Plasmid-encoded initiation protein is required for activity at all three origins of plasmid R6K DNA replication in vitro

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DNA replication of plasmid R6K initiates at three unique sites, *oriα*, *oriβ*, and *oriγ*. Replicating DNA molecules of a deletion derivative of R6K were synthesized in an in vitro system containing π protein fraction from cells carrying a mini-R6K derivative that produced only this initiation protein as an R6K-encoded protein and analyzed by electron miscroscopy. Requirement of π protein for the activity of all these three replication origins in vitro was verified. Frequencies of initiation at the three origins were almost equal.

Plasmid R6K *Initiation protein* *In vitro replication* *Replication origin* *Electron microscopy*

1. INTRODUCTION

DNA replication usually initiates at a unique site on the DNA molecule in prokaryote. However, a few plasmid replicons have been shown to have two or three origins of replication [1–4]. It is very interesting to clarify how multiple origins are activated and how the initiation is controlled, in comparison with the mode of replication at many origins in eukaryotic DNA.

Plasmid R6K, which specifies resistance to ampicillin and streptomycin, is a multicopy plasmid of 38 kilobase pairs [5]. We have found that this plasmid possesses three origins, *oriα*, *oriβ*, and *oriγ* using an in vitro replication system that contains the products encoded by the whole R6K genome [6]. This result has been confirmed in vivo [7]. The in vitro system allowed us to show that an R6K-encoded protein, π protein, is directly required for the initiation of R6K DNA replication [8,9]. This initiation protein interacts with only the *oriγ* region of the DNA as a part of the initiation event [10]. The structural gene for π protein, *pir*, is located in the vicinity of *oriβ* and *oriγ* [11]. We already know that the initiation from *oriγ* is dependent on π protein [10,11]. Therefore, a big question to be answered was whether the initiation from *oriα* and *oriβ* required only π protein or other protein(s) encoded by the *oriα* and *oriβ* regions, in addition to π protein. The in vitro replication system is advantageous to answer this question, because it does not synthesize proteins encoded by template DNA [8], and thus the analysis can be limited to the protein added in the reaction. Therefore, we prepared a π protein fraction from cells carrying a mini-R6K derivative, and used it in the in vitro system containing RSF1040 DNA that is a deletion derivative of R6K, as a template.

Here, we show that π protein, but no other R6K protein, is required for the initiation of replication from all three origins (*oriα*, *-β* and *-γ*) of the R6K derivative in vitro.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and materials*

Escherichia coli K12 YS1 was used as a minicell producing strain [12]. Plasmid pRK419 is a mini-R6K derivative (fig.1) [13], and pMK20 is a mini-*Col*E1 carrying an *Hae*II-Km^r fragment [14]. The π protein fraction for in vitro R6K DNA replication, designated AS(0–45)-pRK419$^+$, was prepared by precipitation of a DEAE-cellulose

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eluent with 45% saturated ammonium sulfate as in [6]. Fractions of *E. coli*-encoded proteins necessary for R6K DNA synthesis, termed AS(0–45)-R⁻ and AS(40–52)-R⁻, were prepared as ammonium sulfate precipitates of YS1 cell extracts according to the procedure in [9].

2.2. *Purification and labelling of minicells, and SDS-polyacrylamide gel electrophoresis*

Minicells were purified from a stationary phase culture of cells carrying plasmids essentially as described by Roozen et al. [15]. Labelling of minicells with [^{35}S]methionine (20 μCi/ml) in M9 medium containing 0.2% glucose and all amino acids except methionine was carried out for 30 min at 37°C. Labelled cells were suspended in the sample buffer [16] and heated at 100°C for 3 min. Gel electrophoresis was run on a 12.5% polyacrylamide gel according to [16].

2.3. *In vitro DNA synthesis, purification of replicative intermediates, and electron microscopy*

Replicative intermediates were purified from the reaction mixture [6,9] containing RSF1040 DNA and a π protein fraction from pRK419$^+$ cells, after synchronization of DNA initiation followed by pulse DNA synthesis for 1.5 min [6]. They were then cleaved with *Eco*RI restriction enzyme, spread and observed with an electron microscope as in [6].

