Interaction of *Escherichia coli* RNA Polymerase with the Ribosomal Protein S1 and the Sm-like ATPase Hfq

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ABSTRACT: We report evidence that ribosomal protein S1 and nucleic acid-binding protein Hfq copurify in molar ratios with RNA polymerase (RNAP). Purified S1 associates independently with RNAP, and Hfq binding to polymerase occurs in the presence of S1. Looking for a functional role of the RNAP−S1−Hfq association, we studied the effects of S1 and Hfq on transcription and coupled transcription−translation. S1 was capable of significant stimulation of the RNAP transcriptional activity from a number of promoters; the stimulatory effect was observed on linear as well as supercoiled DNA templates. In addition, we present biochemical and genetic evidence of ATPase activity associated with the Sm-like hexameric nucleic acid-binding protein Hfq. The limited sequence homology between Hfq and known ATP-utilizing enzymes suggests a new class of ATPases.

The *Escherichia coli* DNA-dependent RNA polymerase (RNAP)[1] core enzyme, consisting of $\alpha_2\beta\beta'$ subunits, is capable of transcription elongation and termination. Protein factors engage the core enzyme at various stages of the transcription cycle, allowing the polymerase to execute the specific stages of the cycle, such as promoter recognition and transcription initiation [sigma factors (*1*, *2*)], transcription termination and RNA transcript management [NusA, GreA, GreB (*3*−*6*)], and the proposed remodeling of the post-termination complex resulting in enhanced RNAP recycling [RapA (*7*)].

The 68 kDa ribosomal protein S1, the largest of the ribosomal proteins, is located at the junction of the head, platform, and main body of the 30S subunit (*8*). It has been suggested that a pyrimidine-rich region upstream of the Shine−Dalgarno (SD) sequence of mRNA interacts with S1, serving as one of the ribosome recognition sites (*9*); S1 has also been reported to be necessary for translation initiation (*10*) and translation elongation (*11*). A recent cryoelectron microscopic study has further indicated that S1 plays a pivotal role in stabilizing mRNA on the ribosome, interacting with 11 nucleotides of the mRNA immediately upstream of the SD sequence (*8*). At the same time, it has been noted that association of S1 with the ribosome is weak and reversible, whereas most other ribosomal proteins are strongly bound; ribosome preparations often have been reported to contain less than stoichiometric amounts of S1 (*12*). Interestingly, the ribosomal protein S1 was also identified as a part of the bacteriophage Qβ replication complex (*13*, *14*), and formation of looped complexes between isolated S1 and the Qβ RNA was demonstrated (*15*).

The nucleic acid-binding protein Hfq was originally identified as a *h*ost *f*actor required for replication of the *Q*β RNA bacteriophage (*16*−*18*). Hfq is an abundant [30000−60000 of Hfq monomers/cell (*19*)] heat-stable, doughnut-shaped hexameric protein (*20*−*23*) consisting of identical 11.2 kDa subunits. Inactivation of the *hfq* gene in *E. coli* causes a variety of phenotypes and alters the expression of many proteins, indicating that Hfq acts as a pleiotropic regulator (*24*, *25*). Recently, it was proposed that the cellular role of Hfq is to mediate RNA−RNA interactions (*20*, *21*). Studies of the cellular localization of Hfq have indicated that while the majority of Hfq is detected in the cytoplasm, a fraction of Hfq associates with the nucleoid (*19*, *26*).

Here we report that the ribosomal protein S1 and the nucleic acid-binding protein Hfq are present in stoichiometric amounts in preparations of RNAP obtained from late exponential phase *E. coli* cell cultures. Purified S1 associates with core RNAP in vitro, and purified Hfq shows little or no affinity to core RNAP but interacts with the RNAP−S1 complex. Purified S1 is capable of significant stimulation of RNAP transcriptional activity, and the RNAP−S1 interaction is downregulated by RapA, the RNAP-associated homologue of the SWI/SNF superfamily. We hypothesize that the RNAP−S1 interaction may be important for transcription−translation coupling.

In addition, we report that Hfq, purified to apparent homogeneity, shows ATPase activity. Biochemical and genetic evidence suggests that the ATPase activity is an intrinsic activity of Hfq rather than that of an Hfq-associated protein; this finding predicts that at least some representatives of the extended family of eucaryotic Sm-like proteins (required for processes as diverse as pre-mRNA splicing, mRNA degradation, and telomere formation) that share sequence homology with Hfq (*20*, *21*) are also ATPases.

MATERIALS AND METHODS

Enzymes. Core RNAP, RNAP holoenzyme, NusA, native S1, and Hfq were purified as follows: 90−110 g of frozen

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PEI−cellulose, poly(ethylenimine)−cellulose; PMSF, phenylmethanesulfonyl fluoride; RNAP, RNA polymerase; SD, Shine−Dalgarno; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography.

