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Novel histone H2A-like protein of *Escherichia coli*

(prokaryotic histone-like proteins/eukaryotic histones/reannealing of single-stranded DNA/immunological crossreaction/structure of *E. coli* chromosome)

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ABSTRACT **A histone-like protein (H) from *Escherichia coli* has been purified to more than 98% homogeneity by using its capacity to inhibit DNA functions. H protein behaves as a dimer of 28,000-dalton subunits. The histone H2A-like properties of H protein are: (*i*) binding to DNA at a stoichiometry of 1 H protein dimer per 75 bases; (*ii*) abundance of about 30,000 molecules per cell, sufficient to bind about 20% of the chromosome; (*iii*) limiting digestion of double-stranded DNA by micrococcal nuclease; (*iv*) reannealing of complementary single-stranded DNA; (*v*) amino acid composition resembling that of eukaryotic histone H2A; (*vi*) neutralization of H protein by antibody specific for H2A; (*vii*) heat stability; and (*viii*) acid solubility. The capacity of H protein to bind DNA prevents its template or substrate functions in several reactions *in vitro*: DNA synthesis by several polymerases; transcription by RNA polymerase; DNA topoisomerase activity; and DNA-dependent ATP hydrolysis by *rep* protein, *dnaB* protein, or protein n′. Together with other histone-like proteins of *E. coli*, H protein may organize the *E. coli* chromosome into nucleosomes, such as in eukaryotic chromatin.**

The *Escherichia coli* chromosome can be isolated in a folded, condensed, supercoiled form (1–4). A search for histone-like proteins in *E. coli* and other prokaryotes has yielded several low molecular weight, basic, DNA-binding proteins that resemble eukaryotic histones (5–9). A pair of HU proteins, also known as protein 2, are near 9000 daltons in size and resemble the eukaryotic histones in (*i*) binding to DNA, (*ii*) having an amino acid composition like that of histone H2B, (*iii*) being heat and acid stable, and (*iv*) forming a nucleosome-like structure with duplex DNA (9, 10). In addition, a histone-like protein of 17,000 daltons in *E. coli* (8) and a low molecular weight protein in thermophilic mycoplasma (*Thermoplasma acidophilum*) similar to histone H4 (11) have been described.

In the course of purifying *E. coli* replication proteins (12) and possible regulatory factors, we encountered an inhibitor that appears to be a novel histone-like protein, provisionally called H protein, that shows immunological crossreactivity with the eukaryotic histone H2A. As a result of tight binding to DNA, H protein inhibits DNA functioning as a template for polymerases, as a substrate for nuclease and topoisomerase, and as an effector for ATPase; it also activates annealing of homologous single strands. H protein may have a significant role in the structure and function of the *E. coli* chromosome.

MATERIALS AND METHODS

Nucleic Acids, Nucleotides, Enzymes, and Proteins. ϕX174 and M13 DNA were prepared as described (13); calf thymus DNA was from Calbiochem; P22 DNA, P22 [^{3}H]DNA, λ precA99 DNA, and *E. coli* RNA polymerase were from K. McEntee (Department of Biochemistry, Stanford University); the hook-shaped template dA_{100}·dT_{25} was from P. Fisher (Stanford University); single-stranded (ss) DNA cellulose was prepared as described (14); *Aspergillus oryzae* S1 nuclease, deoxynucleoside-, and nucleoside triphosphates were from Sigma; ^{3}H-labeled dTTP, UTP, and ATP were from Amersham; and *E. coli* alkaline phosphatase was from Worthington. The following enzymes with specific activities [units (pmol of nucleotide incorporated or hydrolyzed per min)/mg] as described (15) were used: single-stranded-DNA binding protein (SSB), 7×10^4; core DNA polymerase III, 8×10^5; *rep* protein, 4×10^7; *dnaB* protein, 6.2×10^5; and protein n′, 1×10^6. Homogeneous calf thymus histones (H1, H2A, H2B, H3, and H4) were donated by R. Sperling (Stanford University).

Buffers. Buffer A contained 50 mM imidazole·HCl (pH 6.8), 20% (vol/vol) glycerol, 1 mM EDTA, and 1 mM dithiothreitol. Buffer B contained 20 mM Tris·HCl (pH 7.5), 10% (vol/vol) glycerol, 1 mM EDTA, and 5 mM dithiothreitol. Additions of salts were as indicated; all pH measurements were at 20°C.

