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THE SINGLE-STRANDS OF YEAST AUTONOMOUSLY REPLICATING DNA SEGMENTS ARE NOT RECOGNIZED AS ORIGINS OF REPLICATION BY ESCHERICHIA COLI DNA REPLICATION PROTEINS

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SUMMARY

Sequence analysis of yeast DNA segments able to promote autonomous replication suggests that they may be similar in structure to origins of replication recognized by E. coli. This report describes the use of single-stranded circular DNA molecules containing such DNA segments in an in vitro system from E. coli which efficiently initiates DNA synthesis on various phage templates. We found no evidence for specific recognition of these yeast DNA segments as origins of replication by the E. coli DNA synthesis proteins. We conclude that the structural features recognized by the E. coli proteins as origins of replication are not exhibited by the yeast autonomously replicating segments.

INTRODUCTION

Segments of chromosomal DNA have been isolated from the yeast Saccharomyces cerevisiae that are able to promote the autonomous replication of vector DNA plasmids (1,2), thus mimicking DNA segments isolated from bacterial chromosomes, plasmids, and viral DNA molecules which carry specific origins of replication (3). Nucleotide sequence analyses of two such yeast DNA segments, the TRP1 linked ars1 element (4,5) and the autonomously replicating element carried on the 2μ plasmid DNA (6) suggest structural similarities to DNA segments carrying bacterial and viral origins of replication (4,7).

The functional similarities between these yeast and bacterial DNA segments can be assessed directly using in vitro DNA chain initiation assay systems isolated from E. coli. The E. coli cell specifies three different, site-specific DNA chain initiation pathways. The E. coli DNA-dependent RNA

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Table I

Template Recombinant DNA Molecules

Phage Clone	Yeast DNA Inserts			
	Autonomously Replicating Segment[a]	Strand[b]	Size (kB)	Source
fdY3	-	+	2.40	C EcoR1 fragment of 2μ plasmid[c]
fdY4	-	-	2.40	C EcoR1 fragment of 2μ plasmid
fdY5	+	-	2.15	D EcoR1 fragment of 2μ plasmid[c]
fdY6	+	+	2.15	D EcoR1 fragment of 2μ plasmid
fdY11	+	+	1.45	TRP1 EcoR1 fragment of pLC544[d]
fdY12	+	-	1.45	TRP1 EcoR1 fragment of pLC544

[a]Classification of 2μ plasmid DNA segments in this category is based on reference 7.
[b]Classification of strandedness as (+) and (-) is based on hybridization analysis, defining fdY3 and fdY11 as containing the (+) strands from 2μ plasmid and the TRP1 fragment, respectively.
[c]See reference 16.
[d]See reference 1.

polymerase catalyzes the synthesis of a specific RNA primer for DNA synthesis in one of these pathways (8,9), and the E. coli DNA primase in another (10,11). The DNA primase in concert with preinitiation proteins makes RNA primers at any of several different sites in the third pathway (12,13).

All three of these RNA primer synthesis pathways are functional in crude soluble protein extracts of E. coli (14). We used these DNA synthesis extracts to determine if DNA sequences able to promote autonomous replication in the simple eukaryote S. cerevisiae could be specifically recognized as sites for DNA chain initiation by any of the three E. coli RNA primer synthesis pathways.

METHODS

Phage. fd106 was obtained from H. Schaller (15). Yeast DNA fragments of interest were cloned into the single EcoR1 site in the chloramphenicol gene of fd106. The recombinants constructed are listed in Table 1. Each strand of yeast DNA elements carrying autonomously replicating segments was cloned in this vector, as well as each strand of a segment of the yeast 2μ DNA plasmid which cannot promote autonomous replication.

DNA synthesis reactions. A soluble protein extract of E. coli was used as the source of enzymes to convert added single-stranded template DNA to its duplex form, as described previously (14). Synthesis was monitored by incorporation of [^{3}H]dTTP. Each reaction contained 370-450 pmoles (nucleotide) of single-stranded circular DNA template.