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cell pellets, obtained from *E. coli* MG 1655 cell cultures that were grown in super broth (KD Medical) to OD_{600} = 4−6, was mixed with 300 mL of grinding buffer (50 mM Tris-HCl, pH 8, 5% glycerol, 2 mM EDTA, 230 mM NaCl, 1 mM β-mercaptoethanol, 0.023 mg/mL phenylmethanesulfonyl fluoride, 0.015 mg/mL dithiothreitol, 0.26 mg/mL lysozyme) in a Waring blender and blended at low speed for 2−3 min until the cells were completely resuspended and the temperature had increased to 2−5 °C. After 20 min, 8 mL of 10% deoxycholate was gradually added to lyse the cells, and the mixture was blended again for 30 s at low speed. After 20 min at 8−12 °C, the mixture was blended at high speed for 1 min to shear the DNA; then 400 mL of TGED buffer (10 mM *T*ris-HCl, pH 8, 5% *g*lycerol, 0.1 mM *ED*TA, 0.015 mg/mL dithiothreitol) containing 0.2 M NaCl was added, and the mixture was blended again at high speed for 1 min. The cell extract was centrifuged at 4 °C for 1 h at 10000*g*, and the supernatant was collected, typically yielding 730−750 mL. The extract was again placed in a Waring blender, and 29 mL of 10% Polymin P [poly(ethylenimine)], pH 7.8, was gradually added with a constant slow stirring. After 5 min of slow stirring, the mixture was centrifuged for 15 min at 6000*g*, and the supernatant was discarded. The pellet was resuspended in 600 mL of TGED containing 0.5 M NaCl. After 8 min of slow stirring, the suspension was centrifuged again for 15 min at 6000*g*, and the supernatant was discarded. The washed pellet was then again scraped into the blender and resuspended in 500 mL of TGED containing 1 M NaCl with gentle stirring for 8 min. The mixture was centrifuged for 30 min at 10000*g*, and the supernatant was collected, yielding 460−480 mL of eluate. Solid ammonium sulfate (36−38 g/100 mL) was then added to the eluate, and the precipitated protein was left overnight. Approximately 30 mL of single-stranded DNA−agarose (Amersham Pharmacia) was packed in an XK-26 column (Amersham Pharmacia; the following purification steps were performed using the FPLC system) and preequilibrated with TGED buffer containing 0.2 M NaCl. The ammonium sulfate pellets from the previous step were dissolved in TGED buffer to a conductivity corresponding to that of TGED containing 0.2 M NaCl, and the resulting protein solution was loaded on the DNA−agarose column at a flow rate of 1.5−2 mL/min. The DNA−agarose column was washed with 300−400 mL of TGED containing 0.2 M NaCl, and the bound protein (predominantly RNAP, plus some DNA-binding proteins) was eluted with TGED containing 1 M NaCl; the protein peak (80−100 mL) was collected, and the protein was precipitated with ammonium sulfate (36−38 g/100 mL). After a brief centrifugation for 10 min at 6000*g*, the ammonium sulfate pellets were dissolved in TGED containing 0.1 M NaCl (the undissolved protein was removed by centrifugation for 15 min at 10000*g*) and loaded at 1 mL/min on a Mono Q HR 10/10 column (Amersham Pharmacia) preequilibrated with TGED containing 0.1 M NaCl. The column was washed with ∼100 mL of TGED containing 0.1 M NaCl, and the bound proteins were eluted with a shallow linear NaCl gradient, typically 0.1−0.5 M NaCl in 500 mL of TGED; 10 mL fractions were collected. Fractions containing the protein peaks identified from the A_{280} profile were then concentrated; each 10 mL fraction was concentrated individually to 0.5−0.6 mL using Centriprep-10 concentrators (Amicon). Aliquots of 0.15 mL from concentrated Mono Q fractions containing the peaks of interest were then subjected to gel filtration on either Superose 12 10/30 (S1−S2 and Hfq) or Superose 6 10/30 (core RNAP, RNAP holoenzyme, and RNAP−NusA) columns (Amersham Pharmacia) in TGED containing 0.2 M NaCl; the remaining portions of the concentrated Mono Q fractions were mixed with equal volumes of 100% glycerol and kept frozen at −70 °C. The 0.5 mL fractions from Superose columns containing the purified enzymes were concentrated using Microcon 10 concentrators (Amicon) to 20−40 μL, mixed with an equal volume of 100% glycerol, and stored at −20 °C. The core RNAP and RNAP holoenzyme obtained as a result of this purification procedure (Figure 1A) were homogeneous, as judged from silver-stained SDS−polyacrylamide gels. Gel filtration of the RNAP−NusA complex produced NusA of 90−95% purity, with the major contaminant being core RNAP. The specific ATPase activities detected in the Hfq samples, obtained as described above, were 5−20 pmol of ATP hydrolyzed min^{-1} (μg of protein)$^{-1}$.

The ribosomal protein S6, which was present in the S1−S2- and Hfq-containing Mono Q fractions (Figure 1C), was obtained as a byproduct during the final stage of Hfq purification. RapA was purified from the RNAP−RapA complex as previously described (*27*).

NTPase Assays. The NTPase assays were performed in 1× NBT (*N*TPase-*B*inding *T*ranscription) buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 2 mM $MgCl_2$ (unless indicated otherwise in the figure legends). The 15 μL NTPase reactions contained 1.5 μL of 10× NBT buffer, 1.5 μL of 1 mM NTP (either ATP or GTP; low specific NTPase activity) or 1.5 μL of 0.1 mM NTP (high specific activity), 0.1−0.3 μCi of [α-^{32}P]ATP or GTP, and 2−4 μL of the protein of interest (typically, 0.05−0.2 μg of purified Hfq). The reaction mixtures were incubated for 60 min at 37 °C, and the reaction products were separated on PEI−cellulose plates as previously described (*27*).

Glycerol Gradient Ultracentrifugation Binding Assays. Binding reactions containing 1−2 μL of each of the indicated proteins (the final concentrations are given in the Figure 2 legend), 2 μL of 10× NBT buffer, and purified water to a final volume of 20 μL were incubated for 15 min at room temperature and layered on top of a precast (using the FPLC system) 15−30% linear glycerol gradient in 600 μL of 1× NBT buffer in Ultra-Clear $^3/_{16} \times 1^5/_8$ in. centrifuge tubes (Beckman). The reactions were then spun for 24 h at 6 °C in an SW 55Ti rotor at 25000 rpm. Upon completion of the run, each tube was separated into 13 fractions of ∼50 μL each using the Beckman fraction recovery system; the fractions were then stored at −70 °C and analyzed by SDS−PAGE as described in the Results section.

Analysis of the Effects of S1 and Hfq on RNAP Transcriptional Activity. The 16 μL preincubation mix (in 1× NBT buffer supplemented with 0.1 mg/mL bovine serum albumin) contained ∼0.1 mg/mL of the supercoiled DNA template, 0.06 mg/mL RNAP holoenzyme, and Hfq or S1 (if present) at the concentrations indicated in the figure legends. Following a 15 min preincubation at 37 °C, transcription was initiated by the addition of 4 μL of 5× rNTP mix containing 1 mM each ATP, GTP, CTP, and UTP and 0.05−0.1 μCi of either [α-^{32}P]ATP or [α-^{32}P]GTP. After incubation for 60 min (unless indicated otherwise in the figure legends) at 37

