Replicating molecules of plasmid RSF1040, a deletion mutant of R6K, were synthesized in vitro and analyzed by electron microscopy. Initiation of replication occurs at three unique sites, oriA, oriB, and oriC, within a 3900-base pair segment of the R6K genome. These sites are indistinguishable from the origins that are active in vivo. Frequencies of initiation at these three origins, however, are different from those observed in vivo. Replication proceeds unidirectionally in either direction from oriB and oriC and in one direction from oriA. The replication terminus of the R6K genome is inactive in the in vitro system.

Initiation of prokaryotic DNA replication usually occurs at a unique site on the DNA molecule. However, several prokaryotic replicons have been reported to have more than one origin of replication (1-3). In these cases, one of the replication origins is used predominantly with the other origin sites used infrequently or only when the normal origin site is deleted or when the structure of the replicon is otherwise modified. It is highly interesting to determine the genetic and biochemical nature of the control of these multiple origins and their usage in the initiation step of replication.

Plasmid R6K is a conjugal plasmid of molecular weight 38 kilobase pairs and codes for resistance to streptomycin and ampicillin (4). This plasmid and its deletion derivative, RSF1040, have been shown to possess two origins of replication, in vivo. These sites are separated by approximately 3900 base pairs (5, 6). Replication from either one of the two origins, designated α and β, is unusual in that it proceeds unidirectionally towards an asymmetric terminus, followed by replication from the same origin in the opposite direction (5, 7).

An in vitro system capable of replicating R6K DNA has been developed (8, 9). It was established with this system that an R6K-encoded protein, σ protein, is required for the initiation of R6K DNA replication. Preliminary results suggest that this protein associates with the template DNA as part of the initiation event (9). Studies with this in vitro system are providing a very useful approach to understanding the mechanism of replication of R6K and its regulation. A fundamental question with regard to the in vitro system is the correspondence of the origin sites of replication and directionality of replication in the in vitro system with the origin sites and directionality observed in vivo. In this paper, we describe the location of three replication initiation sites and their directionality in an in vitro system for the replication of the R6K derivative, plasmid RSF1040. An accompanying communication (10) describes the activity in vivo of these three origin sites of RSF1040.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Materials—Escherichia coli K12 YSl, free of plasmid DNA, and KE372, a mutant strain carrying an elevated copy number of R6K, were used for the preparation of protein fractions necessary for in vitro DNA synthesis (8, 9). KE414 was constructed by transformation of YSl with RSF1040 DNA.

Closed circular DNA molecules of RSF1040 DNA were prepared by ethidium bromide/CeCl density gradient centrifugation of a Sarkosyl lysate of KE414 cells (11) and used as template DNA. The media and the source of the materials used are described in earlier publications (8, 9).

Preparation of Cell Extracts and Ammonium Sulfate Precipitates—Cell extracts and ammonium sulfate precipitates from cells free of plasmid (R-) were prepared according to the procedure described previously (9). These fractions, designated AS(0-40)-R- and AS(40-02)- R-, were used as the source of least chromosome-encoded proteins necessary for R6K DNA replication. Cell extracts from cells carrying plasmid R6K (R+) were prepared by a small modification of the method previously described (9). In the process of cell lysis, 1 M KCl was added to increase the recovery of σ protein. After cell extracts were passed through a DEAE-cellose column, the protein fraction prepared by precipitation with 45% saturated ammonium sulfate, AS(0-45)-R+, contains plasmid-encoded σ protein.

In Vitro DNA Synthesis and Isolation of Replicative Intermediates—The reaction mixture contained 40 mM 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer (pH 8.0), 80 mM KCl, 7.5 mM MgCl₂, 0.1 mM NAD⁺, 2 mM rATP, 0.5 mM each of rCTP, rUTP, and rGTP, 0.02 mM each of dATP, dGTP, and dCTP, and [3H]-dTTTP (~300 cpm/pmol of TTP), 50 μg/ml of bovine serum albumin, 10 μg/ml of closed circular RSF1040 DNA or 12.5 μg/ml of closed circular R6K DNA, 50 μl each of AS(0-45)-R+ and AS(40-02)-R-, and 6 μl of AS(40-52)-R+ fraction/ml of the reaction mixture. Three milliliters of the above reaction mixture lacking the dNTPs was preincubated for 10 or 15 min at 30°C. The four dNTPs, including [3H]-dTTP, were then added and, after incubation for 15 or 20 min at 30°C, the reaction was stopped by the addition of 20 mM EDTA, 1.25 mg/ml of pronase, and 20 μg of yeast tRNA. The mixture was then incubated at 30°C for 15 min, followed by the addition of 0.25% Sarkosyl and further incubation for another 15 min. After dialysis against 50 mM Tris (pH 8.0), 5 mM EDTA, 50 mM NaCl, 3H-labeled, replicative intermediates were purified by ethidium bromide/CeCl density gradient centrifugation at 40,000 rpm for 4 h in a Ti-50 rotor with 3H-labeled ColE1 DNA as a density marker. The fractions with an intermediate density between closed circular and open circular molecules were pooled and rechromatographed in the same gradient. The purified replicative intermediates were pooled for examination by electron microscopy.

