Proc. Natl. Acad. Sci. USA
Vol. 77, No. 11, pp. 6566–6570, November 1980
Biochemistry

Expression of a DNA strand initiation sequence of ColE1 plasmid in a single-stranded DNA phage

(rifampicin-resistant initiation/*dnaB*/*dnaG*/phage M13)

NOBUO NOMURA AND DAN S. RAY

Molecular Biology Institute and Department of Biology, University of California, Los Angeles, California 90024

Communicated by P. D. Boyer, August 21, 1980

ABSTRACT **In order to investigate initiation of H-strand (lagging strand) replication of the plasmid ColE1, the origin region fragment (*Hae* II-E) of ColE1 was inserted into the intergenic region of filamentous DNA phage M13 and cloned. A site capable of promoting DNA strand initiation on a single-stranded DNA template has been detected on the L-strand (leading strand) of the cloned fragment. The site, named *rri-1* rifampicin-resistant initiation), directs conversion of chimeric phage single-stranded DNA to parental replicative form in the presence of rifampicin, which blocks the function of the complementary strand origin of M13. The function of *rri-1* is dependent on both the *dnaG* and *dnaB* gene products. It is postulated that *rri-1* might be an initiation site for synthesis of the lagging DNA strand during unidirectional replication of ColE1 DNA.**

Plasmid ColE1 replication initiates at a fixed origin and proceeds unidirectionally (1, 2). Although the mechanism of initiation of L-strand (leading strand) synthesis in ColE1 replication has been studied extensively, that of the H-strand (lagging strand) is still unclear. Tomizawa and coworkers have shown that the 6S L-strand fragment is synthesized first, and the transition point from primer RNA to DNA of 6S L-fragment has been mapped in the middle of the *Hae* II-E fragment (Fig. 1) (6–10). RNA polymerase participates in synthesis of the 6S L-strand fragment by making an RNA primer (6, 7). Once the primer is formed, RNA polymerase is not required for further replication (8). Because the *dnaG* function is involved in replication of plasmid DNA (11, 12), RNA priming of H-strand initiation could possibly be catalyzed by the function of the *dnaG* gene product (primase). It has been observed that after 6S L-fragment synthesis, the early replicative intermediate contains a D-loop (displacement loop) structure (13). This suggests that initiation of H-strand synthesis might require a single-stranded DNA (ssDNA) template. A method to assay DNA strand initiation of a fragment in a single-stranded form has been developed (14). This method takes advantage of the fact that the first step of phage M13 replication, conversion of phage ssDNA to the parental replicative form (RF) DNA, requires RNA polymerase for priming (15). Therefore, RNA polymerase-independent RNA primer synthesis due to a cloned fragment can be detected by measuring the efficiency of conversion of the infecting phage DNA to a duplex form under conditions in which RNA polymerase activity is inhibited. The validity of this method has been demonstrated by the successful expression of the complementary strand origin of bacteriophage G4 inserted into the intergenic region of M13 (14). Thus, with the hope of discovering an origin of H-strand replication of ColE1, we have inserted the origin region fragment (*Hae* II-E) of ColE1 into the intergenic space of M13 and cloned it. We present here results that indicate the existence of a site (named *rri-1*) capable of promoting DNA strand initiation on the L-