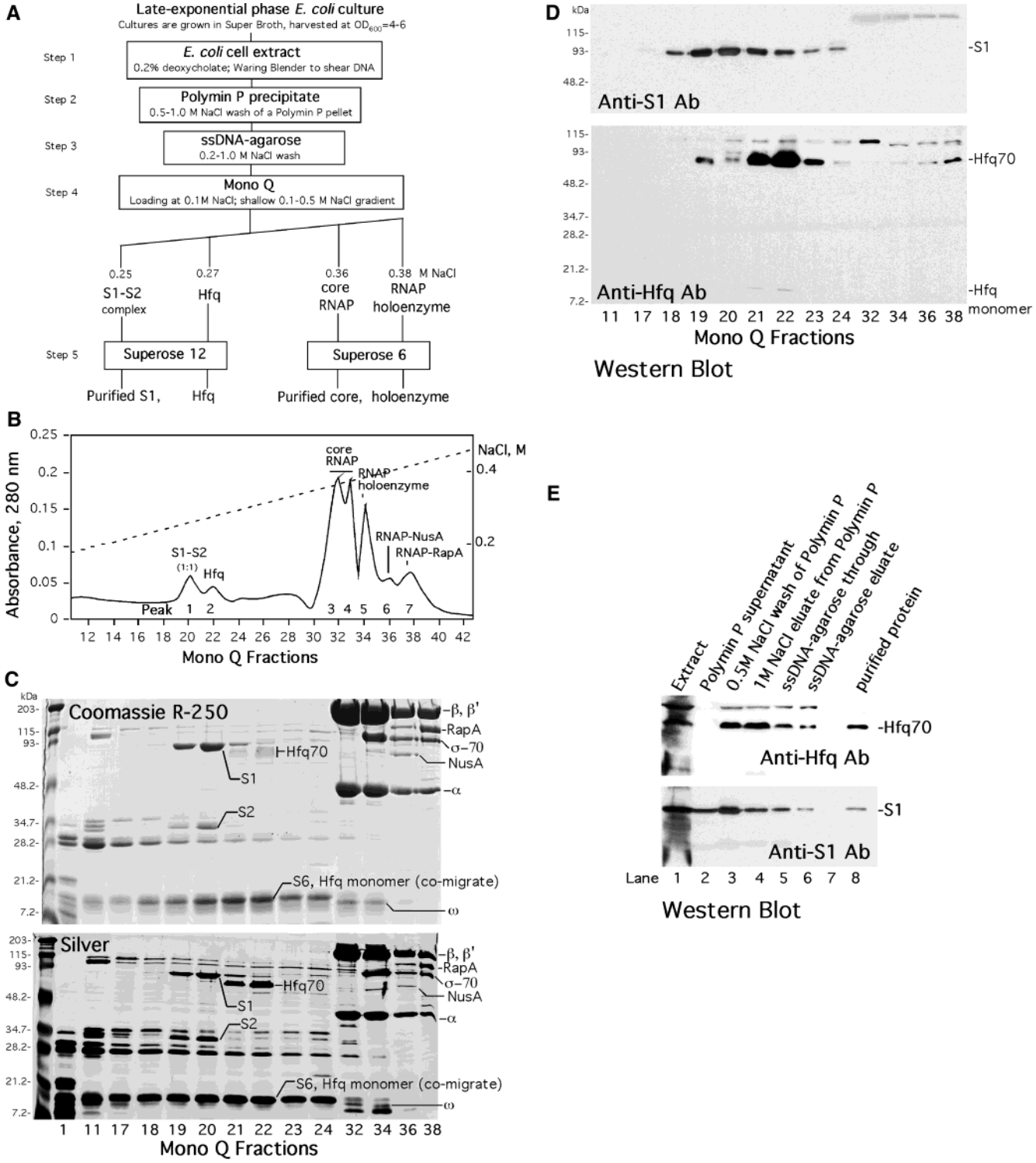


FIGURE 1: Purification of RNA polymerase from late exponential phase *E. coli* cultures yields Hfq and a 1:1 complex of the ribosomal proteins S1 and S2. (A) Schematic for the RNAP, Hfq, and S1 purification procedure. (B) Mono Q column protein profile. (C) Mono Q fractions stained with Coomassie Brilliant Blue R-250 (top; 12.5 μL from each fraction was mixed with an equal volume of 2× Laemmli sample buffer and loaded per lane of 10% SDS−PAG) or silver (bottom; 2.5 μL aliquots from the indicated Mono Q fractions were analyzed). Bio-Rad prestained protein standards (broad range) were loaded in the first lane of each gel. The S1, S2, S6, and Hfq proteins were identified on the basis of the following N-terminal protein sequences: MTESFAQLFE (S1), XTVSMRDMLK (S2), MRHYEIVFM (S6), and AKGQSLQDPFL (Hfq). The identities of Hfq, S6, NusA, and RapA were confirmed using protein-specific polyclonal antibodies. (D) Immunoblots of the Mono Q fractions with S1-specific (top panel) and Hfq-specific (bottom panel) polyclonal antibodies. (E) Copurification of the ribosomal protein S1 and Hfq with RNAP throughout the initial stages of the RNAP purification procedure. Aliquots representing equal fractions of the total volume of the extracts at various stages of the RNAP purification procedure (1 μL was taken per each 100 mL of the indicated fraction) were analyzed on 10% SDS−polyacrylamide gels. Electrophoresis was followed by immunoblotting with Hfq-specific (top) or S1-specific polyclonal antibodies (bottom). The positions of Hfq and S1 are indicated. Quantitation of the fraction of Hfq present in the single-stranded DNA−agarose eluate (lane 6) vs the total amount of the protein (lanes 3 and 4) gave a value of 5−9%; there was a significant, >50% loss of Hfq during the single-stranded DNA−agarose column step. Quantitation of the fraction of S1 present in the single-stranded DNA−agarose eluate (lane 6) vs the total amount of the protein (lanes 2−4) gave a value of 1−2%.

