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Protein HU in the enzymatic replication of the chromosomal origin of *Escherichia coli*

(*oriC*/DNA replication)

NICHOLAS E. DIXON* AND ARTHUR KORNBERG

Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305

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ABSTRACT **A protein that stimulates the enzymatic replication of duplex DNAs of recombinant phages and plasmids bearing the *Escherichia coli* origin of replication (*oriC*) has been isolated from an extract of *E. coli*. The isolated protein and the well-known protein HU, a histone-like DNA-binding protein, have identical polypeptide molecular weights and saturate the *oriC* replication assay at less than 40 dimers per template DNA circle. This level is one-tenth that needed to coat the template. Protein HU from the blue-green alga *Anabaena* is similarly active. Antibody specific for protein HU from *E. coli* inhibits replication promoted both by the reconstituted system and by a crude enzyme extract; in both assays, activity is restored by excess of the isolated protein. Cells lysed in 1 M KCl yield 32,000 dimers of the protein per cell, a number consistent with the reported abundance of HU. These data establish the identity of the isolated factor and protein HU and provide an indication of a function for HU in replication.**

Enzymatic replication of the circular duplex DNA of recombinant phages and plasmids that bear the *Escherichia coli* chromosomal origin of replication (*oriC*) occurs in the presence of a particular ammonium sulfate fraction prepared from rapidly growing cells (1). Replication specifically requires the *oriC* sequence (1) and, with the duplex replicative form of the phage chimera M13*oriC26* (2) or the plasmid pSY317 (3), it begins at or near *oriC* and proceeds bidirectionally (4). Complementation of mutant extracts, inhibition by specific antisera and inhibitors, or direct demonstration of a requirement in reconstituted systems implicate the dnaA (1, 5, 6), dnaB (1, 6), and dnaC (1, 6, 7) proteins, DNA polymerase III holoenzyme (6), primase (6), single-stranded-DNA-binding protein (SSB) (1), RNA polymerase (1, 6), and DNA gyrase (1) in the *in vitro* system. *E. coli* DNA topoisomerase I (ω protein) (8) is required to maintain dependence on dnaA protein and specificity for *oriC*-containing DNA (ref. 6; unpublished data).

At optimal levels, these purified proteins in combination do not sustain maximal DNA synthesis on M13*oriC26* replicative form I (RF I) DNA; activity is maximally stimulated by small amounts of a crude enzyme fraction or mixtures of partially purified components. Physical separation of required components in the crude fraction and their reconstitution should provide assays for their separate purification. In this paper, we report the isolation of one such component by using a reconstitution assay and demonstrate its identity with protein HU, an abundant double-stranded-DNA-binding protein (9).

MATERIALS AND METHODS

Crude Fractions. *E. coli* WM433 (*dnaA204*), the *dnaA*^{ts} mutant from W. Messer, grown at 32°C in L broth supplemented with thymine at 25 mg/liter and 0.2% glucose in a 300-liter fermenter to an OD_{595} of 1.0, and harvested and resuspended as described for strain C600 (1), was used as source of all crude enzyme fractions. Cells were lysed and fraction II was prepared as described (1), except that KCl was present during lysis at 150 mM and protein was precipitated by addition of solid ammonium sulfate to fraction I as specified.

Nucleic Acids and Replication Enzymes. M13*oriC26* RF I DNA (*oriC* DNA) was prepared as described (1). DNA replication proteins purified to homogeneity (unless specified) were DNA polymerase III holoenzyme (fraction V; 2×10^5 units/mg) (10), RNA polymerase holoenzyme (11, 12), primase (fraction IV from the overproducing strain RLM757; 3×10^6 units/mg) (13, 14), SSB (15, 16), dnaA (fraction IIIB; 6×10^4 units/mg) (5), dnaB (17) and dnaC (7) proteins, and proteins i (fraction IV; 1×10^5 units/mg) (18), n (19), and n′ (fraction IV; 1×10^5 units/mg) (20). Partially purified protein n″ was as described (19). Units of enzymatic activity in the appropriate replication assays are defined in the references. DNA gyrase A and B subunits (unpublished data) were purified separately from overproducing strains, using *oriC*-dependent replication assays. The replication assay for DNA topoisomerase I and its purification will be described elsewhere. *E. coli* protein HU (9) and HU antibody were gifts of J. Rouvière-Yaniv (Institut Pasteur). *Anabaena* protein HU (21) was provided by R. Haselkorn (University of Chicago).