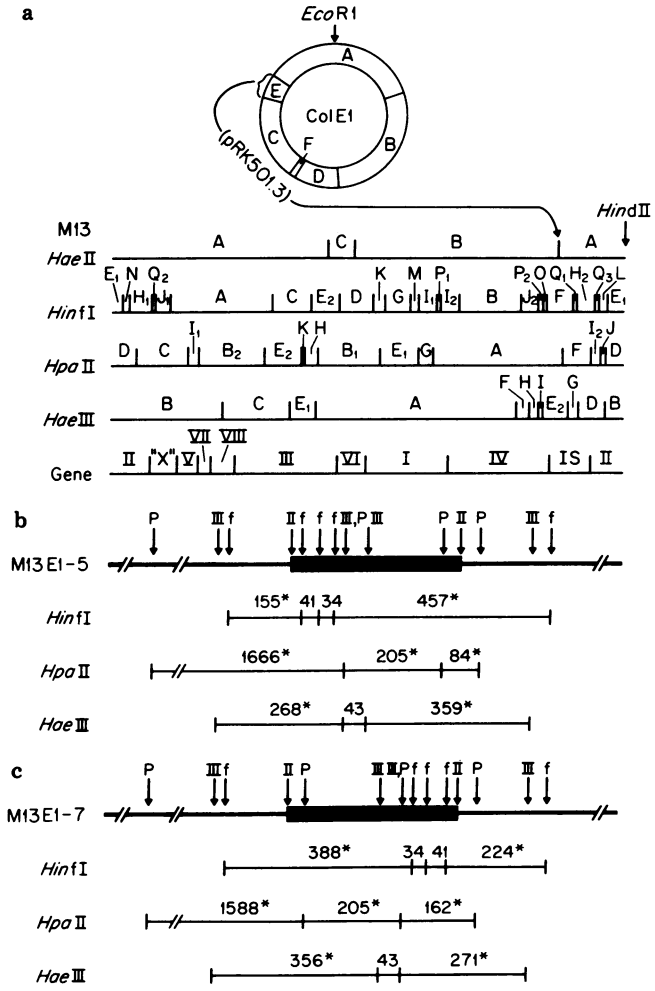

FIG. 1. (*a*) Construction of M13E1 phages. The *Hae* II cleavage map of ColE1 (3) is shown as a circle and the physical and genetic maps of M13 (4, 5) are shown as horizontal lines. ColE1 replication initiates at *Hae* II-E and proceeds counterclockwise in this map (1, 2). Because the 5′→3′ direction of the H-strand is clockwise, the H-strand is a lagging strand in ColE1 replication. The M13 maps are aligned with the single *Hin*dII site. The 5′→3′ polarity of the viral strand of M13 is from left to right. IS denotes an intergenic space between genes *II* and *IV*. The two *Hae* II sites outside of IS are located within genes essential for plaque formation. (*b* and *c*) Restriction endonuclease cleavage sites of M13E1-5 and M13E1-7. An inserted *Hae* II-E fragment of ColE1 (thick line) and M13 DNA adjacent to the inserted fragment (thin line) are shown. The 5′→3′ direction of the viral strand is from left to right. The symbols II, f, p, and III indicate the sites of cleavage by *Hae* II, *Hin*fI, *Hpa* II, and *Hae* III, respectively. Cleavage fragments and their sizes (number of base pairs) are shown. Fragments with asterisks are indicated by arrows in lanes 1 and 2 of Fig. 2 *b*, *c*, and *d*.

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Abbreviations: RF, replicative form; RFI, supercoiled RF; RFII, relaxed RF; ssDNA, single-stranded DNA; ts, temperature-sensitive; pfu, plaque-forming unit.

