Three Origins of Replication Are Active in Vivo in the R Plasmid RSF1040*

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Replicating DNA molecules of RSF1040, a deletion derivative of the conjugative R plasmid R6K, are cleaved at a single site by the Eco RI restriction endonuclease. Microscopic analysis of Eco RI-cleaved RSF1040 replicative intermediates synthesized in vivo indicates that initiation of replication occurs at three unique sites, orα, orβ, and orγ. The relative frequencies of initiations at these three origins are different from those found in vitro.

The R plasmid R6K which confers resistance to ampicillin and streptomycin has several features that make it an attractive system for study. R6K is a 26-megadalton conjugative plasmid which replicates in E. coli K12 cells as a multicopy pool as opposed to the rest of the conjugative plasmids, usually present in low copy number pools in E. coli (1). Nevertheless, R6K shares the ability of the other conjugative plasmids to replicate and be maintained in polA- mutants of E. coli (2, 3).

Generally, plasmid DNA replication is initiated at a unique site on the DNA molecule, although a few plasmids were shown to possess more than one replication origin (4-6). In vivo, R6K and its deletion derivative RSF1040 showed initiation at two sites, orα and orβ, separated by a stretch of about 3900 nucleotides (2, 7, 8). Replication from either orα or orβ first proceeds unidirectionally to a specific terminus and then proceeds from the origin in the opposite direction to complete the replication process at the termination site T. These results suggested the existence of a specific termination site on the R6K genome (3, 8).

Recently, Stalker et al. (9) and Kolter and Helinski (11) reported that they cloned and sequenced a 520-base pair region of R6K that included a functional origin of replication, but the position of this initiation site was not correlated with the location of the previously described orα and orβ (7). I report in this communication that, by using early replicative intermediates of RSF1040 DNA replicating in vivo, it is possible to map a third replication initiation site on RSF1040 DNA which is located between orα and orβ. This third origin, which we named orγ, is used in vivo at a much lower frequency than orα or orβ.

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RESULTS

Purified pulse-labeled RSF1040 DNA was isolated after pulsing with [3H]thymidine and banding the lysates in CsCl/ethidium bromide gradients. Early replicative intermediates (banding at a position very close to the covalently closed circular and open circular DNA) were pooled. The pooled fractions were centrifuged in a propidium iodide/CsCl gradient. The gradient was fractionated, and fractions corresponding to the areas closest to the covalently closed circular DNA peak (likely to contain molecules in which replication has just been initiated) were pooled. After elimination of the propidium iodide and dialysis against 6 mM Tris/HCl, pH 8.0, this pool was ready to be examined in the electron microscope.

Electron Microscopy of Plasmid DNA—Cytochrome c was added to a final concentration of 0.1 mg/ml to Eco RI restriction endonuclease-cleaved replicative intermediate DNA, and the DNA was spread by the method of Davis et al. (10). The DNA was picked up on Parlodion-coated grids, rotary shadowed with PtPd (80:20), and examined with a JEOL 100 B electron microscope.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—Escherichia coli K12 W1485-1 F' thy Nα containing RSF1040 (Ap') was used in this study.

Labeling of RSF1040 Replicative Intermediates—E. coli W1485-1 (RSF1040) was prelabeled with [2-3H]thymidine (0.67 µCi/ml; 1.7 µCi/ 

ml) for several generations in minimal medium (6) to a cell density of 2 x 10^8 cells/ml. The cells were harvested and washed at 25°C and resuspended in fresh thymine-free medium at 37°C. After 30 min, the cells were shifted to a 25°C water bath for 10 min and pulsed for 25 s with [methy-3H]thymidine (10 µCi/ml; 0.05 µCi/ml). Incorporation was stopped by addition of sodium azide (5 x 10^-3 M, final concentration) and the cells were immediately frozen in a dry ice-ethanol bath. Cells were thawed, collected by centrifugation, and resuspended to a density of 5 x 10^8 cells/ml. Cells were lysed as described previously (6), and the lysate was centrifuged to equilibrium in a CsCl/ethidium bromide gradient (6, 7). The gradient was fractionated, and fractions corresponding to replicative intermediate (distributed in the region of the gradient between the prelabeled covalently closed circular and open circular DNA) were pooled. The pooled fractions were centrifuged in a propidium iodide/CsCl gradient. The gradient was fractionated, and fractions corresponding to the areas closest to the covalently closed circular DNA peak (likely to contain molecules in which replication has just been initiated) were pooled. After elimination of the propidium iodide and dialysis against 6 mM Tris/HCl, pH 8.0, this pool was ready to be examined in the electron microscope.