Electron Microscopy—Replicative intermediates were cleaved with restriction endonuclease Eco RI, mixed with open circular ColE1 DNA which was added as an internal length standard, and spread for electron microscopy (12, 13). Photomicrographs were taken on 35-mm film with a Phillips 300 electron microscope, and DNA molecule lengths were measured by overhead projection using a Hewlett-Packard calculator and digitizer.

RESULTS

Purification of Replicative Intermediates—To increase the
level of replicative intermediates in the DNA preparation, the following synchronization process for the initiation of replication in vitro was carried out. The standard reaction mixture lacking all dNTPs was preincubated for 10 or 15 min to form initiation complexes involving 7 protein and to facilitate nascent RNA synthesis that is necessary for the initiation of R6K replication. The four dNTPs including [3H]TTP were then added to initiate DNA synthesis. The reaction was terminated after 1.5 min, and the labeled products were centrifuged to initiation complexes involving preincubation without dNTPs for replication (Fig. 2). R6K DNA also was used as a template in this in vitro system. Unlike RSF1040, R6K DNA contains two Eco RI sites that are asymmetrically located. The origins of replication detected on the larger (A) Eco RI fragment. It has been shown that the in vivo replication origins of RSF1040 correspond to those of R6K (14). Therefore, it is most likely that the replication origins of RSF1040 in vitro are located on the same side near the Eco RI site as shown in Fig. 2.

In order to determine the precise location of the origin sites in Fig. 2, a histogram of the frequency of initiation sites on the genome was made (Fig. 3). It is clear that three replication origins, designated $\alpha$, $\beta$, and $\gamma$, are active in RSF1040 DNA. The $\alpha$, $\gamma$, and $\beta$ origins are located at 25.2% ± 1.3%, 33.9% ± 1.4%, and 39.4% ± 0.9%, respectively, from the Eco RI site of RSF1040 DNA. The three origins are clustered in a region equivalent to 14.2% of the RSF1040 genome and 9.0%, of the R6K genome. Of the 121 replicating molecules, only 1 molecule was found to use both oria and oriy, simultaneously.

As shown in Fig. 4, the sites of the initiation of replication in vitro have been positioned on the R6K and RSF1040 restriction maps. The $\alpha$, $\gamma$, and $\beta$ origins are located on the HindIII fragment 4, around the junction of fragments 9 and 4, and around the junction of fragments 2 and 15, respectively. The in vitro results on the replication of R6K and RSF1040 derivatives have shown that oria is located midway on HindIII fragment 4, while orib is located at the end of fragment 2 that

![Fig. 1. Purification of replicating molecules of RSF1040 synthesized in vitro.](image1)

![Fig. 2. Schematic presentation of replicative intermediates of RSF1040.](image2)
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FIG. 4. Possible locations of the replication origins of RSF1040 on the restriction map. Physical maps shown were published elsewhere (6, 15) and include unpublished data. Numbers on the expanded map indicate the percentage of the genome size of RSF1040 from the Eco RI site. Numbers under the line indicate number designations of fragments of R6K DNA cleaved with HindIII. The dashed line for RSF1040 indicates the region of R6K deleted. kb, kilobase pair; Amp', ampicillin; Sm', streptomycin; Ter, specific terminus which is active in vivo.

is adjacent to fragment 15 (6). Therefore, two of the three origins that have been observed in the in vitro system compare well with the positions of oriA and oriB determined by the in vivo studies. The γ origin of replication on RSF1040 that appears to be used frequently in vitro recently has also been observed during in vitro replication (10).