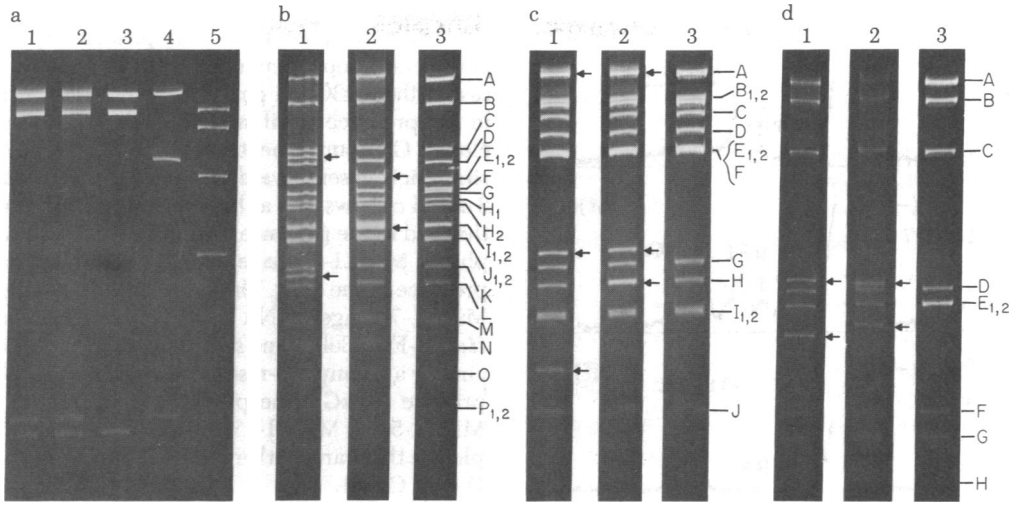

FIG. 2. Restriction endonuclease analysis of M13E1-5 and M13E1-7 RF DNA with *Hae* II (*a*), *Hin*fI (*b*), *Hpa* II (*c*), and *Hae* III (*d*). One microgram of DNA was digested with each restriction endonuclease and analyzed by 5% (*a*, *b*, and *d*) or 6% (*c*) polyacrylamide Tris/borate/EDTA slab gel electrophoresis (22). (*a*) Lane 1, M13E1-5; lane 2, M13E1-7; lane 3, M13 wild type; lane 4, pRK501.3; lane 5, ColE1. (*b–d*) Lane 1, M13E1-5; lane 2, M13E1-7; lane 3, M13 wild type. The bands that are derived from junctions between the inserted *Hae* II-E fragment of ColE1 and the M13 intergenic space or derived from the internal region of *Hae* II-E are indicated by arrows in lanes 1 and 2 of *b–d* and their sizes are shown in Fig. 1 *b* and *c*. The sizes in base pairs of cleavage fragments of M13 wild-type DNA are as follows (5): *Hin*fI-A, 1288; B, 771; C, 486; D, 413; E_1, 345; E_2, 348; F, 328; G, 324; H_1, 274; H_2, 254; I_1, 234; I_2, 232; J_1, 212; J_2, 209; K, 160; L, 137; M, 96; N, 80; O, 63; P_1, 45; P_2, 46. *Hpa* II-A, 1596; B_1, 818; B_2, 829; C, 652; D, 543; E_1, 472; E_2, 454; F, 381; G, 176; H, 156; I_1, 129; I_2, 123; J, 60. *Hae* III-A, 2527; B, 1623; C, 849; D, 313; E_1, 309; E_2, 311; F, 158; G, 142; H, 106.

strand of the *Hae* II-E fragment. We also show that the *dnaB* gene product, as well as that of *dnaG*, is required for the expression of *rri-1*, and we discuss the mode of H-strand replication of ColE1.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The bacterial strains used are derived from *Escherichia coli* K-12. The strains used are PC3 (*dnaG3, leu, thy, str*^{r}) (16), RL114 (F^+ derivative of PC3), RL115 (temperature-resistant revertant of RL114), BT1029 (F^+ *dnaB thy endA polA str*^{r}) (17), RL116 (temperature-resistant revertant of BT1029), W1485 (F^+ *supE*) (18), and LA6 (F^+ *pro thi lacY gal ara*Δ766 *str*^{r} *supE*) (19). The ColE1 derivative plasmid pRK501.3 was a gift of R. Kolter, University of California, San Diego. M13G*ori*1 has been described (14).

Enzymes and Chemicals. *Hae* II restriction endonuclease and phage T4 DNA ligase were purchased from Bethesda Research Laboratories (Rockville, MD). Carrier-free ^{32}P (H_3PO_4) was purchased from ICN. [*Methyl*-^{3}H]thymidine at 20 Ci/mmol and 1 mCi/ml was purchased from New England Nuclear (1 Ci = 3.7×10^{10} becquerels).

^{32}P or ^3H-Labeled Phages. The following labeling protocol was used to produce ^{32}P-labeled phages. W1485 cells were grown at 37°C to 2×10^8 cells per ml in a glucose minimal medium containing thymidine at 2 μg/ml and the 20 L amino acids each at 50 μg/ml (20). Then 0.5 mCi of carrier-free ^{32}P-labeled inorganic phosphate was added to 5 ml of the culture prior to phage infection at a multiplicity of 10. After growth for 5 hr, phages were purified as described (21). ^3H-Labeled phages were prepared as follows: RL116 cells were grown at 37°C to 2×10^8 cells per ml in the medium described above. Then cells were harvested, resuspended in a fresh medium containing [^{3}H]thymidine (20 Ci/mmol) at 200 μCi/ml and infected. After 5 hr of culture at 37°C, phages were purified (21).

