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Enzymatic replication of the origin of the *Escherichia coli* chromosome

(*oriC* plasmids/*dnaA* gene/DNA replication)

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ABSTRACT **An enzyme system that replicates plasmids bearing the origin of the *Escherichia coli* chromosome (*oriC*) has the following physiologically relevant features. The system (*i*) depends completely on low levels of exogenously furnished supercoiled *oriC* plasmids, (*ii*) uses only those plasmids that contain the intact *oriC* region of about 245 base pairs, (*iii*) initiates replication within or near the *oriC* sequence and proceeds bidirectionally, (*iv*) proceeds linearly, after a 5-min lag, for 30–40 min to produce as much as a 40% increase over the input DNA, (*v*) depends on RNA polymerase and gyrase as indicated by total inhibition by rifampicin and nalidixate, (*vi*) depends on replication proteins (e.g., *dnaB* protein and single-stranded DNA binding protein) as judged by specific antibody inhibitions, (*vii*) operates independently from protein synthesis, and (*viii*) depends on *dnaA* activity, as suggested by the inactivity of enzyme fractions from each of two *dnaA* temperature-sensitive mutant strains, and complementation (with a 15-fold overproduction of complementing activity) by a fraction from a strain containing the *dnaA* gene cloned in a multicopy plasmid. Resolution and analysis of factors that control the initiation of a chromosome cycle should become accessible through this enzyme system.**

Initiation of a new cycle of chromosomal replication is the most critical step in the control of replication, and an enormous amount of effort has been expended to understand it (1, 2). Yet little is known about the mechanism of the initiation of chromosomal replication because biochemical data are extremely scant.

In *Escherichia coli*, several genes, including *dnaA*, *dnaI*, and *dnaP*, have been identified as essential for initiating a cycle of replication (1, 2). The denatured *dnaA* polypeptide has been detected (e.g., ref. 3), but nothing is known about the products of *dnaI* and *dnaP*. Genes *dnaB* and *dnaC*, whose products are essential for ongoing replication, are also needed at or near the outset of a cycle of chromosome replication (1, 2). The inhibitory effects of rifampicin at this stage of replication have also implicated RNA polymerase (1, 2).

Once initiated, propagation of the replication fork is relatively well understood through the actions of the multisubunit DNA polymerase III holoenzyme (4) and a primosomal unit composed of perhaps seven discrete proteins (5). Isolation of this cellular machinery came through the use of small, single-stranded DNA phage chromosomes as probes to illuminate the enzymatic components and their detailed operations. Enzyme systems that replicate plasmids have also made it possible to demonstrate direct priming by RNA polymerase and copy-number control by small RNAs for ColE1 (6), and a requirement for positive, *trans*-acting, plasmid-encoded proteins for R6K (7) and R1 (8).

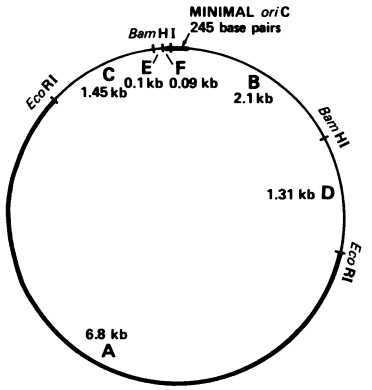

FIG. 1. Restriction endonuclease map of pSY317. This 12-kb *oriC* plasmid constructed by S. Yasuda contains the 5.05-kb *Eco*RI *E. coli* *oriC* fragment of pSY221 and the 6.8-kb *Eco*RI kanamycin resistance fragment of pML21 (11). Restriction endonuclease data are taken from ref. 12. The site of the 245-base pair minimal *oriC* sequence (13), spanning the *Bam*HI site between fragments F and B, is marked.

The availability of the chromosomal origin of *E. coli* (*oriC*) in a functional form as part of a small plasmid (9) and earlier success in the enzymatic resolution of phage replication have sustained our efforts for several years to discover an enzyme system for replication of *oriC* plasmids.