3. RESULTS

3.1. *Mini-R6K produces only π protein as an R6K-encoded protein*

To rule out the possibility that other R6K-encoded proteins as well as π protein participate in R6K DNA replication, the smaller R6K derivative, pRK149, was used to produce π protein. This plasmid carries a 2.85-kilobase pair fragment of the R6K genome and an *Hae*II-Km^r fragment from pMK20, as shown in fig.1.

First, it was confirmed that pRK419 produces active π protein by the in vitro DNA synthesis system as shown in fig.2. Then, pRK419, R6K, and pMK20 DNA were introduced by transformation into YS1 cells in order to analyze the plasmid gene products. Minicells were purified on sucrose gradients, and plasmid-specified polypeptides were

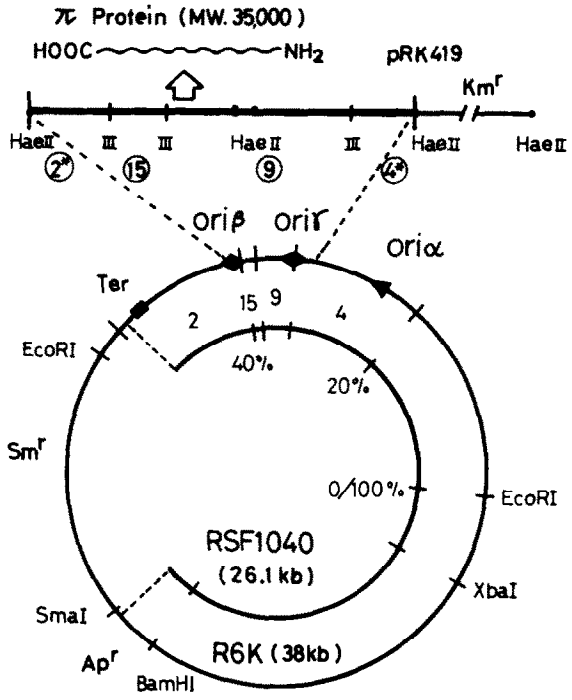

Fig.1. Physical and genetic maps of plasmid R6K, RSF1040 and pRK419. *Oriα*, *oriβ*, and *oriγ* refer to the three initiation sites of DNA replication. Numbers 4, 9, 15 and 2 refer to specific *Hin*dIII (III) fragments in the replication region, and *Hin*dIII sites are only shown in the relevant region. The 2.85-kb fragment that is carried by pRK419 is expanded and contains the *Hin*dIII fragments 9, 15 and parts of 2 and 4 (indicated as 2* and 4*). Percentages inside the RSF1040 map present the relative distance from the *Eco*RI site on the genome [6,10,13].

identified after labelling with [^{35}S]methionine and electrophoretic separation on gels. As shown in fig.3, pRK419 synthesized only two polypeptides in minicells. Additional polypeptides could not be found even under the condition in which those of molecular masses of more than approx. 6 kDa were detected in using a 15% polyacrylamide gel. One has a molecular mass of 28 kDa and can also be produced by pMK20, a mini-*Col*E1-Km^r derivative, which carries the same *Hae*II-Km^r fragment. Therefore, this small polypeptide is a kanamycin-resistant enzyme. The larger polypeptide of 35 kDa from pRK419 can also be found among the R6K-encoded polypeptides. This polypeptide copurified with π protein activity through the initial stage of purification (unpub-