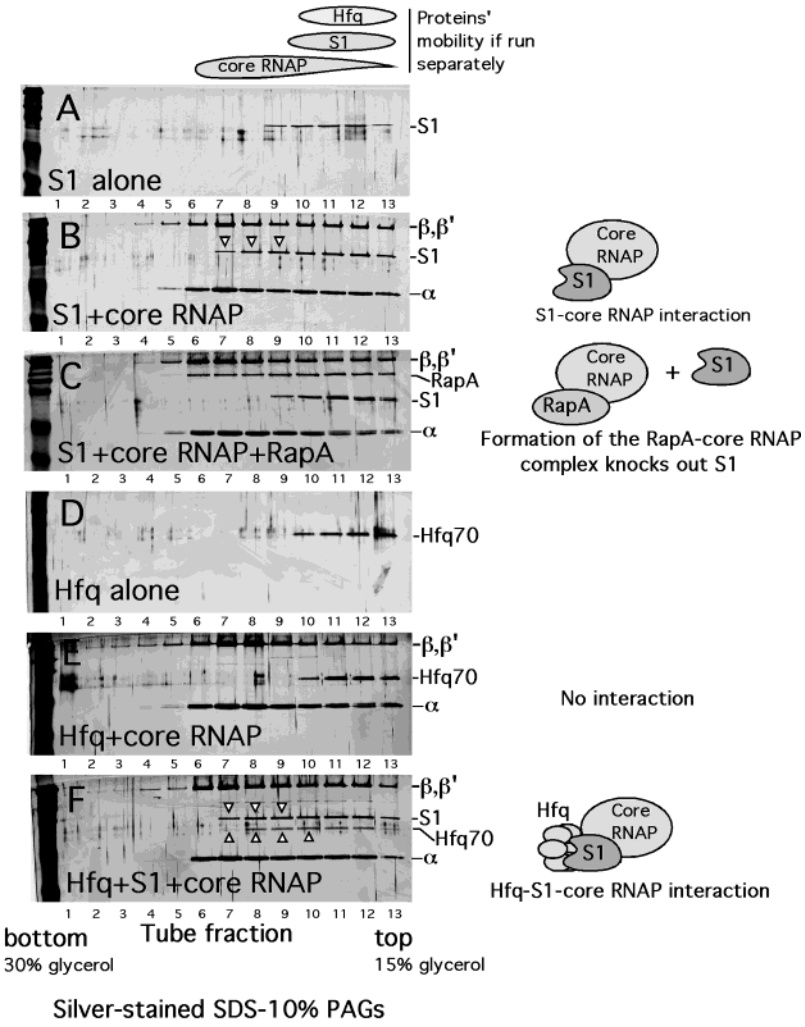


FIGURE 2: Interaction of S1 and Hfq with RNA polymerase studied by glycerol gradient centrifugation of mixtures of the purified proteins. The binding assays were performed as described in Materials and Methods. The figure shows the results of PAGE analysis of the fractions from glycerol gradients. Schematics illustrating the protein−protein interactions are shown at the right. Arrows indicate shifts in the protein peak mobility. Each binding experiment was repeated at least twice with similar results; representative gels are shown. Panels: (A) 2 μM S1; (B) 2 μM S1 plus 1 μM core RNAP; (C) 2 μM S1 plus 1 μM core RNAP plus 2 μM RapA; (D) 2 μM Hfq hexamer; (E) 2 μM Hfq hexamer plus 1 μM core RNAP; (F) 2 μM Hfq hexamer plus 2 μM S1 plus 1 μM core RNAP.

°C, 4 units (4 μL) of RNase-free RQ DNase (Promega) was added to each transcription reaction; the reaction mixtures were incubated for 15 min at room temperature, followed by the addition of 4 μL of 5 mg/mL proteinase K and incubation for another 15 min at room temperature. Ten microliters of loading solution (0.25 M EDTA, 50% glycerol, 0.1% Bromphenol Blue) was then added to each reaction mixture, followed by the addition of 40 μL of formamide, heating at 90 °C for 2 min, and a 3−5 min incubation on ice. Aliquots of 3−5-μL were then analyzed on 8% sequencing gels, or alternatively, 2−3 μL aliquots were spotted on PEI−cellulose plates, which were developed as described above. The sequencing gels (or PEI−cellulose plates) were autoradiographed using BioMax MR2 film and scanned on a phosphorimager (Molecular Dynamics) to quantitate RNA transcript levels or the amounts of NTP hydrolyzed.

For quantitation of the total transcript RNA in the presence and absence of Hfq, aliquots of the transcription reactions were spotted on PEI−cellulose plates, the plates were developed in 1 M LiCl−1 M formic acid, and the labeled RNA that remained at the origin of TLC plate, as seen in Figure 3G, was quantitated as described above. Alternatively, the amount of transcript RNA was estimated via quantitation of entire lanes in 8% sequencing gels loaded with in vitro transcription reactions carried out as described above; the two methods produced similar figures.

Coupled in Vitro Transcription−Translation. Coupled transcription−translation experiments were done using the *E. coli* S30 extract system for circular DNA (Promega). pBEST*luc* supercoiled DNA template was used at a final concentration of 0.1 mg/mL; reactions contained 4 μL of S30 extract/16 μL reaction. The other components, a complete amino acid mixture and S30 premix without amino acids, were added according to the manufacturer's protocol. The Hfq-depleted S30 extract was prepared by mixing 0.5 mL of S30 extract with 0.25 mL of a 1:1 suspension of poly(A)−Sepharose 4B (Amersham Pharmacia) in 10 mM Tris-HCl, pH 7.5, followed by rotation at 4 °C for 30 min. After centrifugation for 10 min at 13000*g*, the supernatant was carefully removed, flash-frozen on dry ice in 50 μL aliquots, and stored at −70 °C.

The S30 extracts from the MC4100 and MC4100*hfq*$^-$ bacterial strains (*21*) were prepared as follows. The overnight bacterial cultures in LB broth were diluted 8−10-fold with