Chromatography Media, Reagents, and Buffers. Phosphocellulose P-11 and Bio-Gel-HTP (hydroxylapatite) were from Whatman and Bio-Rad, respectively. Hepes, polyvinyl alcohol (type II), creatine kinase (type I), creatine phosphate, and ribonucleoside triphosphates were from Sigma. Deoxyribonucleoside triphosphates were from P-L Biochemicals. [α-^{32}P]Thymidine triphosphate (>400 Ci/mmol; 1 Ci = 37 GBq) was from Amersham. Buffer A was 50 mM imidazole·HCl, pH 6.75/1 mM EDTA/2 mM dithiothreitol/20% (wt/vol) glycerol. Buffer B differed only in that EDTA was omitted. Buffer C was 0.3 M sodium phosphate, pH 6.85/2 mM dithiothreitol/20% (wt/vol) glycerol. All manipulations of enzymes and crude fractions were at 0–4°C.

***oriC* Reconstitution Assays.** The basic reconstitution assay mixture (25 μl) contained, in order of addition: Hepes/KOH, pH 7.6 (40 mM); CTP, GTP, and UTP (0.5 mM each); ATP (2 mM); creatine phosphate (6 mM); dCTP, dGTP, and dATP (100 μM each); [α-^{32}P]dTTP (100 μM, 200 cpm/pmol); magnesium acetate (11 mM); bovine serum albumin (0.4 mg/ml); M13*oriC26* RF I DNA (200 ng, 600 pmol as nucleotide); creatine kinase (0.1 mg/ml); SSB (400 ng); DNA polymerase III holoenzyme (160 ng); primase (20 ng); dnaB (100 ng) and

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Abbreviations: RF I DNA, replicative form I DNA (supercoiled covalently closed circular DNA); SSB, single-stranded-DNA-binding protein; kDa, kilodalton(s).

*Present address: Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra 2601, Australia.

dnaC (40 ng) proteins; proteins i and n′ (30 units each) and n (7 ng) and n″ (20 units); RNA polymerase holoenzyme (500 ng); gyrase A (120 ng) and B (800 ng) subunits; dnaA protein (60 units); DNA topoisomerase I (25 ng); and PVA (7%, wt/vol). Crude enzyme fractions to be complemented were added just before PVA, and the complementing fractions were assayed immediately thereafter. After incubation for 30 min at 30°C, reactions were stopped with 1 ml of 10% trichloroacetic acid, 0.1 M in sodium pyrophosphate. Precipitates were collected on GF/C filters (Whatman) and dried, and their radioactivities were measured in a toluene-based fluor with a liquid scintillation spectrometer. One unit is the amount of enzyme promoting incorporation of 1 pmol of nucleotide per min.

The crude fraction to be complemented was prepared by either of two procedures (A and B) (unpublished data) from WM433 fraction II (prepared by addition of 0.28 g of ammonium sulfate per ml of lysate and dissolved to yield 145 mg of protein per ml (1).