Frequency of Origin Usage and Directionality—The analysis of RSF1040 and R6K DNA replicated in vitro has indicated that initiation occurred at oriA more frequently than at oriB (6). In the case of the initiation in vitro, the frequencies of initiation from these two origins is quite different (Table I). Initiation occurs at the frequencies of 24%, 43%, and 37% from the oriA, oriB, and oriγ, respectively. Replication initiated from oriA was observed to proceed only counterclockwise for the molecules examined. Replication from oriB or oriγ, however, proceeds in either direction, but only in one direction at least initially. It appears that replication proceeds counterclockwise from oriA, oriB, or oriγ at almost the same frequency among the molecules examined. Bidirectional replication from any of the three origins was not observed.

Inactivity of the Specific Terminator for R6K Replication in Vitro—R6K and RSF1040 possess a DNA sequence that terminates replication initiated from an R6K origin. This terminus is asymmetrically located with respect to either oriA or oriB (5, 7). The specific terminus appears to be inactive in vitro under the conditions employed since, as shown in Fig. 2, for 34 of the total 121 replicating molecules, the replication fork has passed the terminus site located at 55% from the Eco RI cleavage site.

DISCUSSION

It has been found that plasmid RSF1040 possesses three origins of replication that are functional in replication in vitro. The locations of the three origins are within a 3900-base pair segment of the plasmid genome. Two of the observed origins appear to be identical in position with origins α and β previously shown to be functional in replication in vivo (6). The third origin, γ, recently also has been shown to be active in vivo (10). The γ origin appears to be identical with the 360-base pair origin segment isolated from R6K DNA and functional in E. coli cells when the σ protein is provided in trans (16). The γ origin is located in the region of the junction of Hind III fragments 9 and 4. Initiation of replication at the three origins in vitro required an extract prepared from R6K-containing cells. While initiation of DNA replication at the γ origin required the σ protein in vivo (16) and in vitro, there is no direct evidence as yet whether or not this protein and/or other R6K-encoded proteins are required for initiation of the α and β origins in vivo or in vitro. However, the fact that the α origin requires Hind III fragments 15 and 9 in cis for activity suggests that the σ protein is required for origin activity (6). Hind III fragments 9 and 15 encode σ protein (16).

The reason for the differences in frequency of origin usage and directionality found in the in vitro studies presented here (Table I) and in the in vivo analyses (6, 10) is unclear. It is possible that selectivity of the origins for initiation might be lost due to the preincubation step carried out to synchronize the initiation event. It was found that initiation occurred at oriA more frequently after preincubation for 10 min rather than 15 min. It is also possible that selection of an origin site is influenced by a specific secondary or tertiary structure of this region of R6K that is generated by interaction with other cellular components in intact cells.

Replication of RSF1040 in vitro proceeded unidirectionally from all three origins (Fig. 2). The in vivo results indicate that both oriA and oriB have a potential to initiate replication sequentially in both directions (5, 6). In the case of oriA, only counterclockwise replication was observed in vitro. A similar pattern of replication is observed for oriA in vivo when the terminus is deleted (6). For oriB replication proceeded in both directions at almost the same frequency, while the major initial direction for this origin of replication in vivo is clockwise. Another example of unidirectional replication in both directions comes from the analysis of replicative intermediates of mini-ColE1, (pVH51) in vivo (17), and RSF1010 (18). In these cases, bidirectional replication also was observed.

In the in vitro system employed, the terminus of RSF1040

| Origin | Direction | No. of molecules | Percentage* | Total percentage* |
|--------|-----------|-----------------|-------------|-------------------|
| α      | Counterclockwise | 24              | 20          | 20                |
|        | Clockwise    | 0               | 0           | 0                 |
| β      | Counterclockwise | 28              | 23          | 43                |
|        | Clockwise    | 24              | 20          | 43                |
| γ      | Counterclockwise | 34              | 28          | 37                |
|        | Clockwise    | 11              | 9           | 37                |

* Based on the analysis of 121 replicative intermediate molecules.
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did not function, although previous in vitro results had suggested an active terminus (8). Termination of replication may require additional components, for example a membrane site and/or other proteins that are deficient in this system, in addition to the specific nucleotide sequence. The requirements for an active termination will be examined using this in vitro replication system.

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