MATERIALS AND METHODS

***E. coli* Strains, Plasmids, and Phage.** The strains and sources were: C600 and HB101, laboratory stocks; WM301 (*dnaA*$^+$), WM433 (*dnaA204*), and WM493 (*dnaA5*), from W. Messer (10); HB101(pBF101), this laboratory; ERsm*recA*(pSY317) (*oriC* plasmid-containing strain), from S. Yasuda. The plasmids used were: pSY317 (Fig. 1); pBF101, a 15.5-kilobase (kb) plasmid constructed by ligation of a 5.7-kb *Hin*dIII/*Bam*HI fragment of λ*dnaA*S100 (S. Yasuda, personal communication) containing the *dnaA* and *dnaN* genes and the 10-kb *Hin*dIII/*Bam*HI fragment of the runaway-replication vector pMOB45 (ref. 14; unpublished data). Other plasmids will be described in figure and table legends. Duplex DNAs were prepared as described (15) except that lysis was enhanced with phage T4 lysozyme at 0.1 μg/ml (16).

Reagents and Buffers. Sources were: standard reagents from commercial sources; polyethylene glycol (PEG) 20,000 and polyvinyl alcohol (PVA) 24,000, D. J. Dawson (Dynapol, Palo Alto, CA); [*methyl*-^{3}H]dTTP (300–400 Ci/mmol) and [α-^{32}P]dTTP (600 Ci/mmol), New England Nuclear and Amersham, respectively (1 Ci = 3.7×10^{10} becquerels). Buffer A was 25 mM

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Abbreviations: kb, kilobase(s); PEG, polyethylene glycol; PVA, polyvinyl alcohol; SSB, single-stranded DNA binding protein; ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate.

Hepes, pH 7.6/1 mM EDTA. Buffer B was 25 mM Hepes, pH 7.6/0.1 mM EDTA/2 mM dithiothreitol.

Enzymes and Antibodies. Sources were: standard enzymes and proteins, commercial sources; *Eco*RI and *Bam*HI, J. Carlson and C. Mann (this department); T4 lysozyme, K. Taylor; *dnaB* protein and single-stranded DNA binding protein (SSB) and their antibodies, as described (17–19); antibody against *recA* protein, C. Paoletti.

Assay for DNA Replication. The standard reaction mixture, 25 μl, contained the following components: Hepes (pH 7.6), 40 mM; ATP, 2 mM; GTP, CTP, and UTP, each 500 μM; bovine serum albumin, 50 μg/ml; creatine phosphate, 21.6 mM; dATP, dGTP, dCTP, and dTTP, each 100 μM, with [*methyl*-^{3}H]dTTP at 180 cpm per pmol of total deoxynucleotide; magnesium acetate, 11 mM; creatine kinase, 100 μg/ml; and pSY317 supercoiled DNA, 8.6 μg/ml. PEG 6000, PEG 20,000, or PVA 24,000 was added to final concentrations of 5% or 6% (wt/vol) as indicated in figure and table legends. The mixtures were assembled at 0°C. The reactions were initiated by addition of 200–280 μg of protein (fraction II) and incubation at 30°C. Incubation times are as indicated in figure and table legends. Total nucleotide incorporation was measured by determining radioactivity after trichloroacetic acid precipitation.

RESULTS

The Enzyme Fraction and the *oriC* Template. Although the high-speed supernatant of an *E. coli* lysate was inactive, subsequent ammonium sulfate fractionation yielded a vigorous preparation. Such fractionation was successful only in a rather narrow range of ammonium sulfate additions (Fig. 2) and generally was optimal with about 0.28 g added per ml of supernatant. Brief dialysis was needed to reduce inhibitory effects of salt. The sigmoidal character of the response to the amount of the active ammonium sulfate fraction is consistent with a multifactor system; saturation was achieved at a concentration of about 10 mg of protein per ml.