Bacterial Growth. *E. coli* HMS83 (*polA1, polB1, thy, lys, lac, rha, str*^{r}) from C. C. Richardson (16) was grown as described (15).

Reannealing of Complementary P22 ss DNA. The H protein to be assayed and 120 μl of reaction mixture [20 mM Tris·HCl, pH 7.5/5% (vol/vol) glycerol/10 mM KCl/10 mM $MgCl_2$/1 mM dithiothreitol/2000 pmol of P22 [^{3}H]DNA (as nucleotide; 25,000 cpm/μg; heated at 95°C for 2 min immediately before use)] were incubated at 30°C for 15 min. Sodium dodecyl sulfate was added to 0.5% and calf thymus ss DNA was added to a concentration of 150 μg/ml. To this mixture, 600 μl of 50 mM NaOAc, pH 4.6/150 mM NaCl/1 mM $Zn(OAc)_2$ was added, followed by 100 units of S1 nuclease. Incubation was at 37°C for 30 min. After addition of calf thymus ss DNA to 150 μg/ml and precipitation with an equal volume of 10% (wt/vol) trichloroacetic acid, the acid-insoluble radioactive material was filtered and measured as described (15). Under these conditions, in the absence of H protein, 3% of the input DNA was acid insoluble (S1 nuclease resistant). The acid-insoluble radioactivity of native P22 [^{3}H]DNA was taken as the 100% S1 nuclease-resistant value.

Antisera and Enzyme-Linked Immunosorbent Assay (ELISA). Antisera against calf thymus histones H1, H2A, and H2B were prepared in rabbits by R. Sperling and M. Bustin as described (17), and their specificity was measured by complement fixation (18).

Antiserum against H protein was prepared in female mice [hybrids A/J × BALB/c provided by R. Scibiensky (University of California, Davis)] by intraperitoneal injections of H protein on days 1, 10, 20, and 30. The initial injection was 50 μg of H protein in 100 μl of 0.15 M NaCl emulsified with 100 μl of

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Abbreviations: H, *Escherichia coli* histone H2A-like protein; ss, single-stranded; ds, double-stranded; SSB, single-stranded-DNA binding protein; ELISA, enzyme-linked immunosorbent assay.

complete Freund's adjuvant (Difco no. 0638–60); subsequent injections contained 50 μg of protein in 20 μl of 0.15 M NaCl. Serum was collected before immunization and on days 25 and 35.

ELISA was based on the method of Engvall and Perlmann (19) and performed in a Gilford enzyme-immunoassay system EIA-50 (Gilford Instrument, Oberlin, OH) in final volumes reduced to 50 μl. Goat anti-mouse IgG and goat anti-rabbit IgG conjugated to horse radish peroxidase (Cappel Laboratories, Cochranville, PA) were used in dilutions of 1:500. Enzyme activity retained in the wells was measured colorimetrically, in duplicate, upon incubation with 0.2 mM 2,2′-azino-di(3-ethyl-benzthiazoline-6-sulfonate)/2 mM H_2O_2/50 mM citric acid, pH 4.0, modified according to Saunders and Bartlett (20).