Procedure A. For complementation of a 0.2 M KCl eluate from DEAE-cellulose (100 μg per assay), fraction II (4 ml) was diluted 1:15 with buffer H [25 mM Hepes, pH 7.6/1 mM EDTA/2 mM dithiothreitol/15% (wt/vol) glycerol], and applied at 56 ml/hr to a 50-ml column of DEAE-cellulose (DE52, Whatman) equilibrated with buffer H/50 mM KCl. The column was washed with the same buffer (150 ml); bound protein was eluted with buffer H/200 mM KCl, then precipitated by addition of 0.35 g of ammonium sulfate per ml of eluate. After being stirred for 2 hr at 0°C, the precipitate was collected, resuspended, and dialyzed for 1 hr in buffer H.

Procedure B. A 0.2 M KCl flow-through fraction was prepared by passage (at 16 ml/hr) of fraction II (1 ml), diluted to 13 mg/ml with 50 mM Tris·HCl, pH 7.5/1 mM EDTA/2 mM dithiothreitol/0.2 M KCl/15% (wt/vol) glycerol, through a 10-ml column of DEAE-cellulose equilibrated with the same buffer. Fractions containing protein were pooled, concentrated, and dialyzed as above. This fraction was deficient in another unidentified factor, present in the flow-through from chromatography of fraction II (prepared by addition of 0.35 g of ammonium sulfate per ml of lysate and solution of the precipitate to yield 95 mg of protein per ml; 1 ml diluted 1:10) on a 10-ml column of red A-agarose dye-affinity gel (Amicon) in buffer H. Supplementation of the DEAE-cellulose flow-through (20 μg per assay) with the red A-agarose flow-through (0.5 μg per assay) gave an assay for protein HU similar to that obtained by using procedure A, except that the background was consistently higher.

Other Methods. $NaDodSO_4$/polyacrylamide gel electrophoresis in 15% acrylamide/0.4% bisacrylamide slab gels (13.5 × 12.5 cm × 0.75 mm) was as described (19). Gels were stained with ammoniacal silver nitrate after pretreatment with 10% glutaraldehyde (22). Protein concentrations were measured by the Coomassie brilliant blue method of Bradford (23) with bovine serum albumin as standard. On the basis of the concentration determined by Rouvière-Yaniv and Gros (9), this assay appears to underestimate *E. coli* protein HU by a factor of 3.6.

RESULTS

Purification of a Stimulatory Factor for *oriC* DNA Replication. With a mixture of purified proteins to support *oriC* DNA replication (reconstitution assay), including DNA topoisomerase I to maintain template specificity and dependence on dnaA protein (6), a factor present in fraction II stimulated the reaction 3- to 5-fold. With this assay, the stimulatory factor was purified approximately 400-fold from an ammonium sulfate precipitate of a cleared lysate of *E. coli* WM433 (Table 1). Approximately half of the activity flowed through phosphocellulose, presumably because of its association with nucleic acid; the remainder eluted late in a 0–0.5 M salt gradient. The factor was eluted from hydroxylapatite ahead of the bulk of contaminating proteins (Fig. 1*A*) and had a specific activity of 650,000 units/mg in the peak fractions. Coincidence of activity and a polypeptide with a molecular weight near 10,000 (Fig. 1*B*), together with its chromatographic properties, suggested that the factor might be protein HU (9).

Table 1. Purification of the stimulatory factor

Fraction	Protein, mg	Activity, units × 10^{-4}	Specific activity, units × 10^{-3}/mg	Yield, %
I. Cleared lysate	292	65.6	2.2	100
II. Ammonium sulfate	227	27.2	1.2	41
III. Phosphocellulose	1.76	11.7	66	18
IV. Hydroxylapatite	0.125	6.1	490	9

E. coli WM433 (17.5 g of cell paste) was grown and lysed and fraction II (0.35 g of ammonium sulfate per ml) was prepared. Fraction II (2.0 ml) was diluted 1:20 with buffer A and adsorbed on a column (20 ml) of phosphocellulose equilibrated with the same buffer at a flow rate of 13 ml/hr. The column was washed with buffer A (30 ml), then eluted with a 200-ml linear gradient of 0–0.5 M KCl in buffer A. Active fractions, which eluted near 0.48 M KCl, were pooled (fraction III, 27.5 ml). A portion of fraction III (15 ml) was diluted 1:2 with buffer B and applied at 8 ml/hr to a 2.5-ml column of hydroxylapatite equilibrated with that buffer. After being washed with 10 ml of buffer B, the column was eluted with a 20-ml linear gradient from the composition of buffer B to that of buffer C. The yields at fraction IV were deduced from assays of individual fractions (numbers 22–25, Fig. 1) and have been adjusted to take account of the proportion of fraction III used.