The activity was entirely dependent upon the addition of supercoiled pSY317 plasmid DNA. Nicked DNA was inactive (data not shown). Activity was maximal at a concentration of about 8–10 μg of plasmid DNA per ml (1 nM in plasmid DNA molecules), which is equivalent to about 600 pmol of nucleotide residues in the 25-μl assay. Higher concentrations were less active, presumably due to the dissipation of critical replication proteins bound to the DNA. Synthesis to the extent of 250 pmol, about 40% of the input DNA, was often observed.

Requirements for Replication. Detailed examination of nucleoside triphosphate and other requirements is premature with a relatively crude enzyme fraction, but several features are notable. The optimal Mg^{2+} concentration was rather sharp around 10 mM, a 5-fold dependence on an ATP-regenerating system was observed even with ATP at a starting concentration of 2 mM, and the requirement for a flexible, hydrophilic polymer was absolute (data not shown). Any one of several polymers, such as polyethylene glycol, polyvinyl alcohol, and methylcellulose, with molecular weights ranging from 6000 to greater than 100,000 and at concentrations near 6% sufficed. The polymers may increase the effective concentrations of macromolecular reactants by an "excluded volume" effect (21).

Inhibitors of Replication. Total inhibition by rifampicin implicates an obligatory contribution by RNA polymerase (Table 1). When addition of rifampicin was delayed until the reaction was underway (data not shown), replication was substantial, implying that rifampicin affects the initiation rather than the elongation stages of the reaction.

Inhibition by nalidixate and novobiocin (Table 1) was observed at concentrations consistent with those that affect gyrase action (22, 23). Insensitivity to chloramphenicol and puromycin shows that replication of the *oriC* plasmid, unlike that of R1 plasmids (8) or pSC101 plasmid (data not shown), does not require the concomitant synthesis of protein coupled to transcription of an essential replication gene in the plasmid.

The inhibitory effects (95%) of antibodies against *dnaB* protein and SSB implicate these replication proteins in *oriC* plas-