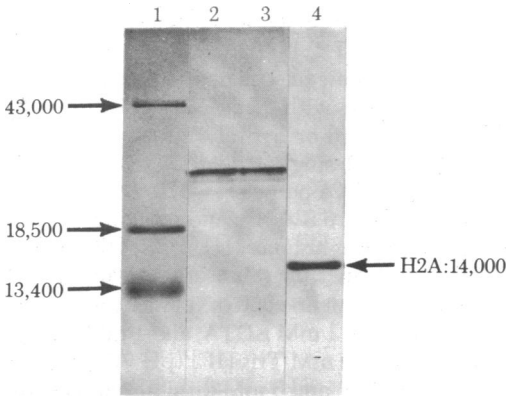

FIG. 1. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of purified H protein. Inhibition of DNA replication was used to monitor the isolation of H protein (see the legend to Fig. 4). Operations were at 0–4°C. Fraction I (600 ml) prepared from 100 g of *E. coli* HMS83 cells (14) was treated with solid ammonium sulfate (0.226 g/ml) added over a 30-min interval with stirring continued for another 30 min. The mixture was centrifuged at 32,000 × *g* for 30 min and the precipitate was resuspended in 30 ml of buffer A (fraction II, 35 ml). Fraction II was diluted with buffer A to a conductivity equivalent to buffer A containing 40 mM ammonium sulfate and was applied at 90 ml/hr to a 300 ml Bio-Rex 70 column (6.4 × 9.3 cm) equilibrated with buffer A containing 40 mM NaCl. The column was washed with 600 ml of buffer A containing 140 mM NaCl and treated further with a 1400-ml gradient of 140–350 mM NaCl in buffer A. Finally, the inhibitory activity was eluted with 1 column volume of buffer A containing 1 M NaCl. Pooled active fractions (fraction III, 20 ml, 3700 units/mg) were concentrated by ammonium sulfate precipitation (0.291 g/ml), resuspended in 1 ml of buffer A containing 30 mM NaCl, and applied to a 210-ml Sephadex G-100 column (1.5 × 120 cm). Filtration was performed at 3–6 ml/hr with buffer A containing 30 mM NaCl. Active peak fractions were pooled (fraction IV, 13 ml, 8500 units/mg, recovery 45%) and applied directly to a 1.5-ml ss DNA-cellulose column (0.9 × 2.8 cm) equilibrated in buffer A containing 40 mM NaCl. The column was washed with 3 ml of equilibration buffer followed by 3 ml each of buffer A containing 100, 250, 500, or 1000 mM NaCl. The enzyme (fraction V, 3 ml, 10,300 units/mg, recovery 40%) was eluted with the 500 mM NaCl buffer, dialyzed against buffer A containing 30 mM NaCl, frozen in liquid nitrogen, and stored at −80°C.

Slab gels with a 2% stacking and a 15% resolving gel were prepared according to Laemmli (22). Purified H protein (fraction V, 10 μg) and reference proteins were reduced and denatured by heating for 3 min at 100°C in 1% sodium dodecyl sulfate/0.04 M dithiothreitol. Electrophoresis was performed at 10 mA until the bromphenol blue marker reached the bottom of the gel. Coomassie brilliant blue (0.25%, vol/vol) in methanol/acetic acid/water, 4.5:1:4.5, was used for staining. Markers (5 μg each) were: lane 1, *E. coli* alkaline phosphatase (43,000), SSB (18,500), and horse heart cytochrome c (13,400); lane 2, denatured H protein (10 μg); lane 3, denatured H protein treated with neutralized iodoacetate at a final concentration of 80 mM; lane 4, calf thymus histone H2A (14,000).

Other Methods. Protein determination was by the Coomassie blue method of Bradford (21).

RESULTS

Isolation of H Protein. In the isolation of replication proteins n, n′, and n″, a fraction tightly bound to an anion exchange resin (Bio-Rex 70) proved inhibitory to the replication assay (R. Low, J. Shlomai, and A. Kornberg, unpublished data). This inhibition, also evident with DNA polymerase III on a defined artificial template (see Fig. 4), was used as an assay to purify the inhibitor, called H protein. Protein of at least 98% homogeneity was obtained by use of Bio-Rex 70 chromatography, gel filtration, and chromatography on DNA cellulose (see legend to Fig. 1). The purified protein was stable for several months at −80 or 4°C.

Measurement of the abundance of H protein in crude enzyme fractions is complicated by the actions of additional inhibitors (such as nucleases) and by factors (such as nucleic acids) that may antagonize these inhibitors. The heat stability of H protein was exploited to assay the crude extract (fraction I) after extensive dialysis against buffer A. The cellular content of H protein was grossly estimated to be 30,000 dimer molecules, based on the assay value for the extract of 28,000 units/g of *E. coli* cell paste.