Localization of the gap in synthetic duplex DNA. After synthesis of the complementary DNA strand, proteins were extracted, and nucleic acids were precipitated and resuspended in 20 µl water. Gap filling was performed at 15°C for 45 minutes in an incubation mixture of 50 µl containing: 50 mM Tris-HCl, pH 7.5; 10 mM $MgSO_4$; 10 mM β-mercaptoethanol; 50 µg/ml bovine serum albumin; 24 µM each dATP, dCTP, and dGTP; 1 µM [^{32}P]dTTP; 10 µl of resuspended DNA sample; and Klenow fragment of DNA polymerase I (17). The reaction was terminated by heating 10 minutes at 65°C. The sample was adjusted to 50 mM NaCl, and EcoR1 was added. After incubation at 37°C for 30 minutes, the reaction was stopped by addition of EDTA to 20 mM. DNA species were then resolved by zone sedimentation.

RESULTS

DNA synthesis dependent upon DNA primase. DNA synthesis on single-stranded DNA templates was first carried out in the presence of the RNA polymerase inhibitor rifampicin. Both DNA primase-dependent RNA primer synthesis pathways are active in the presence of this inhibitor, while the DNA-dependent RNA polymerase pathway is inhibited. Since primer synthesis at the fd106 vector DNA origin of replication requires the latter pathway (8,9), this allows the selective inhibition of initiation at the vector origin of replication.

The data in Figure 1a demonstrate that the two rifampicin-resistant pathways are active in the protein extract used in these experiments. DNA synthesis was readily detectable on phage G4 DNA, which is recognized by the DNA primase site specific initiation pathway (10,11), and on phage ØX174 DNA, which is specifically recognized by the preinitiation protein-DNA primase initiation pathway (12,13). In addition, these data demonstrate that the DNA-dependent RNA polymerase pathway for RNA primer synthesis was active in the absence of rifampicin and that in its presence, fd106 vector DNA showed little DNA synthesis. This low amount of synthesis is not above the background seen in the absence of added DNA (data not shown).

Figures 1b, 1c, and 1d show the amounts of DNA synthesis seen on fd106 DNA molecules carrying yeast DNA inserts. All reactions included rifampicin. No rifampicin-resistant DNA synthesis was observed on DNA molecules carrying any of the cloned yeast DNA segments. All DNA molecules were able to function as templates for DNA synthesis in the absence of rifampicin (data not shown). We conclude that neither those yeast DNA strands from segments able to promote

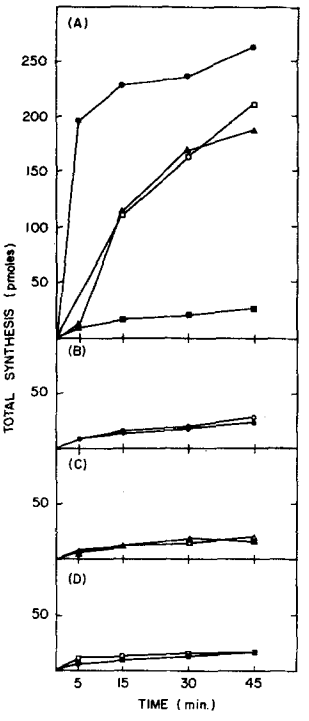

Figure 1. DNA synthesis directed by single-stranded circular template molecules. In (A) fd106 DNA, G4 DNA, and ØX174 DNA were incubated in standard reactions and treated as described in Methods. (●) G4 DNA + rifampicin; (▲) ØX174 DNA + rifampicin; (■) fd106 DNA + rifampicin; and, (□) fd106 DNA without rifampicin.

In (B), (C), and (D), synthesis was carried out in the presence of rifampicin. (●) fdY3; (O) fdY4; (▲) fdY5; (Δ) fdY6; (■) fdY11; and (□) fdY12.

autonomous replication in the yeast cell nor the control yeast DNA strands were recognized by either one of the two E. coli DNA primase dependent RNA primer synthesis pathways as carrying specific sites necessary for RNA primer synthesis.

DNA synthesis dependent upon RNA polymerase. Given the lack of recognition by the E. coli DNA primase dependent DNA chain initiation pathways, we asked if any of the yeast DNA inserts could be specifically recognized as sites for priming by RNA polymerase. Since single-stranded fd106 DNA is itself primed by RNA polymerase at a specific site, we determined whether chain initiation during DNA synthesis occurred only at the fd origin, or within the yeast insert DNA segment as well. This was accomplished by locating the gap