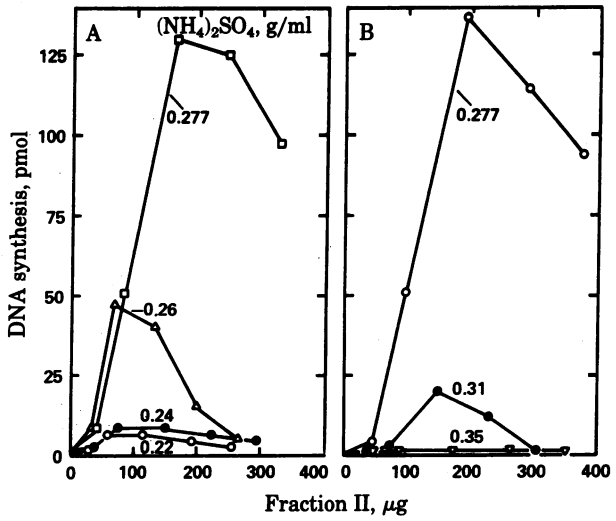

FIG. 2. Ammonium sulfate fractionation of *oriC* replication activity. *E. coli* C600 was grown in L broth plus 1.0% glucose in a 300-liter fermenter at 37°C, doubling in 25 to 30 min, and harvested in a Sharples continuous flow centrifuge at OD_{595} = 0.5–1.5. Pellets were rinsed with a small volume of buffer A and resuspended in buffer A to OD_{595} = 325, frozen in liquid nitrogen, and stored at −70°C. Frozen-cell suspensions were thawed at 0–5°C and adjusted to 80 mM KCl, 2 mM dithiothreitol, egg lysozyme at 300 μg/ml, and T4 lysozyme at 0.1 μg/ml. After incubation at 0°C for 20 min, the cells were again frozen and thawed. Lysates were clarified by centrifugation at 200,000 × *g* for 20 min at 0–5°C (fraction I). Ammonium sulfate in the amounts indicated in the figure was added slowly to fraction I at 0°C with stirring over a 20-min period; suspensions were stirred an additional 20 min. Precipitates were collected by centrifugation at 48,000 × *g* for 20 min at 0°C and packed, after removal of supernatants, by recentrifugation for 5 min. The pellets were resuspended in a minimal volume of buffer B and dialyzed against a 100-fold volume of buffer B at 0°C for 30–45 min, until the conductance was equivalent to that of 100–300 mM KCl. The dialysate (fraction II), with a protein concentration of 80–140 mg/ml, distributed as 50-μl aliquots, was frozen in liquid nitrogen and stored at −70°C. Active preparations remained stable after repeated freezing and thawing. Protein determinations were made by the method of Bradford (20), using bovine serum albumin as a standard. Reactions were in the presence of PEG 6000 at 5% for 50 min (*A*) or 30 min (*B*).

Table 1. Sensitivity of *oriC* replication *in vitro* to inhibitors of transcription, translation, and replication

Inhibitor	Concentration	DNA synthesis, pmol
None	—	134
Rifampicin	20 μg/ml	1.7
Puromycin	200 μg/ml	132
Chloramphenicol	200 μg/ml	133
Nalidixate	0.2 mM	66
	0.4 mM	34
	1.0 mM	4.3
Novobiocin	10 nM	109
	40 nM	75
	100 mM	36

Reactions were in the presence of PEG 20,000 at 5% for 40 min.

Table 2. Antibody effects on *oriC* replication

Exp.	Gamma globulin added	Protein added	DNA synthesis, pmol
A	None	None	144
	Anti-*dnaB* protein	None	11
	None	*dnaB*	102
	Anti-*dnaB* protein	*dnaB*	102
B	None	None	59
	Anti-SSB	None	4
	None	SSB	61
	Anti-SSB	SSB	44
C	None	None	74
	Anti-*recA* protein	None	87
D	None	None	87
	Anti-pol I	None	72

Assays were as described in *Materials and Methods* except that purified proteins and gamma globulin were added as indicated. Exp. A, reaction mixtures were incubated for 20 min with 6% PVA 24,000. Anti-*dnaB* gamma globulin (3 μg) and *dnaB* protein (0.5 μg) were added where indicated. Exp. B, reaction mixtures were incubated for 20 min with 5% PEG 6000. Anti-SSB gamma globulin (3.6 μg) and SSB (2 μg) were added where indicated. Exp. C, reaction mixtures were incubated for 30 min with 5% PEG 6000. Anti-*recA* protein gamma globulin (4.6 μg), sufficient to inhibit (by 70%) DNA-dependent ATPase activity of 0.2 μg of *recA* protein, was added where indicated. Exp. D, reaction mixtures were incubated for 20 min with 6% PVA 24,000. Anti-DNA polymerase I (pol I) gamma globulin (1 μg) was added at a level that inhibited ColE1 DNA replication by 90%.

mid replication (Table 2). Replication activity was restored to a considerable extent by respective additions of pure *dnaB* protein or SSB. Antibodies against *recA* protein and DNA polymerase I failed to inhibit the reaction (Table 2).

Specificity of Replication for *oriC* Sequences. In addition to pSY317, another template, M13*oriC*26 replicative form DNA containing *oriC* was also active (Table 3). M13*oriC*26 is an *in vitro* recombinant containing the *E. coli* chromosomal origin inserted into an M13 vector, and it has been shown *in vivo* to contain a functional *oriC* sequence (25). In contrast, an *oriC*$^-$ derivative of M13*oriC*26, M13*oriC*26Δ221, which has a deletion of the *oriC* sequence [position 21 to 352 (25)] is inert as a template, indicating that the *oriC* sequence is required for *in vitro* replication. The deletion template did not inhibit pSY317 replication when incubated in the same mixture (data not shown).