Physicochemical Properties. Under denaturing conditions in sodium dodecyl sulfate/polyacrylamide gels, over 98% of the H protein preparation migrated as a single band of 28,000 daltons (Fig. 1). The protein emerged slightly behind hemoglobin from a gel filtration column, suggestive of a dimeric structure in the native state. H protein is heat and acid stable; no more than 10–20% of its inhibitory activity was lost after

Table 1. Amino acid composition of H protein*

Amino acid	Amino acid composition, mol %: H protein	Eukaryotic histone H2A†	Eukaryotic histone H2B†	*E. coli* HU protein†
Lys	10.7	10.2	14.1	14.0
His	1.7	3.1	2.3	1.5
Arg	9.9	9.4	6.9	5.1
Glx	11.6	9.8	8.7	9.6
Asx	7.6	6.6	5.0	8.1
Ser	4.3‡	3.4	10.4	4.4
Thr	4.5‡	3.9	6.4	6.0
Ala	11.3	12.9	10.8	16.3
Val	8.3	6.3	7.5	6.0
Ileu	6.5	3.9	5.1	6.0
Leu	7.2	12.4	4.9	6.6
Met	—§	—	1.5	1.5
Phe	1.3	0.9	1.6	3.0
Tyr	0.4	2.2	4.0	—
Pro	4.8	4.1	4.9	3.0
Gly	9.7	10.8	5.9	7.4
Cys	—§	—	—	—
Trp	—¶	—	—	—
Lys/Arg	1.08	1.08	2.0	2.7
Gly × Arg	96	101	41	38

* H protein, dialyzed against quartz-distilled water and lyophilized, was hydrolyzed at 110°C in 5.7 M HCl for 24, 48, and 72 hr at a protein concentration of 0.1 mg/ml. After lyophilization, the amino acids were dissolved in 100 μl of 0.2 M sodium citrate (pH 2.2) containing 25 nmol of norleucine as standard and analyzed by Alan Smith (U. California, Davis) in a Beckman amino acid analyzer.

† Data from (5, 23).

‡ Hydrolysis data extrapolated to zero time.

§ Analysis according to ref. 24.

¶ Analysis according to ref. 25.

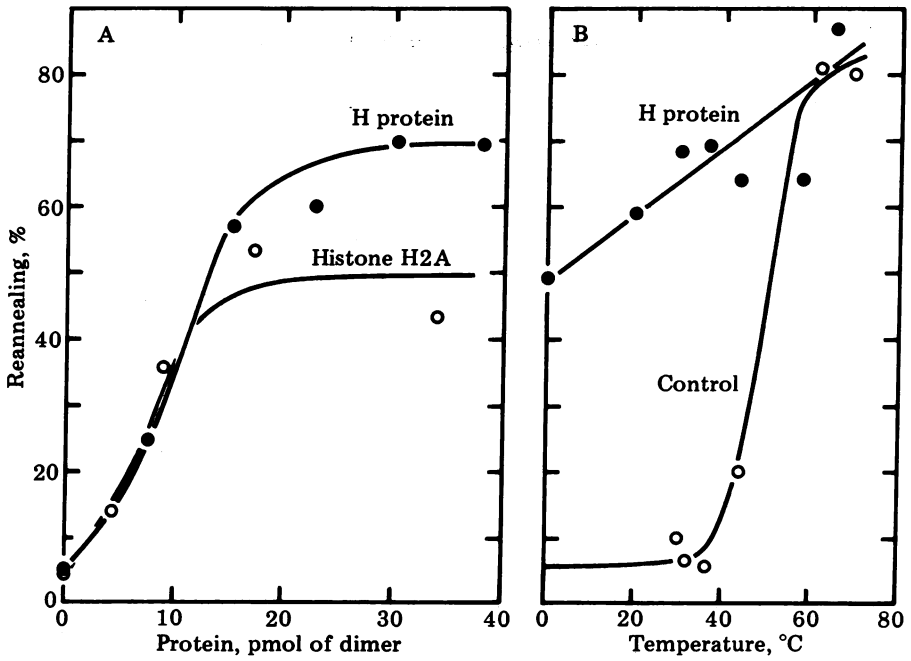

FIG. 2. Reannealing and thermal renaturation of P22 ss DNA by H protein. Reannealing was performed as described, with 2 nmol (as nucleotide) of P22 ss DNA in *A* and 13.2 pmol of H protein dimer and 600 pmol of P22 ss DNA in *B*.

heating at 100°C for 5 min or after exposure to pH 2, even at 100°C.

H protein binds to ss and double-stranded (ds) DNA at NaCl concentrations up to 100 mM and limits DNA digestion by staphylococcal nuclease (unpublished data). At a ratio of 1 H protein dimer to 75 nucleotides, DNA fragments of 150–520 bases were obtained after nuclease treatment.