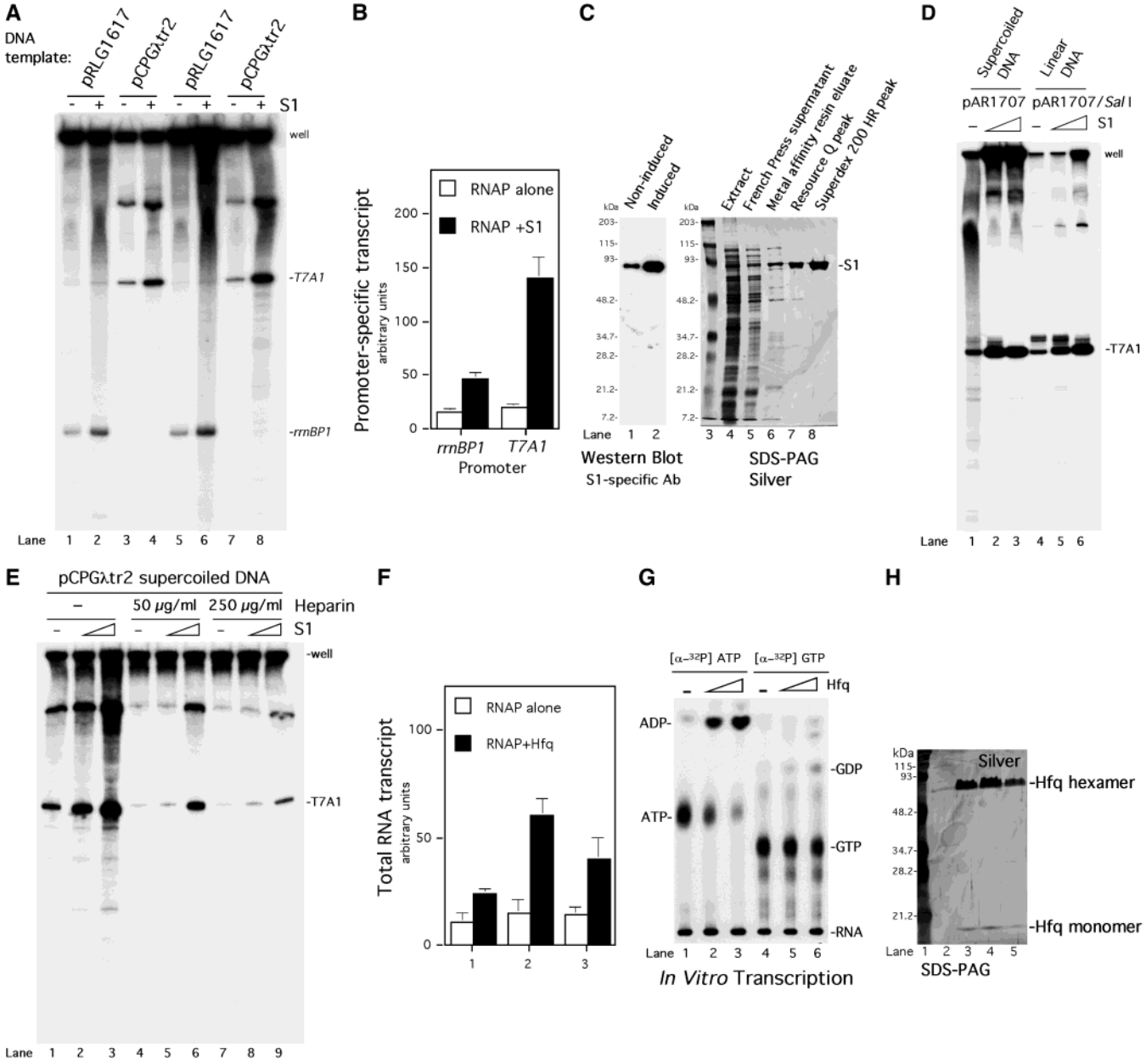


FIGURE 3: Effects of S1 and Hfq on the RNAP transcriptional activity. (A) Stimulation of RNAP transcriptional activity by purified endogenous S1. Reactions in the absence (lanes 1, 3, 5, 7) and in the presence (lanes 2, 4, 6, 8) of 0.2 μM purified endogenous S1. In vitro 15 min (lanes 1−4) and 120 min (lanes 5−8) transcription reactions were performed as described in Materials and Methods. The supercoiled plasmid DNA templates used in the corresponding transcription reactions are indicated. (B) Quantitated levels of the promoter-specific transcripts in the absence (open columns) or presence (black columns) of 0.2 μM purified endogenous S1. Data represent the average of two independent experiments. (C) Overexpression and purification of recombinant S1. 10 μL from each of the indicated fractions corresponding to various stages of the S1 purification procedure was analyzed by SDS−PAGE, followed by staining of the gel with silver (lanes 3−8). The detailed purification procedure is described in Materials and Methods. Samples from induced and noninduced bacterial cultures transformed with pQE31S1 were analyzed by immunoblotting with S1-specific antibodies (lanes 1 and 2). (D) Stimulation of RNAP transcriptional activity by recombinant S1. In vitro transcription assays with supercoiled pAR1707 plasmid DNA (*44*; lanes 1−3) and linear pAR1707/*Sal*I DNA templates (lanes 4−6) were as described in Materials and Methods except that DNase and proteinase K treatments were omitted. The S1 concentrations were 0.22 μM (lanes 2 and 5) and 1.1 μM (lanes 3 and 6). (E) Stimulation of RNAP transcriptional activity by recombinant S1 in the absence and in the presence of the DNA competitor heparin. Supercoiled pCPG*λtr2* DNA template (*44*). The conditions were as described in panel D. The heparin concentrations are indicated. (F) Moderate stimulation of RNAP transcriptional activity by high concentrations of Hfq. The quantitated levels of total RNA transcripts in the absence (open columns) or in the presence (black columns) of ~1 μM purified endogenous Hfq (hexamer) are shown. Data represent the average of two independent experiments. The supercoiled plasmid DNA templates were (1) pλPR (see Materials and Methods), (2) pCPG*λtr2*, and (3) pCPG*trpA*+ (*44*). (G) ATP hydrolysis resulting from the addition of Hfq to in vitro transcription reactions. Reactions were performed in the absence (lanes 1 and 4) or in the presence of 0.17 μM (lanes 2 and 5) or 0. 67 μM (lanes 3 and 6) Hfq hexamer with the supercoiled DNA template pRLG1617 (*28*). (H) Overexposed silver-stained gel illustrating virtual homogeneity of the Hfq protein used in the in vitro transcription reactions above. Lane 1, Bio-Rad prestained protein standards; lanes 2−5, 10 μL from Superose 12 (the final stage of the endogenous Hfq purification procedure) fractions 23−26, respectively. The protein bands labeled Hfq hexamer and Hfq monomer from the Hfq preparation illustrated here produced the same N-terminal sequence, AKGQSLQDPFL, of Hfq.

fresh LB broth and allowed to grow for 60 min at 37 °C. Bacterial pellets, obtained by centrifugation of the cultures for 5 min at 4000*g*, were washed once with 20 mM Tris-HCl, pH 8, 14 mM magnesium acetate, 200 mM KCl, 1 mM DTT, and 0.05 mg/mL PMSF (buffer A) and resuspended in 10 volumes of buffer A supplemented with 0.2 mg/mL lysozyme. Following a 30 min incubation at 4 °C, bacterial pellets were collected by centrifugation for 5 min at 4000*g*, resuspended in 4 volumes of buffer A, and broken in a French press. The extracts were then spun for 30 min at 30000*g*. The supernatants from this centrifugation were removed, avoiding any pellet, aliquoted, and stored frozen at −70 °C. The 4 μL aliquots of the S30 extracts were used in coupled transcription−translation reactions; the reactions were carried out as described above.

