesis observed with the isolated complex of YEp3 DNA and the R complex.

The binding assay described here depends on the determination of DNA synthesis directed by the replicating activity present in a complex with the DNA. The levels of DNA synthesis were lower (generally 10–20%) than those obtained in reactions that did not

involve prior isolation of protein–DNA complexes, which resulted typically in the replication of 5–10% of the input template in 1 h. One possible explanation for this difference is that, in the binding assay, the protein–DNA complexes must survive the rigours of isolation. Nevertheless, the results obtained with the binding assay were reproducible, and the product of the reaction was the same as that resulting from the replication of the DNA without isolation of preformed protein–DNA complexes. Label was incorporated into full-length plasmid DNA. In addition, DNA synthesis was linear for at least 4 h in both reactions. As reported earlier [24], DNA replication *in vitro* is insensitive to prior treatment of the template with alkali, and the plasmid DNA is always prepared without prior amplification with chloramphenicol. This and the fact that the amount of DNA synthesis obtained in this system was not simply proportional to the size of the template indicates that the DNA synthesis observed was not due to extension of pre-existing primers, as seen in certain other systems *in vitro* [31]. Finally, the high- M_r replicative fraction initiates DNA synthesis at the origins used *in vivo* by 2- μ m plasmid and at a unique site in *ARS1*, as determined by electron microscopy [22,23], and the DNA synthesized on the 2- μ m plasmid template YEp3 in a brief pulse was found to be associated preferentially with restriction fragments from regions of the DNA spanning the origins of replication (Table 2a). All of these results suggest that initiation of DNA synthesis takes place in the origin-region *in vitro*.

The results presented here raise the possibility that binding of replicating activity (R complex) to the DNA occurs in more than one stage. This suggestion is consistent with the apparent differences in the requirements for detection of protein–DNA complexes by electron microscopy [24] and by the biochemical assay (Table 1) discussed above. These differences reside in the presence or absence of a requirement for ATP and the deoxynucleoside triphosphates. As pointed out above, ATP appears to play a role at some stage of binding, even though it did not appear to be required for recovery of an active protein–DNA complex (Table 1). It is possible *a priori* to categorize the protein–DNA complexes according to sites of binding on the DNA, according to whether they are weak or strong (tight) and according to functional criteria. With regard to the first, it can be hypothesized that the binding observed in the presence of Mg^{2+} alone represents an interaction of R complex with a binding or 'loading' site on the DNA, while in the presence of ATP, and possibly also deoxynucleoside triphosphates, the association found involves the region containing the actual DNA initiation site(s). On the other hand, from a functional standpoint, the protein–DNA complex found at the origin of replication in the presence of Mg^{2+} alone may represent a complex at an early stage of the replication process preceding, for example, initiation, while that with ATP, and even more so with deoxynucleoside triphosphates, present additionally would involve a distinct complex at a later stage of replication. Obviously, these three possibilities for classification of the protein–DNA complexes are not mutually exclusive. Indeed, the synthesis of a primer that was postulated [24] to prevent the dissociation of protein–DNA complexes, as detected in the electron microscope, is consistent with this view of R complex binding rather than excluding it.

It should be stressed that binding in the presence [24] or in the absence (Fig. 2) of ATP and deoxynucleoside triphosphates was template (origin)-specific, and, thus, the interactions under discussion involve the origin region. The binding, even in the presence of Mg^{2+} alone, was relatively tight (Table 3). The observed lag in DNA synthesis as compared with the rapid binding of R complex to DNA (Fig. 1) is consistent with the existence of more than one stage in protein-DNA complex formation, although other explanations exist for this observation. Similarly, the apparent requirement for sequences in YEp3 DNA restriction fragment B for efficient replication that initiated close-by in the adjacent fragment G (Table 2) lends support to the existence of more than one stage in protein-DNA complex formation. One possible function of fragment B in initiation may be the contribution of the 11 base-pair core consensus sequence that is present at all yeast *ARS*s (origins) [3,15] and which can be found in the vicinity of the *Ava*I site separating fragments G and B [23]. This sequence could operate as a loading site that functions in one of the stages of binding of R complex to the DNA.

What is the possible role of ATP in the initial interaction of R complex with DNA? The results in Fig. 3 indicate that the nucleotide lends stability to R complex. The preferential inhibition of binding by the drug coumermycin suggests that ATP may exert its effect at least partially through DNA topoisomerase II, which appears to be a component of R complex [24]. At any rate, the results indicate this enzyme is likely to be an early participant in the interaction of R complex with DNA, whether or not the effect of ATP on binding is mediated in whole or in part by this enzyme. During initiation of replication at the *Escherichia coli* origin (*ori* C), the bacterial topoisomerase, DNA gyrase, performs an early function prior to primer synthesis [32].