Table 3. Specificity of replication to *oriC*

Template	DNA synthesis, pmol
pSY317	87
M13*oriC*26 replicative form	77
M13*oriC*26Δ221 replicative form	3
ColE1	18
pBEU17	5
pSC101	3
φX174 replicative form	4
φX174 viral single strand	72
None	3

Reactions were in the presence of PVA 24,000 at 6% for 20 min. Supercoiled DNAs were added to standard reaction mixtures to 8.6 μg/ml. φX174 single-stranded DNA was added to 3.2 μg/ml. Each number is an average of duplicates. A strain containing pBEU17, a 13-kb runaway-replication mutant plasmid derived from pKN402 (itself derived from R1) and containing a gene for ampicillin resistance, was provided by B. E. Uhlin (24). A strain containing pSC101 was provided by P. Meacock.

Supercoiled pBEU17 (an R1 miniplasmid with a runaway-replication mutation), pSC101, and φX174 replicative form DNAs were inert as templates (Table 3). The slight activity of ColE1 was inhibited by a level of antibody against DNA polymerase I that had no effect on pSY317 replication (Table 2). The capacity of the pSY317 system to convert φX174 single strands to the duplex form attests to the presence of an active set of priming and chain elongation proteins.

Replication Begins at or near the *oriC* Sequence. To localize the actual site of initiation of replication *in vitro* on pSY317, replicative intermediates were analyzed. Intermediates of decreasing extents of replication were generated by using an increasing ratio of the chain terminator 2′,3′-dideoxythymidine 5′-triphosphate (ddTTP) to dTTP in a series of reactions (26). The reaction products were analyzed by restriction endonucleolytic cleavage and agarose gel electrophoresis (Fig. 3). The order of replication of the fragments may be deduced from the order of disappearance of the fragments as a function of the con-