Cloning, Overexpression, and Purification of His-Tagged Hfq. Hfq was cloned into the expression vector pQE31 (Qiagen) as follows. A DNA fragment containing the *hfq* gene was amplified (from MG1655 chromosomal DNA) by polymerase chain reaction using the primers MS79 (5′-CAACGCAAGGATCCAGCTAAGGGGCAATCTTTAC-AAGATCCGTTCCTG) and MS80 (5′-GCACCAAGCA-AGCTTTTATTCGGTTTCTTCGCTGTCCTGTTGCGC). The amplified DNA fragment was purified, digested with the restriction enzymes *Bam*HI and *Hin*dIII, and ligated with pQE31 vector DNA which had been digested with *Bam*HI and *Hin*dIII. The resulting plasmid pQE31Hfq was confirmed by DNA sequencing of the entire *hfq* gene. M15 (pRep4) cells, transformed with the plasmid pQE31Hfq, were grown overnight at 37 °C in 50 mL of LB medium supplemented with 100 μg/mL ampicillin and 50 μg/mL kanamycin. In the morning, 450 mL of LB supplemented with 100 μg/mL ampicillin was added to the culture. At an OD_{600} of 0.6−0.8, the expression of the protein was induced with 1 mM IPTG, and after 4 h of shaking at 37 °C, the cells were collected by centrifugation for 10 min at 5000*g*. About 1 mL of the cell pellet was resuspended in 30 mL of extraction buffer (1× PBS, 0.5 M NaCl, pH 7.2) and lysed in a French press. Fifty microliters of ribonuclease A (2 units/μL) was then added to the suspension, and after a 1 h incubation at room temperature, the extract was spun for 30 min at 20000*g*. Talon metal affinity resin (2−3 mL) (Clontech) was added to the clear supernatant, and the suspension was rotated for 30 min at 4 °C. The suspension was then placed in a column and extensively washed with 400−500 mL of extraction buffer. Following an additional wash with 50 mL of extraction buffer containing 1 mM imidazole, the bound protein was eluted with 15 mL of extraction buffer containing 200 mM imidazole. Purified His-tagged Hfq was concentrated to 0.5−0.6 mL using Centriprep 10 concentrators (Amicon), and 150 μL aliquots of the concentrated protein were subjected to an additional purification step using a Superose 12 column to separate RNA-bound complexes containing >1 Hfq hexamer. Superose 12 chromatography and storage of the purified protein were carried out as described above in the purification procedure for native Hfq. The identity of the purified His-tagged protein was confirmed using Hfq-specific polyclonal antibodies.

Cloning, Overexpression, and Purification of His-Tagged S1. S1 was cloned into the *Bam*HI and *Sph*I restriction sites of the expression vector pQE31 (Qiagen). A DNA fragment containing the *rpsA* gene was amplified from MG1655 chromosomal DNA by polymerase chain reaction using the primers MS73 (5′-CAACGCAAGGATCCAACTGAA-TCTTTTGCTCAACTCTTTGAAGAGTCC) and MS74 (5′-GCACCAAGCGCATGCAGAGAATTACTCGCCTTTAGC-TGCTTTGAAAGC). The N-terminal part of the S1 gene, the sequence located upstream from the intrinsic *Hin*dIII site, including the junction region, in the resulting plasmid pQE31S1 was confirmed by DNA sequencing. The expressed protein product was also confirmed using S1-specific antibodies (Figure 3C). Initial stages of purification of the recombinant S1, including the bacterial culture growth and induction, extraction of the protein, and the metal affinity resin chromatography, were performed in a manner similar to that described above for the purification of His-tagged Hfq. The protein fractions eluted from the metal affinity resin were dialyzed overnight against 500 mL of TGED buffer and loaded on a 1 mL Resource Q column (Amersham Pharmacia), preequilibrated with TGED buffer. The column was operated at a flow rate of 1 mL/min. The column was washed with 10 mL of TGED buffer, and the bound protein was then eluted with a linear gradient of NaCl in TGED buffer (typically 0−0.4 M NaCl in 40 mL of TGED buffer). The S1 peak eluted at approximately 0.2 M NaCl. The 2 mL Resource Q column fractions containing S1 (identified by Western blot using S1-specific antibodies) were then concentrated to 50−100 μL using Centricon 10 concentrators (Amicon) and subjected to an additional purification step: gel filtration on a Superdex 200 HR 10/30 column (Amersham Pharmacia). The column was preequilibrated with TGED containing 0.1 M NaCl and operated at a flow rate of 0.5 mL/min; 0.5 mL fractions were collected. The S1 protein thus purified was virtually homogeneous, judging from silver-stained protein gels (Figure 3C). The column fractions containing purified S1 were concentrated and stored as described above in the purification procedure for native Hfq.

Site-Directed Mutagenesis. Hfq mutagenesis was performed using the Quick Change site-directed mutagenesis kit (Stratagene) in accordance with the manufacturer's instructions using the wild-type *hfq* clone in pQE31Hfq as a DNA template. To construct the *hfq* Q35K mutation, the mutagenic primers MS105 (5′-GGTATTAAGCTGCAAGG-GAAAATCGAGTCTTTTGATCAG) and MS106 (antiparallel to MS105) were used. To construct the *hfq* K56W mutant, the primers MS103 (5′-CAGATGGTTTACTG-GCACGCGATTTCT) and MS104 (antiparallel to MS103) were used. In both cases, the entire *hfq* sequence containing the desired mutation was confirmed by DNA sequencing. The mutant proteins were overexpressed in M15(pRep4) cells (Qiagen) and purified as described above for His-tagged wild-type Hfq.

Plasmid Constructs. The plasmid pλPR containing the λPR promoter, which replaced the *rrnBP1* promoter in pRLG1617 (*28*), was constructed by digesting the annealed primers MS51(5′-CTGATAAGCTTGAATTCGATATCTAACACCG-TGCGTGTTGACTATTTTACCTCTGGCGGTGATAAT-GGTTGCATGATATCAAGCTTCCCGGGACTGAG) and MS52 (antiparallel to MS51) and the vector pRLG1617 with *Eco*RI and *Hin*dIII, followed by ligation of the resulting DNA fragment into pRLG1617. The final plasmid constructs containing the λPR promoter were verified by DNA sequencing.