Construction and Isolation of M13E1 Phages. The origin region fragment (*Hae* II-E) of ColE1 plasmid was excised from a ColE1 derivative plasmid pRK501.3 and inserted into the M13 duplex RF *in vitro*. Fragments from a *Hae* II digest of pRK501.3 DNA (Fig. 2*a*, lane 4) were ligated with a partial *Hae* II digest of M13 RF DNA in which approximately 40% of the material was unit-length linear molecules (data not shown). Calcium chloride-treated LA6 cells (23) were then transfected with DNA from the ligation reaction and plated for M13 plaque formation, and the resulting plaques were screened by the plaque hybridization technique of Benton and Davis (24). Plaques that were positive in this assay (about 1% of the total plaques) were further purified, and several chimeric phages in which the DNA was slightly larger (about 400 bases) than that of wild-type M13 were identified by gel electrophoresis of phage ssDNA prepared from the supernatant of an infected culture (25). Extensive restriction endonuclease analysis has shown that these chimeric phages, termed M13E1 phages, carry the *Hae* II-E fragment of ColE1 as a cloned fragment at the *Hae* II site within the 507-base-pair M13 intergenic space in both orientations (Fig. 1*a*). Fig. 2 shows the gel electropherogram of representatives of M13E1 phages, M13E1-5 and M13E1-7, in which either the H-strand or the L-strand of the *Hae* II-E fragment has been ligated with the viral strand of M13, respectively. The *Hae* II-E fragment of ColE1 was excised from M13E1-5 and M13E1-7 RF DNAs with restriction endonuclease *Hae* II (Fig. 2*a*). Because the *Hae* II site within the intergenic space is located within the M13 *Hin*fI-F, *Hpa* II-A, and *Hae* III-E_2 fragments (Fig. 1*a*), these bands are not seen in digests of M13E1-5 and M13E1-7 (Fig. 2 *b–d*). In addition, new bands derived from junctions between the inserted *Hae* II-E fragment of ColE1 and the M13 intergenic space or derived from the internal region of *Hae* II-E appear. The orientation of the inserted fragment was determined by measuring the size of these bands, which migrate at the positions predicted from DNA sequence data (Fig. 1 *b* and *c*). Also, the conclusion drawn from restriction endonuclease analysis was confirmed by hybridization with the L-strand or H-strand of whole ColE1 DNA (data not shown).

RESULTS

M13E1-7 Phage ssDNA Is Converted to Parental RF DNA in the Presence of Rifampicin. As described in *Materials and*