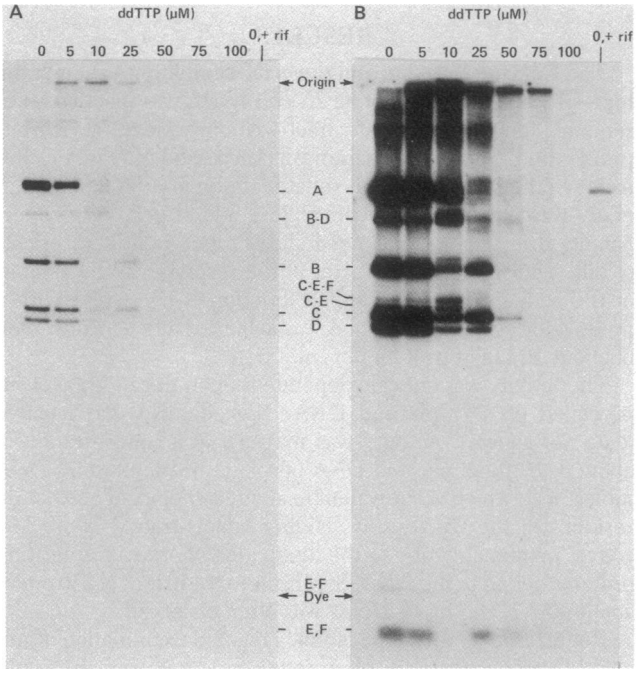

FIG. 3. Restriction endonuclease analysis of initiation of replicative intermediates. Reactions were in the presence of PVA 24,000 at 6% for 30 min; [α-^{32}P]dTTP was included at 500 cpm/pmol of total nucleotide. Rifampicin (rif), when added, was 20 μg/ml. Reactions were terminated with 225 μl of 25 mM EDTA (pH 7.5), the mixtures were extracted twice with phenol and five times with ether, and products were precipitated twice with ethanol in the presence of 300 mM sodium acetate and 5 μg of yeast tRNA as carrier. Precipitates were resuspended in 25 μl of 10 mM Tris·HCl (pH 7.5)/1 mM EDTA containing pancreatic ribonuclease at 1 μg/ml and incubated at 37°C for 30 min. Samples (10 μl) were digested first with a 100-fold excess (10 units) of restriction endonuclease *Bam*HI in 50 mM Tris·HCl (pH 7.5)/1 mM dithiothreitol/20 mM $MgCl_2$/50 mM NaCl then with 10 units of *Eco*RI in the same buffer except with 100 mM NaCl. Fragments were separated by electrophoresis in a horizontal 1.4% agarose gel in 100 mM Tris·borate/1 mM EDTA (pH 8.3). Gels were dried and autoradiographed at −70°C. The autoradiographic exposure in *B* is about 10-fold greater than in *A*. Size standards, an *Eco*RI and a *Bam*HI/*Eco*RI digest of pSY317 and *Hin*dIII and *Eco*RI/*Hin*dIII digests of bacteriophage λcI857 duplex DNA, were used to identify radioactive bands, including partial fragments.

Table 4. Restriction endonuclease analysis of initiation of replicative intermediates

ddTTP, μM	Molar ratios of fragments E + F / A	C / A	B / A	D / A
0	1.0	1.5	1.4	1.1
5	1.1	2.1	1.6	1.2
10	3.4	6.7	4.2	2.3
25	27	23	13	3.8
75	48	NM	NM	NM
0, + rifampicin	NM	0.5	0.5	0.3

Autoradiograms in Fig. 3 and those of a similar experiment (data averaged) were scanned with a Quick Scan Jr. (Helena Laboratories) densitometer. Peak magnitudes were measured by weight; ratios of values for fragments B, C, D, and E plus F to that of fragment A were calculated for each lane. These ratios were normalized by dividing them by the molecular size ratio of the fragments—e.g., the molecular size ratio of B to A = 2.1 kb/6.8 kb = 0.31; these data are presented in the table. NM, not measurable.

centration of ddTTP.* Fragment A (Fig. 1), most remote from *oriC*, is decreased in intensity relative to the other fragments at 5 μM ddTTP and virtually disappears at 25 μM ddTTP, and so is the last to be replicated. Fragment D disappears next, followed by B and C. An intentional autoradiographic overexposure (Fig. 3*B*) allows visualization of fragments E and F, treated together because they run together ahead of the dye, bromophenol blue. Labeling of these fragments persists up to the highest concentration of ddTTP; thus they are the first to be fully replicated.

A quantitative analysis (Table 4) shows the relative extents of replication of the fragments, normalized by the ratio of molecular size. In the absence of ddTTP, these ratios are close to unity, indicating that most initiations have led to complete replication. Inspection of the data again indicates the following order of replication: E plus F, followed by C, by B, by D, and finally by A. This implies that initiation occurs within or near fragments E and F, that is, at a site consistent with the location of the *oriC* sequence (Fig. 1). This also implies that either replication is bidirectional or it proceeds with approximately equal probability in both directions from the site of initiation. Electron microscopic analysis of replicative intermediates supports the conclusion that replication is bidirectional in a large percentage of individual molecules (data not shown).

A small amount of incorporation occurs in the presence of rifampicin (Fig. 3), but this incorporation uniformly labels the fragments and probably reflects a background of repair-like DNA synthesis.