Abundance of the Stimulatory Factor. Extraction by freeze-thaw lysis in 150 mM KCl yielded 3.7×10^4 units/g of cells (Table 1). Assuming a specific activity of 7×10^5 units/mg for the pure protein and a molecular weight of 19,000 for the dimer, the lysate activity corresponds to 2,800 molecules per cell. The yield was dramatically improved by lysis in 1 M KCl; 4.3×10^5 units were extracted per gram of cells, corresponding to 32,000 dimers per cell. This lysate had a specific activity of 1.6×10^4 units/mg of protein and was used for isolation of the more highly purified factor employed in subsequent experiments; the ammonium sulfate step (Table 1) could be replaced by chromatography on double-stranded DNA-cellulose as described for protein HU (9).

Protein HU Is Active in the *oriC* Reconstitution Assay. *E. coli* protein HU replaced the isolated factor in the reconstitution assay, with the same specific activity (Fig. 2). On the basis of its specified concentration, protein HU would have a specific activity of 7.2×10^5 units/mg, as determined in either of the two reconstitution assays. Approximately 8 ng, or 17 dimers per DNA circle, produced half-maximal stimulation in the assay (Fig. 2). Thus the requirement for HU is estimated to be near 40 dimers per DNA molecule replicated. The stimulatory activity is not restricted to *E. coli* protein HU, since the *Anabaena* sp. protein also substitutes in the assay. Silver-stained $NaDodSO_4$/polyacrylamide gels (Fig. 3) of the purified factor and protein HU from *E. coli* at equivalent concentrations as assessed by enzymatic activity show that the two proteins comigrate and are slightly smaller than protein HU from *Anabaena*, as reported (21). The staining intensities of the two *E. coli* proteins are essentially identical, suggesting little difference in their specific enzymatic activities. *Anabaena* protein HU appears to be approximately half as active.

Inhibition of *oriC* Replication by Protein HU Antibody. The activity of the stimulatory factor in the reconstitution assay was inhibited by antibody directed against protein HU in