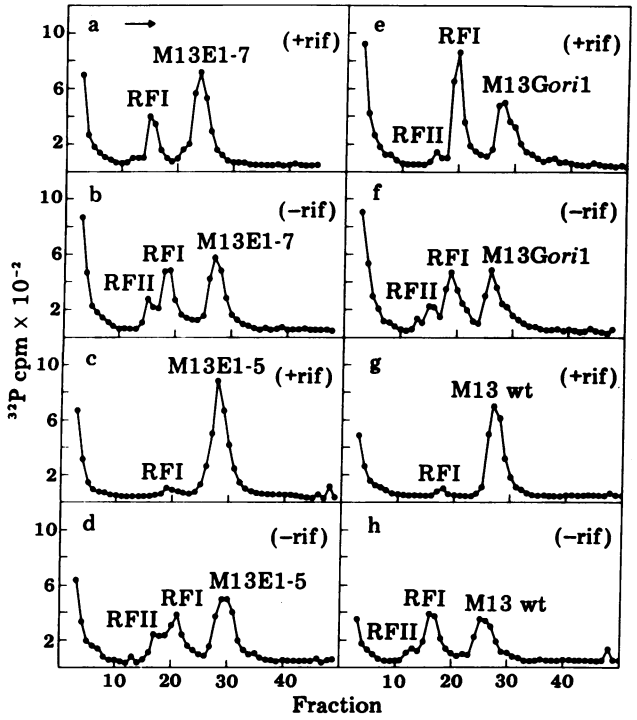

FIG. 3. Conversion of M13E1-7 viral DNA to the duplex RF *in vivo* in the presence of rifampicin (rif). A culture of *E. coli* LA6 was grown in an M9 glucose minimal medium containing thymidine at 2 μg/ml and 0.5% casamino acids at 37°C. At an optical density of 0.5 at 595 nm, the culture was divided into aliquots (5 ml each) and 50 μl of rifampicin at 40 mg/ml in dimethyl sulfoxide was added to *a*, *c*, *e*, and *g* or 50 μl of dimethyl sulfoxide alone was added to *b*, *d*, *f*, and *h*. After 5 min at 37°C, cultures were infected with ^{32}P-labeled phages at a multiplicity of 100. After an additional 10 min at 37°C, an equal volume of ice-cold NaCl/EDTA/Tris/NaCN buffer (20) was added and the mixture was kept in ice for 10 min. The bacteria were centrifuged and washed twice. Cell pellets were resuspended in 0.5 ml of NaCl/EDTA/Tris buffer (20) and incubated with 50 μl of lysozyme at 4 mg/ml for 10 min at 37°C. Then, 25 μl of 10% Sarkosyl NL97 was added and incubation was continued for 10 min at 37°C. The resulting clear and highly viscous lysate was layered on top of 5–20% sucrose gradients containing 1 M NaCl, 10 mM Tris·HCl (pH 8.0), and 1 mM EDTA. Sedimentation was for 4.5 hr at 5°C and 40,000 rpm in a Beckman SW 40 rotor. Fractions were collected from the top directly into scintillation vials for measurement of radioactivity (20). The direction of sedimentation is from left to right as indicated by a horizontal arrow in *a*. Cells were infected with (*a*) ^{32}P-labeled M13E1-7 in the presence of rifampicin at 400 μg/ml; (*b*) ^{32}P-labeled M13E1-7; (*c*) ^{32}P-labeled M13E1-5 in the presence of rifampicin at 400 μg/ml; (*d*) ^{32}P-labeled M13E1-5; (*e*) ^{32}P-labeled M13G*ori*1 in the presence of rifampicin at 400 μg/ml; (*f*) ^{32}P-labeled M13G*ori*1; (*g*) ^{32}P-labeled M13 wild type (wt) in the presence of rifampicin at 400 μg/ml; (*h*) ^{32}P-labeled M13 wild type. RFI, supercoiled RF; RFII, relaxed RF.

Methods, the origin region fragment (*Hae* II-E) of ColE1 was inserted into the intergenic region of the duplex form of the single-stranded DNA phage M13 in both orientations. M13E1-5 and M13E1-7 are two representative isolates of M13E1 phages in which the H-strand or the L-strand of the *Hae* II-E fragment has been ligated to the viral strand of M13, respectively. In order to test the capability of a cloned ColE1 fragment to direct DNA strand initiation by a rifampicin-resistant mechanism, M13E1-5, M13E1-7, M13G*ori*1, and M13 wild-type phages were labeled with ^{32}P and used to infect *E. coli* cells in the presence and absence of rifampicin. If DNA synthesis from the complementary strand origin of M13 or an origin within a cloned fragment occurs, ^{32}P label contained within the infecting viral DNA will be converted to RF DNA. Fig. 3 *b*, *d*, *f*, and *h* shows that viral ssDNA of these phages is converted to RF DNA in the absence of rifampicin. In agreement with previous results (15), essentially no parental RF DNA synthesis of M13 was observed in the presence of rifampicin, as expected for an RNA polymerase-dependent initiation mechanism (Fig. 3*g*). In the case of the M13G*ori*1 phage, the synthesis of parental RF DNA in the presence of rifampicin (Fig. 3*e*) is dependent on the cloned G4 complementary strand origin, which utilizes the rifampicin-insensitive *dnaG* primase for initiation (26). Fig. 3 *a* and *c* shows that although parental RF DNA was not synthesized in the presence of rifampicin in the case of M13E1-5 phage, M13E1-7 phage ssDNA was converted to RF in the presence of the drug. These results indicate that conversion of M13E1-7 phage ssDNA to RF is due to the cloned L-strand of *Hae* II-E of ColE1 and suggest that RNA primer synthesis occurs by a rifampicin-resistant mechanism, probably involving primase (*dnaG* gene product). Results similar to those for M13E1-5 and M13E1-7 were also obtained with other chimeric phages that carry either the H-strand or the L-strand of *Hae* II-E of ColE1.