Dependence on *dnaA* Activity. Enzyme fractions prepared from two different *dnaA* mutants were inactive in *oriC* replication, whereas the isogenic wild-type (*dnaA*$^+$) strain yielded an active enzyme system (Table 5). The small amount of activity observed with the mutant fractions was insensitive to rifampicin

* Radioactivity in a fragment measures the extent to which the replication of that fragment has been completed. Restriction of partially replicated molecules generates branched fragments (electron microscopic evidence, data not shown), which migrate anomalously or fail to enter the gel; such anomalous bands and material at the (gel) origin do appear (Fig. 3) in the presence of ddTTP. However, the analysis in Table 4 treats only fully replicated fragments, and the contribution of material trapped at the origin has been ignored. Digestion of samples failed to reach completion despite purification of the replicative products and use of excess restriction endonuclease (e.g., *Bam*HI in the 10 μM ddTTP lane). Whenever partial products could be identified by sizes (e.g., the B-D partial product, the C-E partial product, etc.), their contribution was included in the analysis in Table 4.

Table 5. Activity of fractions from *dnaA* mutants and the isogenic parent

Allele	DNA synthesis: pSY317, pmol/30 min, Without rif	pSY317, pmol/30 min, With rif	φX ssDNA, pmol/5 min, With rif
dnaA$^+$	134	17	48
dnaA204	4.6	5.9	65
dnaA5	2.8	4.9	58

WM301 (*dnaA*$^+$) and isogenic mutant strains WM433 (*dnaA204*) and WM493 (*dnaA5*) were grown in L broth (plus thymine at 25 μg/ml and 0.2% glucose) in flasks at 32°C. The doubling time was 30 min and harvest was at OD_{595} = 1.0. Enzyme fractions were as described in the legend to Fig. 2. Reactions were in the presence of PVA 24,000 at 6%. Rifampicin (rif) was added at 20 μg/ml where indicated. Reaction mixtures with φX174 single-stranded DNA (φX ssDNA) contained 200 pmol of template and 0.5 μg of SSB in 20 μl.

inhibition and therefore does not represent *oriC*-specific replication. Growth of both mutant strains from which the enzyme fractions were obtained was rapid and indistinguishable from that of the wild type. Furthermore, conversion of φX174 single strands to the duplex form was equally active in the mutant and wild-type enzyme fractions, indicating that the replication proteins responsible for priming and chain elongation were not wanting in the mutant fractions (Table 5) and that a novel factor (or factors) was needed.

Complementation of the mutant fractions by fractions of the *dnaA*$^+$ strains WM301 or C600 was not convincing; generally, activities were essentially additive, presumably because the *dnaA*$^+$ activity was limiting. However, complementation was impressive with fractions from strains containing the *dnaA*$^+$ gene cloned in a multicopy plasmid (Fig. 4). The complementing *dnaA* activity was 15-fold greater than that of the plasmidless strain, indicating that degree of overproduction. The *dnaA5* mutant fraction behaved much like *dnaA204* (data not shown), indicating that complementation is not limited to a single class of *dnaA* mutants (10). The rifampicin sensitivity of the activities observed by complementation indicates that the replication is *oriC*-specific.

DISCUSSION

Chromosomal replication has been linked to cellular membranes, and some indications of initiation of chromosomal DNA synthesis have recently been observed in membrane-containing crude lysates on cellophane discs (28). Nevertheless, from the data presented in this initial report, it is clear that a soluble enzyme system can specifically recognize the *E. coli* origin sequence for initiation of replication.

The system depends on at least two of the elements anticipated from *in vivo* studies, RNA polymerase and *dnaA*. RNA polymerase function can be inferred from the total inhibitory effect of rifampicin, but whether the enzyme produces a primer or participates in some less direct way is still unknown. Dependence on *dnaA* activity seems clear from the inactivity of extracts of temperature-sensitive mutant cells, complementation of mutant-cell extracts by those from the cells containing the cloned *dnaA* gene, and the overproduction of *dnaA* activity in the *dnaA* plasmid-containing cells. The need for at least two additional components has been predicted from mutations in distinct loci (*dnaI* and *dnaP*) that cause a slow stop in replication upon exposure of mutant cells to a restrictive temperature. Identification of the protein products of these genes and perhaps other novel proteins should emerge from future enzyme