The Amino Acid Composition. The amino acid composition of H protein bears a striking resemblance to that of histone H2A [and is distinct from that of H2B and *E. coli* protein HU (Table 1)]. Lysine and arginine contents are identical; tryptophan, cysteine, and methionine are absent. As expressed by the lysine/arginine ratio and the glycine × arginine product, the compositions of H protein and H2A histone appear to be similar.

Annealing of Complementary ss DNA. H protein facilitated reannealing of complementary ss DNA (Fig. 2*A*). The reannealing limit (S1 nuclease-resistance of 70–80% of the input DNA) occurred at a ratio of 1 H dimer per 75 nucleotides. The rate of annealing was rapid: 325 pmol/min. Under these conditions, H protein did not affect ss M13 DNA (less than 3% became S1 nuclease-resistant), which indicated the need for complementary strands. Reannealing did not require ATP and was effective at pH 6–10; it occurred even at low temperature, with 50% renaturation being observed at 0°C (Fig. 2*B*). Renaturation has been observed also under these conditions with each of the five calf thymus histones (M. Cox and I. R. Lehman, personal communication); indeed, the titration of H protein and histone H2A were almost identical (Fig. 2*A*).

Neutralization by Antiserum Specific for Histone H2A. Reannealing of complementary ss DNA was used as a functional assay to measure the effect of various antisera. An antiserum specific for histone H2A neutralized the reannealing activity of H protein (Table 2); antisera specific for histones H2B and H1 did not. However, antiserum prepared against H protein

Table 2. Antiserum specific for histone H2A neutralizes H protein in a functional assay

Antiserum	Reannealing, %*			
	H	H1	H2A	H2B
Rabbit control	67	71	75	51
Anti-H1	52	7	74	65
Anti-H2A	5	68	1	44
Anti-H2B	53	49	63	2
Mouse control	44	ND	91	ND
Anti-H	8	ND	73	ND

Sera were diluted 1:200 in 0.14 M NaCl/1 mM EDTA/0.01 M Tris·HCl, pH 7.5. Each protein (2.5 μg) was incubated for 3 hr at 0°C with the amount of diluted serum needed for neutralization of the corresponding protein. After heating for 3 min at 70°C, the immunoprecipitate was pelleted by centrifugation and the supernatant was analyzed for reannealing capacity; histones and H protein withstand the heating. ND, not determined.

* The S1 nuclease-resistance of native P22 [^{3}H]DNA in the absence of protein was taken as 100% reannealing.

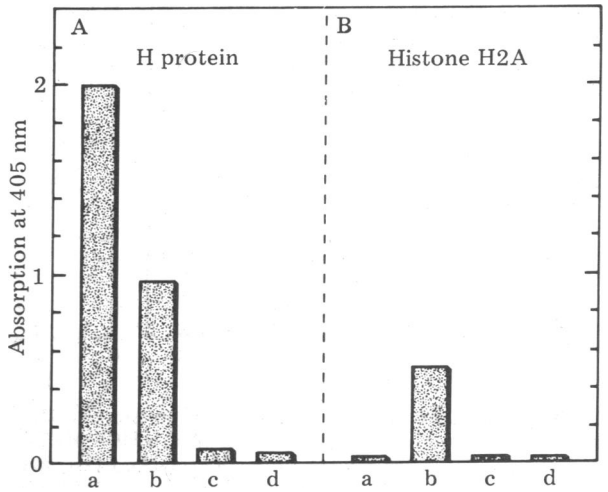

FIG. 3. Antiserum against H2A reacts with H protein as measured by ELISA. Wells were coated with 2 μg of H protein (*A*) or histone H2A (*B*). Sera were diluted 1:100 in buffer containing 0.15 M NaCl, 1 mM EDTA, 1 mg of bovine serum albumin per ml, 0.02% NaN_3, 0.05% Tween 20, and 0.05 M Tris·HCl (pH 7.5). Results were the same with 0.5 μg of H protein. Bar a, mouse anti-*H*; bar b, rabbit anti-H2A; bar c, mouse control; and bar d, rabbit control.

did not affect the reannealing activity of H2A. These results were substantiated by the ELISA test (Fig. 3). Antiserum induced by H2A reacted with both H2A and H protein, whereas antiserum raised against H protein reacted with H protein only. Reactivity of control sera was no more than 1–2%.