dnaG and _dnaB_ Gene Products Are Required for the Rifampicin-Resistant Conversion of M13E1-7 Phage DNA to Parental RF. In order to confirm that the *dnaG* gene product is involved in rifampicin-resistant conversion of chimeric phage ssDNA to RF, a *dnaG* temperature-sensitive (ts) strain (RL114) and a temperature-resistant revertant derivative (RL115) of RL114 were infected with ^{32}P-labeled chimeric phage in the presence of rifampicin. The cells were simultaneously coinfected with ^{3}H-labeled M13 phage to ensure that DNA strand initiation from the M13 complementary strand origin was inhibited under these conditions. The results are shown in Fig. 4. Parental RFs of M13E1-7 and M13G*ori*1 are synthesized in the revertant strain at 42°C (Fig. 4 *c* and *d*). Conversion of the chimeric phage ssDNAs to parental RF is blocked in RL114 at the nonpermissive temperature (Fig. 4 *a* and *b*). Therefore, it is concluded that the *dnaG* gene product is required for rifampicin-resistant conversion of both M13E1-7 and M13G*ori*1 phage DNAs to parental RF.

It has been shown that not only the *dnaG* but also the *dnaB* gene product is required for *in vitro* replication of the early replicative intermediate of ColE1 (27, 28). To determine whether the *dnaB* gene product is involved in rifampicin-resistant conversion of M13E1-7 phage ssDNA to RF, a *dnaB*^{ts} strain BT1029 and a temperature-resistant revertant (RL116) of BT1029 were infected with ^{32}P-labeled chimeric phage in the presence of rifampicin. The cells were also infected with ^{3}H-labeled M13 phage as in Fig. 4. As shown in Fig. 5*b*, conversion of M13G*ori*1 to parental RF is not influenced by the *dnaB* mutation, consistent with the known initiation mechanism at the complementary strand origin of G4 (26). However, conversion of M13E1-7 to parental RF is blocked in BT1029 at the nonpermissive temperature (Fig. 5*a*). Parental RFs of M13E1-7 and M13G*ori*1 are synthesized in the revertant strain at 42°C (Fig. 5 *c* and *d*). It is concluded that the *dnaB*, as well as the *dnaG* gene product, is required in rifampicin-resistant conversion of M13E1-7 phage single strand to a double-stranded form.

DISCUSSION

The origin region fragment (*Hae* II-E) of ColE1 has been inserted into the 507-base-pair intergenic region of bacteriophage M13 and cloned. M13E1-7 phage ssDNA, in which the L-strand of the *Hae* II-E fragment has been ligated to the viral strand of M13, is converted to parental RF DNA in the presence of rifampicin, indicating the presence of a site capable of promoting DNA strand initiation of the L-strand of the *Hae* II-E fragment. Because the locus promotes DNA strand initiation by a rifampicin-resistant mechanism, we propose to name the site *rri-1* (rifampicin-resistant initiation). We have shown here