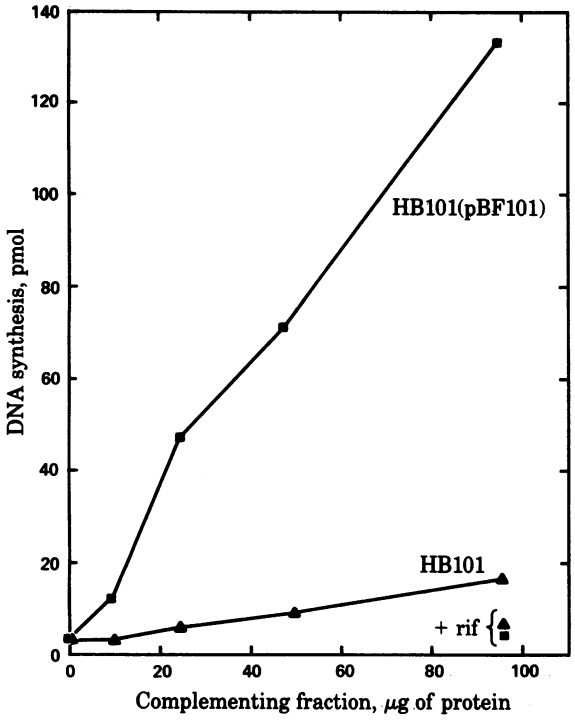

FIG. 4. Complementation of *dnaA* mutant fractions. HB101(pBF101) and HB101 were grown in L broth (plus thymine at 25 μg/ml and 0.2% glucose) in flasks at 32°C; doubling times were 50 and 35 min, respectively. Enzyme fractions were as described in legends to Table 5 and Fig. 2, respectively, except that nucleic acid was removed from fraction I with a 3% streptomycin sulfate precipitation prior to ammonium sulfate precipitation (27), 0.25 g of ammonium sulfate was added per ml of fraction I, and the fractions were not dialyzed. Reactions were in the presence of PVA 24,000 at 6% for 30 min. WM433 (*dnaA204*) fraction II (250 μg) was added to each reaction. HB101(pBF101) (95 μg) alone yielded 26 pmol; HB101 (95 μg) alone yielded 10 pmol. Where indicated, rifampicin (rif) was present at 20 μg/ml in the reaction mixtures containing 95 μg of HB101 or HB101(pBF101).

fractionation and complementation studies. Requirements for the replication proteins needed for the priming of lagging strands and elongation of chains are expected. Thus far the need for *dnaB* protein and SSB has been demonstrated by specific antibody inhibition, and DNA gyrase function can be inferred from inhibitory actions of nalidixate and novobiocin *in vitro*.

The extensive analysis of the structure and *in vivo* function of the *oriC* region has disclosed that a 245-base pair sequence is required and that within this region certain recognition and spacer sequences must be strictly maintained (13, 29). Whether these rules apply *in vitro* can now be examined. Several proteins may be needed to recognize and exploit the various features of the 245-base pair *oriC* sequence, with RNA polymerase being merely one of the components. The role, if any, of sequences adjacent to the minimal *oriC* should now be amenable to study in the isolated *in vitro* system.

Ultimately, the exploitation of this system should allow a direct biochemical approach to the regulation of replication. Availability of an authentic enzyme system for replication of the *E. coli* chromosomal origin should allow the characterization of factors and processes that control the frequency of origin initiation and couple the rate of chromosomal duplication to that of the cell as a whole.

We particularly wish to record our indebtedness to Dr. Seiichi Yasuda, who (with Dr. Y. Hirota) constructed our first *oriC* plasmids and whose extensive studies on these and ColE1 plasmids as a Fellow of the American Cancer Society (California Division) in our laboratory in 1978 and 1979 facilitated the work reported here. The present work was supported in part by grants from the National Institutes of Health and the National Science Foundation. J.M.K. is a Fellow of the Damon Runyon–Walter Winchell Cancer Fund.

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