Other Biochemical Techniques. Protein concentrations were determined using the method of Bradford (*29*), with bovine serum albumin as a standard. Western blots were done using Fastblot semidry electroblotter (Biometra) and Immobilon-P membranes (Millipore). The protein bands were visualized using goat anti-rabbit peroxidase-conjugated secondary antibodies (Calbiochem) and ECL Western blotting detection reagents (Amersham Pharmacia). Amounts of proteins on immunoblots were estimated using Eagle Eye software (Stratagene) after the integrated density of individual protein bands were determined on X-ray films; serial dilutions of the individual purified proteins were used to establish exposures that produced acceptable errors. Amino acid sequencing was performed at the Columbia University Protein Chemistry Core Facility.

Antibodies. Hfq-specific antibodies were a gift from Aixia Zhang and Gisela Storz, NIH; NusA-specific antibodies were a gift from David Friedman. Antiserum against S1 was raised in a rabbit injected with ~0.2 mg of the purified S1 protein by Spring Valley Laboratories, Inc.

RESULTS

The RNA Polymerase Purification Procedure Yields Stoichiometric Amounts of the Ribosomal Proteins S1 and S2 and the Nucleic Acid-Binding Protein Hfq. A number of studies that utilized *E. coli* RNAP purification procedures based on the protocol developed by Hager et al. (*30*) have indicated copurification with RNA polymerase accessory proteins; notable among these are the 110 kDa RNAP-associated protein RapA, a homologue of the SWI/SNF protein superfamily (*7*, *27*), and an unidentified ~70 kDa ATPase (*31*). Using a procedure similar to the protocol developed by Hager et al. (Figure 1A) for purification of RNA polymerase from *E. coli* late exponential phase cell cultures, we noted the consistent appearance of an ~70 kDa protein that eluted in the Mono Q fractions preceding the core RNAP and the RNAP holoenzyme. However, detailed resolution of the Mono Q fractions by a shallow NaCl gradient revealed two closely migrating protein peaks (Figure 1B): the first peak (peak 1 in Figure 1B) harbored stoichiometric amounts of the ribosomal proteins S1 and S2 (Figure 1B,C), as determined from quantitated Coomassie Blue-stained gels and N-terminal protein sequencing, respectively. The second peak's ~70 kDa protein (which stained well with silver but appeared as a broad, pale blue band on Coomassie-stained gels; Figure 1C) produced an N-terminal sequence identical to that of the 11.2 kDa hexameric protein Hfq (Figure 1C). We then confirmed the results of the N-terminal protein sequencing using Hfq-specific polyclonal antibodies (Figure 1D).

Immunoblotting of fractions from the RNAP purification with Hfq- and S1-specific antibodies indicated that the fraction of Hfq and S1 that copurified with RNA polymerase throughout the step of chromatography on a single-stranded DNA column represented respectively 5−9% and 1−2% of the total amount of the protein in the cell (Figure 1E).

Interaction of S1 and Hfq with RNA Polymerase. Because the initial stages of the RNAP purification procedure involved nonspecific fractionation and chromatography steps, we asked if Hfq and S1 were capable of specific interaction with RNAP. For this purpose the purified proteins were subjected to binding assays using ultracentrifugation of the protein mixtures in glycerol gradients. In the presence of high concentrations of glycerol, in 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and 2 mM $MgCl_2$ (thus, under relatively stringent conditions that minimize hydrophobic interactions), purified Hfq and S1 ran respectively as hexamer and monomer with similar molecular masses of 70−90 kDa. The binding assays revealed interaction of S1 with the RNA polymerase core enzyme (Figure 2B; note the shift in the S1 profile and the fractions of S1 comigrating with the core RNAP peak). No complex formation between either S2 or S6 and RNA polymerase was detected (data not shown). Interestingly, the addition of the RNAP-associated homologue of SWI/SNF, RapA (*7*, *27*, *32*), completely abolished the RNAP−S1 interaction (Figure 2C); the effect was distinctly RapA-specific, since the RNAP−S1 complex stability was not altered significantly when RapA was replaced by another RNAP-binding protein, NusA, or when the core RNAP was replaced by the RNAP holoenzyme. Although purified Hfq showed little or no affinity to the core RNAP under these conditions (Figure 2D,E), it associated with the S1−core RNAP complex, interacting with S1 in an approximately 1:1 (1 Hfq hexamer/mol of S1) molar ratio (Figure 2F).

Effects of S1 and Hfq on the RNAP Transcriptional Activity. Addition of purified native S1 to in vitro transcription reactions resulted in a significant increase in the levels of promoter-specific transcripts (Figure 3A,B); the effect was observed with various DNA templates. To confirm that the transcription−stimulatory effect was due to S1 rather than an S1-associated protein impurity, we overexpressed His-tagged S1 (Figure 3C and Materials and Methods) and obtained virtually homogeneous S1 (Figure 3C, silver-stained gel) using a purification procedure dissimilar to that used for endogenous S1 (Figure 1A). The recombinant S1 thus obtained showed a transcription−stimulatory effect similar to that seen with endogenous S1 (Figure 3D,E); therefore, we conclude that the transcription−stimulatory effect is S1-specific. The S1-mediated stimulation of transcription was detected with linear as well as supercoiled DNA templates (Figure 3D); the effect was also evident during single-round transcription reactions (Figure 3E, lanes 4−9), a property dissimilar to the previously described transcription−stimulatory effect of RapA (*7*).

The addition of excess Hfq to in vitro transcription reactions showed a mild stimulatory effect on the overall yield of RNA transcripts (Figure 3F); however, the formation of virtually nondissociable Hfq−transcript RNA complexes hindered the analysis of the levels of promoter-specific transcripts with most of the DNA templates we used (data not shown). The addition of highly pure Hfq (Figure 3H) to the in vitro transcription reactions was accompanied by ATP hydrolysis; ATP was hydrolyzed with about 7-fold higher efficiency than GTP (Figure 3G).