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RESULTS

Purified pulse-labeled RSF1040 DNA was isolated after pulsing with [3H]thymidine and banding the lysates in CsCl/ethidium bromide gradients. Early replicative intermediates (banding at a position very close to the covalently closed circular Form I peak) were selected in order to determine accurately the position of possible origin(s) of replication (Fig. 1).

Eco RI treatment of these molecules rendered linear molecules which, when examined in the electron microscope, showed an internal loop of replicated DNA and two unreplicated branches. In these molecules, approximate positions for origins of replication could be obtained by measuring the distance from one of the Eco RI-generated ends to the repli-
Three Origins of Replication in Vivo on RSF1040

FIG. 2. Diagram of measurements of Eco RI-cleaved replicating RSF1040 DNA molecules at early stages of replication. In this diagrammatic representation, the cleaved molecules are aligned with the short unreplicated length of DNA to the left. The replicated region between the forks is represented by a heavy line, while the light lines correspond to the unreplicated region. Measurements are presented in terms of percentage of total molecular length. The percentages not shown (50 to 100) correspond to unreplicated regions.

TABLE I

| Experiment | No. of molecules analyzed | oriα | oriβ | oriγ |
|------------|---------------------------|------|------|------|
| 1          | 44                        | 0.48 | 0.34 | 0.18 |
| 2          | 40                        | 0.47 | 0.38 | 0.15 |
| 3          | 52                        | 0.49 | 0.35 | 0.16 |
| 4          | 43                        | 0.50 | 0.32 | 0.18 |

FIG. 3. Electron micrographs of Eco RI-cleaved replicating RSF1040 DNA molecules at early stages of replication. Replicating RSF1040 DNA was obtained from cells of E. coli 1485-1 (RSF1040) and cleaved with Eco RI restriction endonucleases. a and b, oriα; c and d, oriβ; e and f, oriγ.

The majority of RSF1040 molecules initiated replication from either oriα or oriβ; some molecules were observed in which both origins were operating simultaneously (7).

Molecular cloning experiments (11) suggested the presence of an origin of replication near the junction of HindIII fragments H4 and H9, but the position of this origin did not correspond to the positions of either oriα or oriβ as previously reported (7, 8). When we became aware of Kolter and Helinski's (11) results, we reexamined the replicative properties of RSF1040 by using replicative intermediates which were at very early stages of replication. These molecules were cleaved with Eco RI and examined in the electron microscope. Although most of the initiations occurred at 21.4 ± 1.3% and 40.8 ± 2.0%, respectively, from one of the Eco RI ends, there is also very clear evidence of initiations occurring at a site 30.9 ± 1.9% from the Eco RI end, suggesting the presence of an additional origin of replication which we named oriγ. Presumably, in past in vivo experiments, oriγ was overlooked because initiations at this origin are rare in vitro and molecules initiating from oriα or oriβ after replicating but for a short time would have masked this site. This is also a problem when one wants to determine directionality for molecules initiating replication at oriγ. However, inspection of Fig. 2 suggests that, at
Three Origins of Replication in Vivo on RSF1040

least initially, replication from oriy must be counterclockwise, that is towards or$. The initial component of the sequential mode of replication exhibited by R6K and derivatives in vivo is counterclockwise from oria and clockwise from orip (3,7,8). It remains to be seen whether replication from oriy is bidirectional in vivo or unidirectional as is the case in vitro (12).

Our assignment of the γ origin of replication would place it around the junction of fragments HindIII H4 and H9, while oria is located on HindIII fragment H4 and orip is located on HindIII fragment H2 near the junction with HindIII fragment H15 (see Fig. 4). The location of oriy at the junction of HindIII fragments H4 and H9 suggests that oriy must be the origin cloned and sequenced in Helinski's laboratory (9,11).

It is interesting that initiation of DNA replication at oriy requires the π protein both in vivo (11) and in vitro (2), and that initiation of DNA replication at oria or orip requires an intact sequence of HindIII fragments H9 and H15 which encodes π protein (2). Despite these similarities, our findings indicate that the frequency of origin usage in vivo is quite different from that in vitro (12). In vivo, oria is the origin used at highest frequency, while in vitro oriy and orip are the origins used at highest frequency. The reason for these differences in frequency of origin usage is not clear as yet. Lack of specific cellular components in the in vitro system could lead to a difference in selection of initiation origins. We are currently examining RSF1040 replication under a variety of physiological conditions to define the parameters that lead to selection of the origins of replication in RSF1040 DNA.

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