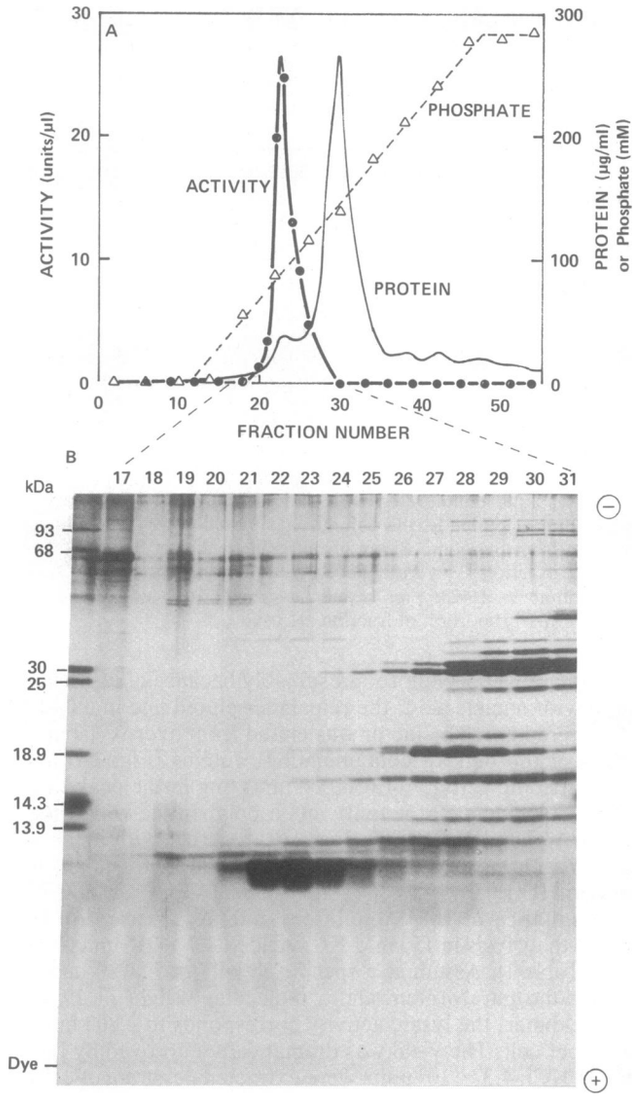

FIG. 1. Isolation of the stimulatory factor. (*A*) Profile of elution from hydroxylapatite as described in Table 1. Fractions (0.5 ml) were assayed for protein (—) by the method of Bradford (23) or for enzymatic activity (●) by a partially reconstituted *oriC* replication assay (procedure A). (*B*) $NaDodSO_4$/polyacrylamide gel electrophoresis of 5-μl samples of individual fractions as indicated. Standards were phosphorylase *b* [93 kilodaltons (kDa)], bovine serum albumin (68 kDa), carbonic anhydrase (30 kDa), chymotrypsinogen (25 kDa), SSB (18.9 kDa), ribonuclease A (14.3 kDa), and egg white lysozyme (13.9 kDa). Bands in the region 55–65 kDa are artifacts of the silver staining due to dithiothreitol in the sample buffer (24).

proportion to the quantity of antibody added; the inhibition was overcome by an excess of the purified factor (Fig. 4). Specific antibody inhibition was also observed in the crude enzyme fraction II (5), with restoration of activity upon addition of the purified factor (Fig. 5).

DISCUSSION

Protein HU (DNA-binding protein II) is an abundant small basic protein that binds both single- and double-stranded DNA (9, 25) and is associated with the bacterial nucleoid (26). It is made up of two homologous 90-amino acid peptides, whose sequences have been determined (27–29) and that exist in solution as a heterodimer (30) or a tetramer (31). The two subunits co-electrophorese on $NaDodSO_4$/polyacrylamide gels but are separated electrophoretically or chromatographically at low pH in urea (29). The genes encoding

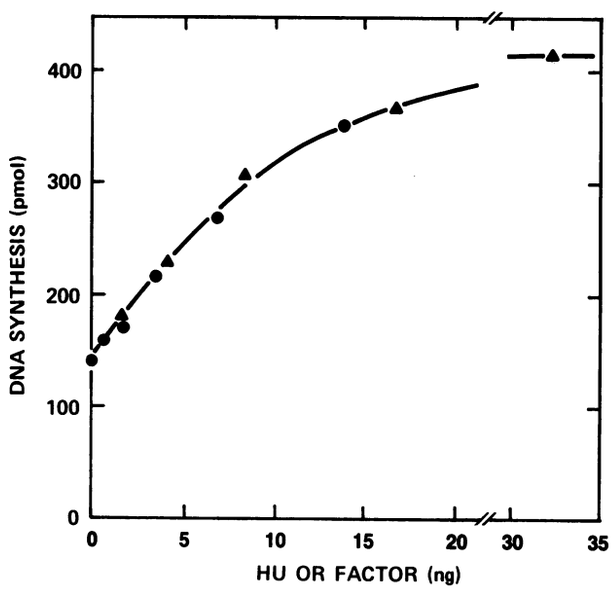

FIG. 2. Titration of the stimulatory factor (●) and *E. coli* protein HU (▲) in the *oriC* reconstitution assay (procedure B). Concentrations of the two proteins were determined by a Coomassie blue dye-binding assay (23) using bovine serum albumin as standard and were adjusted for the relative insensitivity of protein HU (see *Materials and Methods*).

the two polypeptides have not been identified. HU has been isolated from DNase I-treated lysates of *E. coli* by chromatography on double-stranded DNA-cellulose (9) and was found associated with native 30S ribosomal subunits (and termed NS1, NS2) (31). The homologous *B. stearothermophilus* DNA binding protein II has been sequenced (32) and crystallized (33).