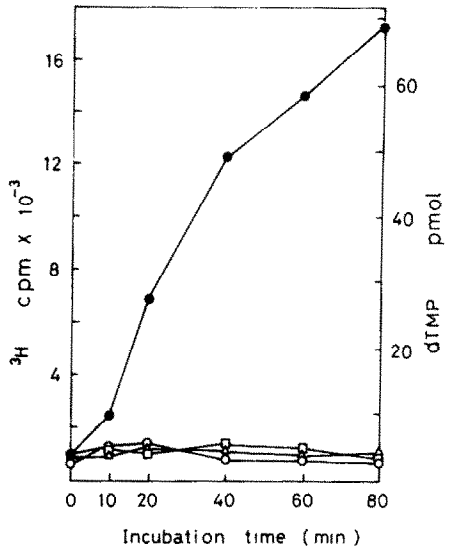

Fig.2. Replication of R6K DNA in the reaction mixture containing a protein fraction prepared from pRK419$^+$ cells. The standard reaction mixture contained CC-R6K DNA, AS(0–45)-pRK419$^+$ fraction, AS(40–52)-R$^-$ fraction, [^{3}H]TTP, the other dNTPs, 4rNTPs, and buffer system [9]. The reaction was carried out at 30°C [9]. (●) Standard reaction mixture, (△) with the addition of rifampicin (20 μg/ml), (□) without template DNA, (○) with AS(0–45)-R$^-$ instead of AS(0–45)-pRK419$^+$.

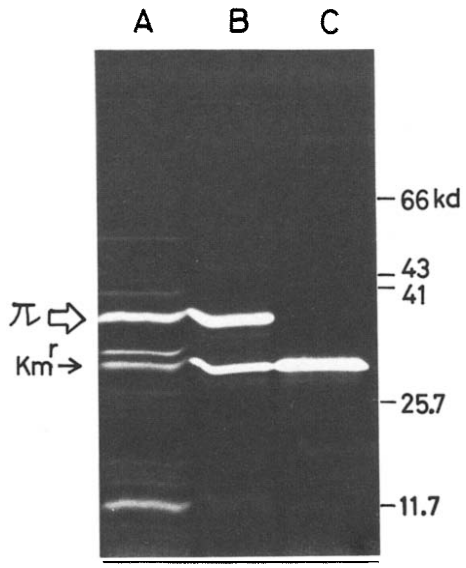

Fig.3. SDS-polyacrylamide gel electrophoresis of π protein synthesized in minicells. ^{35}S-labelled samples were run on 12.5% gel which was dried and then placed on an X-ray film. Lane A, R6K; B, pRK419; C, pMK20. Standard molecular mass markers are indicated on the right side in kDa. From the top: bovine serum albumin; ovalbumin; alcohol dehydrogenase; chymotrypsinogen A and cytochrome *c*.

lished). It is known that the nucleotide sequence of the *pir* gene region encodes a polypeptide of 305 amino acids [17,18], which is in good agreement with the size estimate of the above polypeptide. Other than π, the largest polypeptide predicted from the nucleotide sequence of this region is 47 amino acid residues in length, but the region encoding this hypothetical peptide has no DNA sequence resembling ribosome binding sites [17]. The larger polypeptide must therefore be the π protein and seems to be only one protein expressed by the R6K segment carried on pRK419.

3.2. *Analysis of replicative intermediates synthesized in vitro*

We were able to exclude the possibility that any one of the three origins on the R6K genome initiated replication using the usual cell replication proteins but no R6K-encoded π protein, because none of R6K origins was active in a reaction mixture which contained only the protein fraction prepared from cells free of the plasmid (R$^-$) (fig.2).

The π protein fraction was prepared from YS1 cells carrying pRK419, which produces π protein and a kanamycin-resistant enzyme. As described in section 2, the replicative intermediates were synthesized in vitro and purified. *Eco*RI treatment of these molecules yielded linear molecules with an internal bubble of replicated DNA and two unreplicated segments. The analyses of the data obtained by electron microscopy are presented in fig.4, in which the replicative intermediates were classified into three populations depending on the origins used according to [6]. These results clearly show that all of the three origins (*oriα*, *oriβ*, and *oriγ*) are active in the in vitro system containing only π protein as an R6K-encoded protein. The locations of *oriα*, -γ and -β are 25.2, 32.7 and 40.0%, respectively, from the *Eco*RI site of RSF1040 DNA. These results are in good agreement with origin sites previously determined for