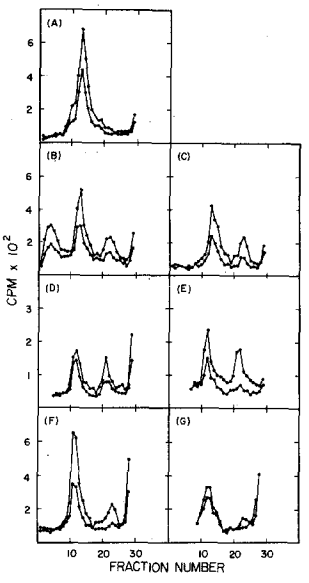

Figure 2. Localization of the gap in duplex molecules synthesized in vitro. After isolation of the duplex DNA from the reaction mixtures, samples were layered onto isokinetic, neutral pH sucrose gradients and spun 4.5 hours at 55,000 rpm at 15°C in a Beckman SW56 rotor. Fractions were collected from the bottom of the tubes, and the total [^{3}H] and [^{32}P] radioactivity was determined for each 0.1 ml fraction. (O–O) [^{32}P] label; (●–●) [^{3}H] label. (A) fd106; (B) fdY3; (C) fdY4; (D) fdY5; (E) fdY6; (F) fdY11; and (G) fdY12.

left, after DNA synthesis in these extracts, between the origin and terminus of the product DNA strand. After completion of DNA chain elongation, the [^{3}H] labeled double-stranded DNA molecules were isolated, and the gaps were filled in with [^{32}P] labeled nucleotides. This synthesis was catalyzed by the Klenow fragment of E. coli DNA polymerase I at 15°C to minimize nick translation and strand displacement (17,18). The DNA molecules were then digested with EcoR1 endonuclease to liberate the yeast DNA insert from the fd106 vector, and the two DNA segments were separated by zone sedimentation.

The results of these analyses are shown in Figure 2. The sedimentation profile of the linear fd106 DNA segment resulting from the control DNA synthesis reaction on fd106 DNA template without a yeast DNA insert is illustrated in Figure 2a. The [^{32}P] label marking the gap adjacent to the fd origin cosedimented with the uniformly distributed [^{3}H] label. In all other cases a

slower sedimenting [^{3}H] labeled yeast DNA insert band was also visible after endonuclease digestion. Given the amount of [^{3}H] label in each DNA band, the known sizes of each DNA segment, and the assumption that a gap left adjacent to an initiation site is identical in size no matter where chain initiation occurs on the DNA template, the extent to which gaps were left in yeast DNA segments versus the fd segment could be estimated. These values were less that 7% for fdY6, fdY11, and fdY12, and no more than 15% for fdY3, fdY4, and fdY5; that is, the [^{32}P] label marking the gap was always greater in the fd106 DNA segment. Thus, the yeast DNA segments were primed by RNA polymerase, if at all, at frequencies far less than those on the fd106 DNA segment, and the yeast DNA segments which support autonomous DNA replication in the yeast cell showed no higher specific activity for priming than the control yeast DNA segments. Therefore, the RNA polymerase priming pathway does not specifically recognize sites on those yeast DNA segments able to replicate autonomously in yeast.

DISCUSSION

Yeast DNA segments that promote autonomous replication of plasmid DNA in yeast cells have some of the properties of bacterial and viral DNA segments carrying origins of DNA replication. Nucleotide sequence analyses have suggested structural similarities between these kinds of DNA segments (4,7). Despite these similarities, Stinchcomb et. al. (19) have demonstrated that the duplex *E. coli* chromosomal origin of replication does not promote autonomous replication of plasmid DNA in the yeast cell. Yeast cell DNA replication proteins apparently do not recognize the duplex *E. coli* origin of replication as being functionally equivalent to a yeast origin of DNA replication.

The experiments presented here were designed to test the possibility that the *E. coli* DNA chain initiation proteins could specifically recognize yeast DNA segments capable of promoting autonomous DNA replication in yeast. The three kinds of chain initiation pathways active in the *E. coli* soluble protein extracts used in the experiments each recognize specific sites on single-

stranded template DNA. We observed no specific recognition of single-stranded clones of yeast DNA segments by any of these three E. coli RNA primer synthesis pathways. Our results suggest that these yeast DNA segments do not carry sites which act as origins for RNA primer synthesis in the manner recognized by these E. coli enzymes. If these yeast DNA elements do indeed carry specific origins of replication recognized by site-specific proteins in the yeast cell, these sites must be different from those recognized by the E. coli DNA initiation proteins.

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