Inhibition of Enzymes Dependent on DNA and the Stoichiometries of H Protein to DNA. H protein inhibits DNA synthesis by core DNA polymerase III in the presence of SSB (Fig. 4*A*). It also inhibits DNA replication with reconstituted enzyme systems that convert ϕX174 ss DNA to the replicative form or generate ss DNA from the replicative form (data not shown). H protein inhibited transcription by *E. coli* RNA polymerase on phage λ DNA (Fig. 4*B*) and prevented DNA-dependent ATP hydrolysis with each of three homogeneous DNA-dependent ATPases: *rep* protein, *dnaB* protein, and protein n′ (Fig. 4*C*). H protein also inhibited the nicking–closing enzyme obtained from *Drosophila melanogaster* (T. Hsieh, personal communication).

The stoichiometry of these inhibitions—about 1 H dimer per 70 nucleotides—was the same as that observed for binding to ds DNA, for reannealing of ss DNA, and for limiting digestion by nuclease (Table 3); an exception was the DNA-dependent ATP hydrolysis, which required twice as much H protein for inhibition with the same amount of DNA.

Table 3. Stoichiometry of H protein to DNA in various assay systems

Assay system	Stoichiometry,* DNA/H protein
Reannealing of ss DNA	75
Inhibition of replication	73
Inhibition of transcription	60
Inhibition of DNA-dependent ATP hydrolysis	30
Protection from staphylococcal nuclease digestion†	75
Binding to ds DNA†	70

* Stoichiometry represents the moles of DNA (as nucleotide) affected by mol of H protein (as dimer) titrated in the assays described in *Materials and Methods* or in the legend to Fig. 4.

† U. Hübscher and A. Kornberg, unpublished observations.

DISCUSSION

H protein, isolated from *E. coli*, resembles histones (28–30) in its (*i*) abundance, estimated at 30,000 dimers per cell [an amount similar to that of other *E. coli* histone-like proteins (8, 9)]; (*ii*) stability to heat and acid; (*iii*) binding to DNA and limiting of staphylococcal nuclease digestion; and (*iv*) reannealing of complementary ss DNA at low temperature. H protein resembles histone H2A in particular in its amino acid composition and reactivity with an antiserum specific for histone H2A. A clear discrepancy between these two proteins is the subunit molecular mass of H protein of 28,000 daltons, a value twice that of histone H2A. Sequence determination of H protein is needed to learn the extent of its homology with histone H2A.

H protein is unlike *E. coli* protein HU (9). It differs in size and amino acid composition (Table 1), is not neutralized by an anti-HU serum (unpublished data), and inhibits transcription, which protein HU stimulates (5). The inhibitory effects of H protein on DNA replication, DNA-dependent ATP hydrolysis, and DNA topoisomerase activity have not been described for protein HU.

The multiple actions of H protein can be attributed to binding DNA; the capacity of one protein molecule to bind a