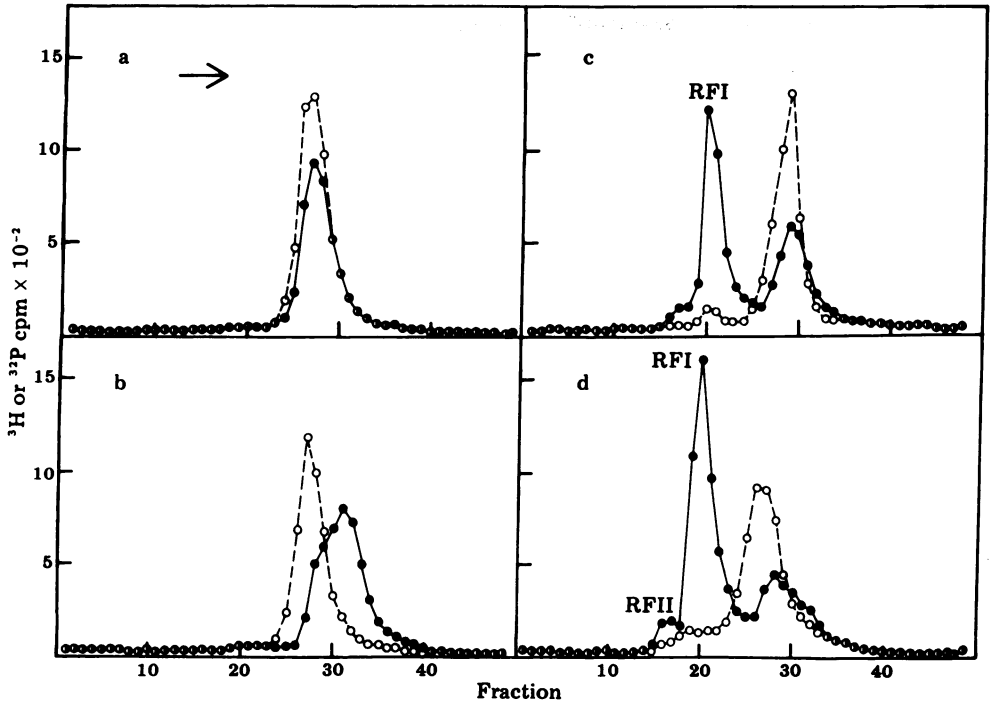

FIG. 4. *dnaG*-dependent conversion of M13E1-7 viral DNA to RF. RL114 and RL115 cells were grown in a M9 glucose minimal medium supplemented with thymidine at 2 μg/ml and 0.5% casamino acids at 32°C. At an optical density of 0.5 at 595 nm, each culture was divided into aliquots (5 ml each) and 50 μl of rifampicin at 40 mg/ml in dimethyl sulfoxide was added to all samples. Then the temperature of the RL114 culture (*a* and *b*) and the RL115 culture (*c* and *d*) was shifted to 42°C. After 10 min of incubation, cells were infected with (*a* and *c*) ^{32}P-labeled M13E1-7 [1.35 × 10^{10} plaque-forming units (pfu), 3.2 × 10^5 cpm] or (*b* and *d*) ^{32}P-labeled M13*Gori*1 (1.7 × 10^{10} pfu, 3.2 × 10^5 cpm). At the same time, ^{3}H-labeled M13 wild-type (3.1 × 10^9 pfu, 3.2 × 10^5 cpm) was also added to all samples. After an additional 20 min at the elevated temperature, the cultures were harvested and cell lysates were analyzed as described in the legend of Fig. 3. The direction of sedimentation is from left to right. ●, ^{32}P; ○, ^{3}H.

that the function of *rri-1* is dependent on both the *dnaG* and *dnaB* gene products.

Two mechanisms of *dnaG*-dependent primer synthesis have been identified by *in vitro* replication studies of small single-stranded DNA phage (29, 30). In φX174 complementary strand synthesis, primer RNA is polymerized at various positions, presumably at random. Primer synthesis follows the prepriming event, which is catalyzed by several *E. coli* proteins including the *dnaB* and n′ proteins. The recognition site of protein n′, in the untranslated region between genes *F* and *G*, has been postulated to be the "loading site" of the *dnaB* protein on φX174 ssDNA (31). After binding to DNA, the *dnaB* protein appears to move along the template strand, presumably in the 5′→3′ direction. The *dnaG* primase interacts with bound *dnaB* protein, which functions as a "mobile promoter," and initiates synthesis of RNA transcripts. This mechanism probably accounts for the multiple initiation sites of RNA primers. Alternatively, in G4 complementary strand synthesis, primer RNA is synthesized at a unique DNA sequence (origin) without participation of the *dnaB* protein (32). Because the function of *rri-1* in ColE1 H-strand initiation is dependent on both the *dnaG* and *dnaB* gene products, the mechanism of the expression of *rri-1* resembles the "φX174 pathway" rather than the "G4 pathway." We postulate that *rri-1* is a loading site for the *dnaB* protein for H-strand synthesis. Although it is not clear whether both strands of ColE1 are synthesized discontinuously (33), at least the H-strand must be synthesized discontinuously (34), because it is the lagging strand in ColE1 DNA replication.