The physiological function of protein HU is uncertain. *In vitro*, at levels near stoichiometric by weight with DNA, it enhances transcription of phage λ DNA, showing no specific preferences among promoters (9). At a similar protein-to-DNA ratio, in the presence of a eukaryotic type I DNA topoisomerase, HU resembles histones in condensing simian

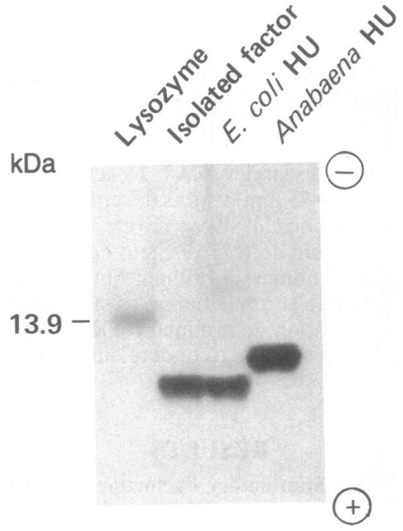

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis of protein HU. Samples (50 units of each) of the isolated factor and *E. coli* and *Anabaena* proteins HU were electrophoresed on a 15% polyacrylamide gel in the presence of $NaDodSO_4$ together with egg white lysozyme as a molecular weight marker. The upper half of the silver-stained gel is not included in the photograph.

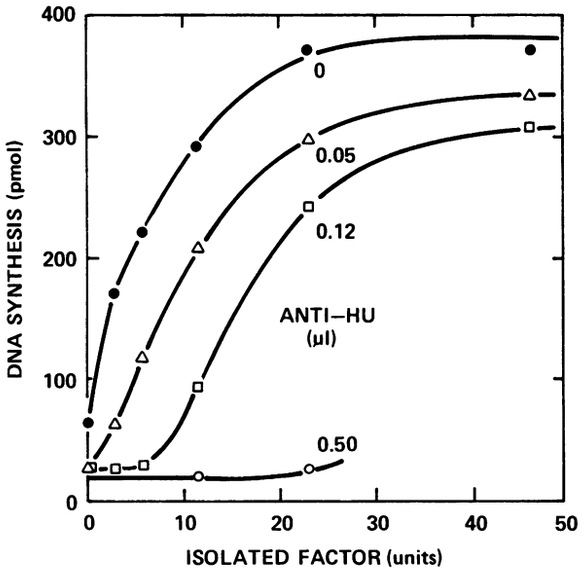

FIG. 4. Inhibition of the *oriC* reconstitution assay by protein HU antibody. The isolated factor (Fig. 1, fraction 23) was titrated into the *oriC* reconstitution assay (procedure A) in the absence (●) or presence of 0.05 (△), 0.12 (□), or 0.50 (○) μl of protein HU antibody.