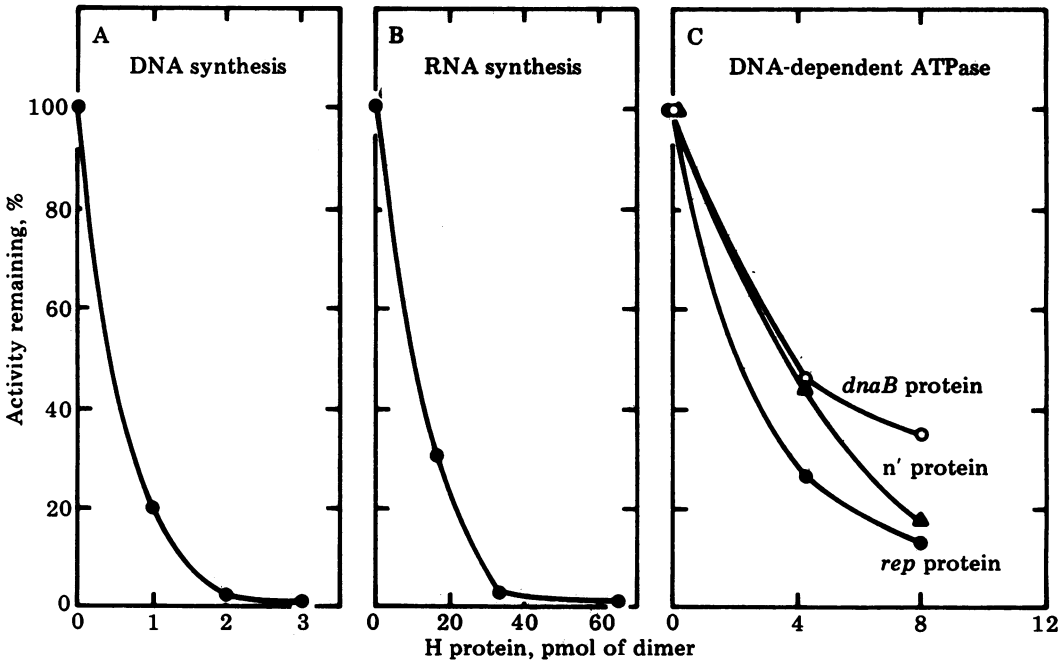

FIG. 4. Inhibition of enzyme functions by H protein. (*A*) DNA synthesis by core DNA polymerase III on the hook-shaped template, dA_{100}-dT_{25}. The 25-μl reaction mixture contained 50 mM Tris·HCl (pH 7.5), 6% (wt/vol) sucrose, 10 mM dithiothreitol, 0.1 mg of bovine serum albumin per ml, 10 mM $MgCl_2$, 225 pmol (as nucleotide) of dA_{100}-dT_{25}, 20 μM [^{3}H]dTTP (100–200 cpm/pmol), 0.5 μg of SSB, 60 ng of core DNA polymerase III, and the H protein to be assayed. Incubation was at 30°C for 20 min. The reaction was stopped by addition of 100 μl of 0.1 M sodium pyrophosphate; after addition of 100 μg of denatured calf thymus DNA, DNA was precipitated, filtered, and measured for radioactivity as described (15). One unit is defined as the amount needed to give 50% inhibition under these assay conditions. (*B*) RNA synthesis. The 100-μl reaction mixture contained 20 mM Tris·HCl (pH 7.5); 10 mM magnesium acetate; 0.1 mM EDTA; 0.1 mM dithiothreitol; 0.02 mg of bovine serum albumin per ml; 100 mM KCl; 1800 pmol (as nucleotide) of λ precA99 DNA; ATP, GTP, and CTP each at 400 μM; 40 μM [^{3}H]UTP (1000 cpm/pmol); 4 μg of *E. coli* RNA polymerase; and the H protein to be assayed. After 20 min at 37°C, the reaction was stopped and a precipitate was formed, filtered, and measured for radioactivity as described (15). (*C*) DNA-dependent ATPases. Assays with 250 pmol (as nucleotide) of ϕX174 DNA were as reported elsewhere for *dnaB* protein (26), protein n′ (27), and *rep* protein (14).

length of 75 nucleotides suggests that the DNA may wind about the protein. A highly organized structure has been proposed for the *E. coli* chromosome (1–4). Now, at least three proteins (protein HU, protein 1, and H protein) are known that may represent part of a "histone set" of *E. coli* "chromatin." Whether the beaded structure of *E. coli* DNA (4) is due to a chain of nucleosomes organized by these proteins is speculative at this point.

In view of the high abundance of H protein in *E. coli*, one wonders how its inhibitory activity is overcome *in vivo* and *in vitro*. A similar question has been raised with regard to the destabilization of histones in eukaryotic chromatin for replication, transcription, repair, recombination, and other DNA events. Activation of DNA might be achieved by altering ionic strength; by modification of histones through phosphorylation, methylation, acetylation, ADP ribosylation, or isopeptide linkage of another protein (29); or by action of specific proteins that alter histone affinity for DNA. With regard to H protein, preliminary observations identify one or more proteins in crude extracts that prevent or reverse the inhibitory and reannealing reactions of H protein (unpublished data). Several transcriptional regulatory proteins have already been described (31–33), and it also has been suggested that DNA-binding proteins and chromatin-associated enzymes may contribute to regulation of DNA replication (34), DNA repair, and genetic recombination (35), and to the conformation of the DNA (36). Isolation of these proteins may identify novel histones and nonhistones with important roles in the regulation of the numerous DNA transactions.

Note Added in Proof. H protein, obtained in a large-scale purification, appears as an equimolar doublet at 28,000 daltons in sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

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