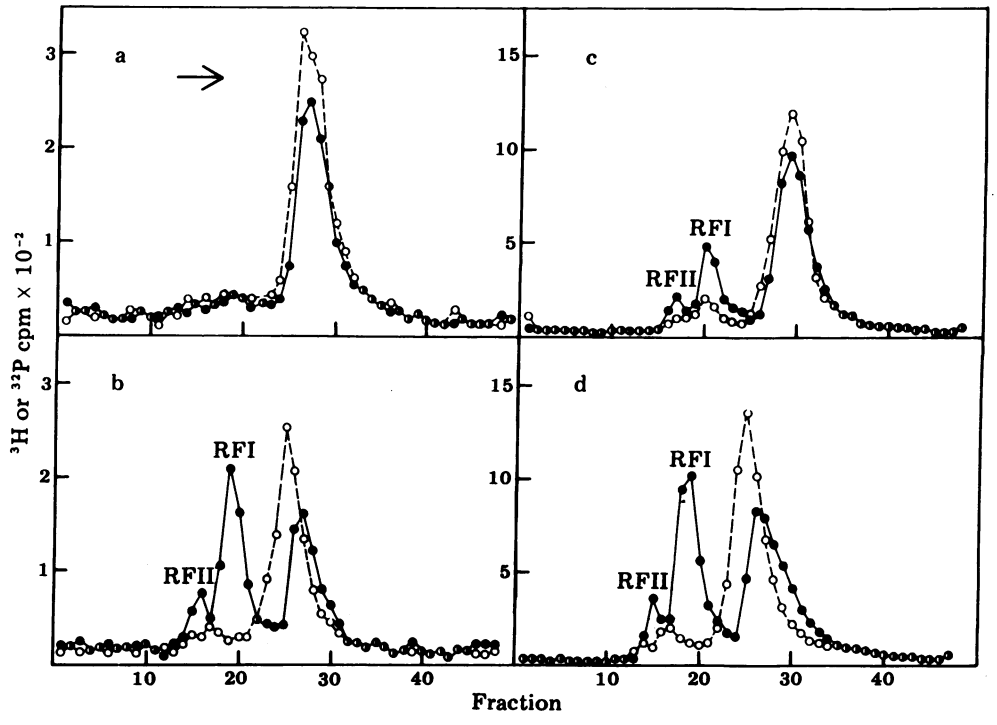

FIG. 5. *dnaB*-dependent conversion of M13E1-7 viral DNA to RF. BT1029 and RL116 cells were used as host strains. Conditions of cell growth, infection and lysis were the same as in Fig. 4. (*a*) BT1029 infected with ^{32}P-labeled M13E1-7 (1.35 × 10^{10} pfu, 3.2 × 10^5 cpm) and ^{3}H-labeled M13 wild type (3.1 × 10^9 pfu, 3.2 × 10^5 cpm) at 42°C. (*b*) BT1029 infected with ^{32}P-labeled M13*Gori*1 (1.7 × 10^{10} pfu, 3.2 × 10^5 cpm) and ^{3}H-labeled M13 wild type (3.1 × 10^9 pfu, 3.2 × 10^5 cpm) at 42°C. (*c*) RL116 infected with ^{32}P-labeled M13E1-7 and ^{3}H-labeled M13 wild type at 42°C. (*d*) RL116 infected with ^{32}P-labeled M13*Gori*1 and ^{3}H-labeled M13 wild type at 42°C. ●, ^{32}P; ○, ^{3}H.

The discontinuous replication of the H-strand might be dependent upon the function of the *dnaB* protein as a mobile promoter. Recently, Staudenbauer *et al.* (12) have succeeded in uncoupling L-strand from H-strand synthesis in a ColE1 *in vitro* replication system. They have shown that the *dnaG* and *dnaB* proteins are required for H-strand synthesis, which is consistent with our results.

Subdividing a replication origin of a duplex DNA molecule into its single-stranded DNA components might be successfully undertaken in other systems. Also, because DNA replication of *dnaG* and *dnaB* ts *E. coli* cells ceases immediately after a shift to the nonpermissive temperature, the *dnaG* and *dnaB* proteins are believed to be involved in DNA chain elongation during chromosome replication, that is, synthesis of an "Okazaki fragment" (16, 17). It may be possible to identify sites analogous to *rri-1*, which are involved in Okazaki fragment synthesis, in the host chromosomal DNA (35) by the methods used here.

We are greatly indebted to Ms. Jane C. Hines for her important preliminary work. This work was supported by Research Grant AI 10752-08 from the National Institutes of Health.

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