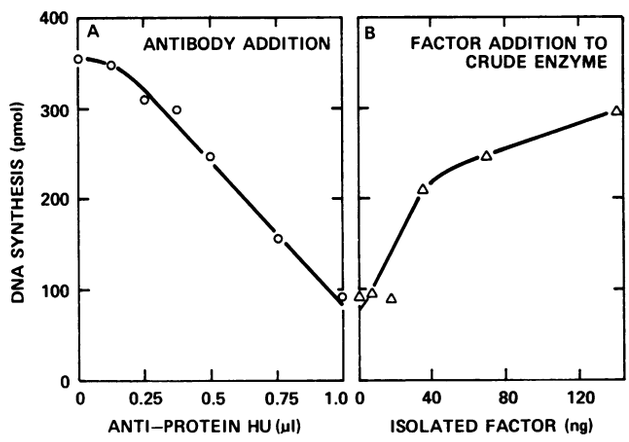

FIG. 5. Inhibition of the dnaA-complementation assay by protein HU antibody. The assay mixtures contained purified dnaA protein and WM433 (*dnaA204*) fraction II as a source of all other replication proteins (5). The reaction was allowed to proceed for 20 min. (*A*) Inhibition of the reaction by the antiserum (○). (*B*) Recovery of activity by addition of the isolated factor to assay mixtures containing 1 μl of protein HU antibody (△). The concentration of the isolated factor was determined as described in the legend of Fig. 2.

virus 40 closed-circular DNA into beaded structures reminiscent of nucleosomes (34). On the basis of these data, a reasonable estimate for the stoichiometry of binding of HU to DNA is one protein dimer per 30 base pairs, although noncooperative binding of up to one dimer per base pair has been observed in velocity sedimentation experiments (35).

A protein isolated from *E. coli* that stimulates a partially reconstituted *oriC*-specific reaction on M13*oriC26* RF I DNA by 3- to 5-fold is identical to protein HU (9) by several criteria. (*i*) It replaces protein HU in the reconstituted assay system and possesses the same specific activity. (*ii*) It has a subunit molecular weight near 10,000 and migrates on $NaDodSO_4$ gel electrophoresis with protein HU. (*iii*) Antibody specific for protein HU inhibits the reaction in the reconstituted system and the dnaA-complementation assay; and the inhibition is overcome in each case by addition of excess of the purified factor. (The dnaA-complementation assay utilizes a crude extract, deficient in and supplemented with dnaA protein, as the source of replication proteins and therefore more closely resembles *in vivo* conditions.) (*iv*) The abundance of the stimulatory factor (32,000 dimeric molecules per cell) agrees with the estimate of 30,000 dimers for the cellular content of HU (25).

Role of Protein HU in *oriC* Replication. Enzymatic replication of M13*oriC26* RF I DNA in partially reconstituted systems requires novel factors that act either in a positive sense (e.g., protein HU) or as "specificity proteins" [e.g., DNA topoisomerase I (unpublished data)] that suppress replication of plasmids that lack *oriC* and permit the dnaA protein-dependent replication of *oriC* plasmids. The specificity role bears some analogy to that of SSB in replication of single-strand phage DNA, in which SSB maintains specificity of priming at complementary strand origins and dependence on specific priming mechanisms.

Forty dimers of HU per template circle (Fig. 2) stimulate replication of M13*oriC26* (12.2 kilobases) at least 3-fold. This represents one molecule of the protein per 300 base pairs, or about 1/10th the level required for stimulation of transcription or extensive condensation of the DNA. The more stringent requirement for HU in replication and its apparent lack of positive effect on dnaA-independent reactions (data not shown) suggest its action is at the *oriC* locus. Its binding to double-stranded DNA, production of nucleosome-like structures in the presence of a type I topoisomerase, and stimulation of transcription of λ DNA indicate two possible functions in replication. One is in preparation of the template, where HU, in concert with gyrase and topoisomerase I, could introduce and preserve some favorable structure in the DNA in the vicinity of *oriC*, a process perhaps directed by the dnaA protein. The second possibility is that HU specifically binds and stabilizes the nascent RNA transcript destined for use as primer for leading strand synthesis.

Further study of the mechanism of the actions of HU will become accessible with more complete reconstitution of replication of M13*oriC26* RF I and other *oriC*-containing DNAs. Assays in hand suggest that additional positively acting factors have roles in the enzymatic replication of M13*oriC26* DNA.

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