It may prove possible to further distinguish the stages in protein-DNA complex formation by using kinetic criteria. Differential sensitivity to salt of complexes formed in the presence and absence of ATP and deoxynucleoside triphosphates has not as yet been detected [24] (Table 3; S. M. Jazwinski, unpublished work). However, it may be possible to extend this analysis by examining the effects of heparin. A more definitive resolution of this issue awaits more precise mapping of binding sites and their comparison with initiation sites determined at the single-nucleotide level.

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REFERENCES

1. Baserga, R. (1985) *The Biology of Cell Reproduction*, Harvard University Press, Cambridge

2. Kornberg, A. (1980) *DNA Replication*, Freeman, San Francisco
3. Newlon, C. S. (1987) in *The Yeasts* (Rose, A. H. & Harrison, J. S., eds.), vol. 3, Academic Press, New York, in the press
4. Johnson, L. M., Snyder, M., Chang, L. M. S., Davis, R. W. & Campbell, J. L. (1985) *Cell* **43**, 369–377
5. Plevani, P., Badaracco, G., Augl, C. & Chang, L. M. S. (1984) *J. Biol. Chem.* **259**, 7532–7539
6. Singh, H. & Dumas, L. B. (1984) *J. Biol. Chem.* **259**, 7936–7940
7. Jazwinski, S. M. & Edelman, G. M. (1985) *J. Biol. Chem.* **260**, 4995–5002
8. Banks, G. R. & Barker, D. G. (1985) *Biochim. Biophys. Acta* **826**, 180–185
9. Goto, T. & Wang, J. C. (1982) *J. Biol. Chem.* **257**, 5866–5872
10. LaBonne, S. G. & Dumas, L. B. (1983) *Biochemistry* **22**, 3214–3219
11. Durnford, J. M. & Champoux, J. J. (1978) *J. Biol. Chem.* **255**, 1086–1089
12. Sugino, A., Ryu, B. H., Sugino, T., Naumovski, L. & Friedberg, E. C. (1986) *J. Biol. Chem.* **261**, 11744–11750
13. Struhl, K., Stinchcomb, D. T., Scherer, S. & Davis, R. W. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1035–1039
14. Hsiao, C.-L. & Carbon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3829–3833
15. Williamson, D. H. (1985) *Yeast* **1**, 1–14
16. Fangman, W. L., Hice, R. H. & Chlebowicz-Sledziewska, E. (1983) *Cell* **32**, 831–838
17. Pringle, J. R. & Hartwell, L. H. (1981) in *The Molecular Biology of the Yeast *Saccharomyces*: Life Cycle and Inheritance* (Strathern, J. N., Jones, E. W. & Broach, J. R., eds.), pp. 97–142, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
18. Jazwinski, S. M. & Edelman, G. M. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3933–3936
19. Jazwinski, S. M. & Edelman, G. M. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1223–1227
20. Taketo, M., Jazwinski, S. M. & Edelman, G. M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3144–3148
21. Jazwinski, S. M. (1982) *Acta Biochim. Polon.* **29**, 159–173
22. Jazwinski, S. M. & Edelman, G. M. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3428–3432
23. Jazwinski, S. M., Niedzwiecka, A. & Edelman, G. M. (1983) *J. Biol. Chem.* **258**, 2754–2757
24. Jazwinski, S. M. & Edelman, G. M. (1984) *J. Biol. Chem.* **259**, 6852–6857
25. Jazwinski, S. M. & Edelman, G. M. (1984) *Ann. N.Y. Acad. Sci.* **435**, 161–163
26. Kojo, H., Greenberg, B. D. & Sugino, A. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7261–7265
27. Celniker, S. E. & Campbell, J. L. (1982) *Cell* **31**, 201–213
28. Noguchi, H., Reddy, G. P. V. & Pardee, A. B. (1983) *Cell* **32**, 443–451
29. Decker, R. S., Yamaguchi, M., Possenti, R. & DePamphilis, M. L. (1986) *Mol. Cell. Biol.* **6**, 3815–3825
30. Diffley, J. F. X. & Stillman, B. (1986) *Mol. Cell. Biol.* **6**, 1363–1373
31. Jong, A. Y. S. & Scott, J. F. (1985) *Nucleic Acids Res.* **13**, 2943–2958
32. Baker, T. A., Sekimizu, K., Funnell, B. E. & Kornberg, A. (1986) *Cell* **45**, 53–64