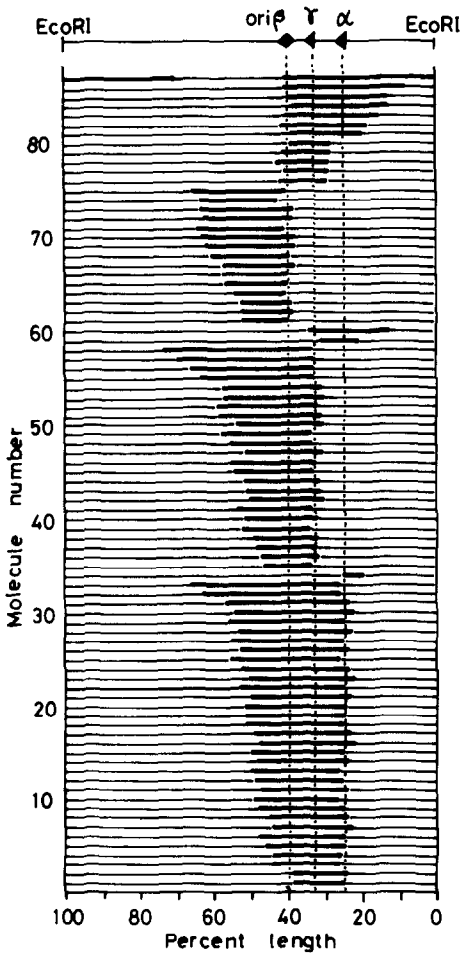

Fig.4. Schematic presentation of *Eco*RI-cleaved replicating RSF1040 DNA molecules. The replicated region between the forks is indicated by the heavy line. Linear replicating molecules were arranged in order of increasing extent of replication from the three origins. Measurements are presented in terms of percentage of total molecular length.

oriα, *-γ* and *-β* (25.2, 33.9 and 39.4%, respectively) [6], as shown in fig.1.

3.3. *Frequency of origin usage*

No preferential use of one of the three origins was observed in this in vitro system. That is, the frequencies of origin usage were approximately equal: 40 ± 2% for *oriα*, 28 ± 4% for *oriβ*, and 32 ± 3% for *oriγ* (out of 87 molecules examined). These values are almost the same as those obtained in the previous in vitro system containing the proteins encoded by the whole R6K genome [6]. Unclassified molecules including those of bidirectional replication type were also observed at several percents. For the molecules examined, replication from *oriα* and *-γ* proceeds predominantly counterclockwise, whereas replication from *oriβ* proceeds in either direction with almost the same frequency.

4. DISCUSSION

A mini-R6K derivative, pRK149, produces only one R6K-encoded protein detectable in minicells, the π protein. The same result was obtained by the analysis of the gene products of pRK419 in maxicells (unpublished). Electron microscopic study of replicative intermediates has demonstrated that all three origins of replication are functional only when the in vitro replication system contains a π protein fraction prepared from cells carrying pRK419. Furthermore, all three origins are inactive, in vivo, in R^- cells; this is shown by the fact that plasmid P15A derivatives carrying the *oriα*, *oriβ*, or *oriγ* region cannot replicate in R^- *polA*$^-$ cells [10]. From these results, we can show that the R6K-encoded π protein is sufficient to initiate the replication from *oriα*, *-β* and *-γ* in the presence of necessary cellular proteins. It remains to reconfirm this fact by the in vitro replication system containing a purified π protein.

Recent observations of *oriα* and *oriβ* activity in vivo suggest that the *oriγ* region must be located in *cis* with the *oriα* and *oriβ* regions even when π protein is supplied in *trans* [10,19]. In addition, π protein always interacts with the *oriγ* region for the initiation of replication, but not with the *oriα* and *oriβ* regions [10,20]. Based on these results, we are conducting further experiments to determine how all three origins are activated by the interaction of the initiation protein with the direct repeats in the *oriγ* region, including the analysis of transcription from the *oriγ* to the *oriα* or *oriβ* regions.

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