Effects of Hfq and S1 on the Coupled Transcription−Translation Reaction. Whereas deletion of the S1 gene (*rpsA*) is lethal for the bacterial cell due, almost certainly, to the critical role of S1 in translation initiation, viable Hfq deletion mutants have been reported (*24*, *25*). To study the effects of Hfq and S1 on the coupled transcription−translation reaction, we first prepared Hfq-depleted S-30 extracts. The poly(A)−Sepharose treatment (the rationale for the chosen strategy is

outlined in the Discussion section) removed >80% of Hfq from the extract (Figure 4A) and resulted in a significant loss of its ability to efficiently catalyze multiple rounds of translation (Figure 4B; compare the 24−96 min time points). However, the data indicate a rapid synthesis of the initial copies of luciferase in the poly(A)−Sepharose-treated extract, as evidenced by comparable levels of luciferase generated in the poly(A)−Sepharose-treated and nontreated extracts at early time points (Figure 4B). The addition of excess RNAP to the coupled reaction (which resulted in a >10-fold increase in stationary levels of transcript RNA; data not shown) produced only a marginal, <2-fold effect on the translation of luciferase (Figure 4B), indicating that translation rather than transcription is a rate-limiting step under the chosen conditions. The addition of purified Hfq to the depleted system produced a moderate, 2−3-fold stimulatory effect; addition of excess S1 had an inhibitory effect (Figure 4D). Note that the level of intrinsic S1 remained unaltered by poly(A)−Sepharose treatment (Figure 4A). Again, addition of Hfq to extracts harboring excess S1 produced a mild stimulatory effect, thus rescuing the extract from a near shutdown in luciferase production caused by excess S1 (Figure 4D).

To explore an alternative strategy, we prepared S30 extracts from *hfq*$^+$ and *hfq*$^-$ bacterial strains (see Materials and Methods) and monitored the kinetics of luciferase translation in the two strains. Consistent with the previous set of experimental data, the S30 extract obtained from the *hfq*$^+$ strain yielded overall higher levels of *luc*-specific translation products than the S30 extract from the *hfq* deletion mutant (Figure 4E); however, the *hfq* deletion mutant showed a more efficient synthesis of the full-length product at early time points (Figure 4E, Western blot).

Hfq Is an ATPase. The ATPase activity detected in the in vitro transcription reactions in the presence of Hfq prompted us to ask if Hfq is an ATPase. This finding was particularly surprising, taking into account the small, 11.2 kDa size of the protein and seeming lack of homology to enzymes incorporating known ATPase motifs. The association of an ATPase activity with a component of the RNAP−S1−Hfq complex also could shed light on the functional significance of this protein−protein interaction in vivo.

First, we asked if the ATPase activity comigrates with Hfq in chromatography during the final stage of the Hfq purification procedure (Figure 1A; see also Materials and Methods). As seen in Figure 5A, during gel filtration on a Superose 12 column, the maximum ATPase activity was in fraction 24, thus comigrating with the peak of Hfq. (Both peaks comigrated with the 68 kDa molecular mass standard for gel filtration, bovine serum albumin.)

We then used a bacterial strain containing an *hfq* deletion mutation (*21*) and performed two parallel purifications of RNAP from *hfq*$^+$ and *hfq*$^-$ bacterial strains, comparing the ATPase activities in the Mono Q (step 4 in Figure 1A) fractions. While the Mono Q fractions obtained from the *hfq*$^+$ cells showed a characteristic peak of ATPase activity that comigrated with the Hfq-containing fractions, the *hfq* deletion mutant's Mono Q fractions lacked the corresponding ATPase activity peak (Figure 5B).

To explore the possibility that the ATPase activity in the Hfq preparations was due to a contaminating protein, we then cloned and overexpressed His-tagged Hfq in the pQE31 expression vector. Overall, tagged protein showed properties similar to those of the purified endogenous Hfq, such as stability of the hexameric ring under strong denaturing conditions (Figure 5C, SDS gel) and high-affinity RNA binding. The purified recombinant Hfq, which was obtained using a purification procedure dissimilar to the one described above for the native protein (see Materials and Methods), showed ATPase activity; the purified protein vs ATPase activity profiles showed a perfect alignment during chromatography on a Superose 12 column (Figure 5C).

Furthermore, we also obtained Hfq using another independent purification procedure described by Zhang et al. (*21*), which includes 80 °C heat treatment of the bacterial lysate and denaturation−renaturation of the protein after nearly irreversible adsorption on poly(A)−agarose. Hfq purified by this method also showed ATPase activity (data not shown).

Although the Hfq sequence does not show classic Walker A/B ATPase motifs, close examination reveals a slightly modified Walker A box [Figure 5D; the alignment shows homology between Hfq and the part of the heat-shock protein ClpB that has been identified as the ATP-binding site (*33*)]. To demonstrate further that the ATPase activity is an intrinsic property of Hfq, rather than an Hfq-associated unidentified protein impurity, we also constructed site-specific Hfq mutants, mutating Q35, a residue highly conserved among Hfq proteins from other organisms, to lysine (to achieve a consensus with the Walker A box) and K56 (a highly conserved lysine in Sm-like proteins) to tryptophan. The kinetic parameters of the purified Q35 mutant were (perhaps, predictably) similar to those of the purified wild-type Hfq; however, the K56 mutation increased K_m for ATP by 3−4-fold and, interestingly, altered the $k_{cat,Mn}/k_{cat,Mg}$ ratio (Figure 5E). In addition, we found that purified S1 had a moderate (2−3-fold) stimulatory effect on the Hfq ATPase (Figure 6B,C).

DISCUSSION

Hfq: the "RNAP-Associated ATPase". The ∼70 kDa RNAP-associated ATPase has been previously noted in studies that have employed a similar procedure for the purification of RNAP (*31*, *34*). The incredible stability of the Hfq hexamer resulted in its appearance as a broad ∼70 kDa protein band in SDS−polyacrylamide gels (Figures 1C,D and 3H); purified to apparent homogeneity, Hfq showed an ATPase activity. Similarities such as (a) the molecular mass of the protein and the position of the protein peak in the Mono Q gradient in similar purification procedures and (b) the comparable efficiencies of ATP/GTP hydrolysis suggest that the protein in question is Hfq. On the other hand, the poor staining of Hfq with Coomassie Blue, taken together with the copurification and near comigration of Hfq with S1 in the Mono Q gradient, does not rule out the possibility that the ∼70 kDa band in the gels referred to previously (see references above) actually belongs to S1, while the ATPase activity is that of Hfq.

Cellular Levels of Hfq and S1. The fact that stoichiometric amounts of S1, S2, and Hfq copurified with RNAP throughout the single-stranded DNA chromatography step, the purification step that typically yields RNAP of 80−90% purity, prompted us to investigate further their binding to RNAP, ATPase activities, and effects on in vitro transcription

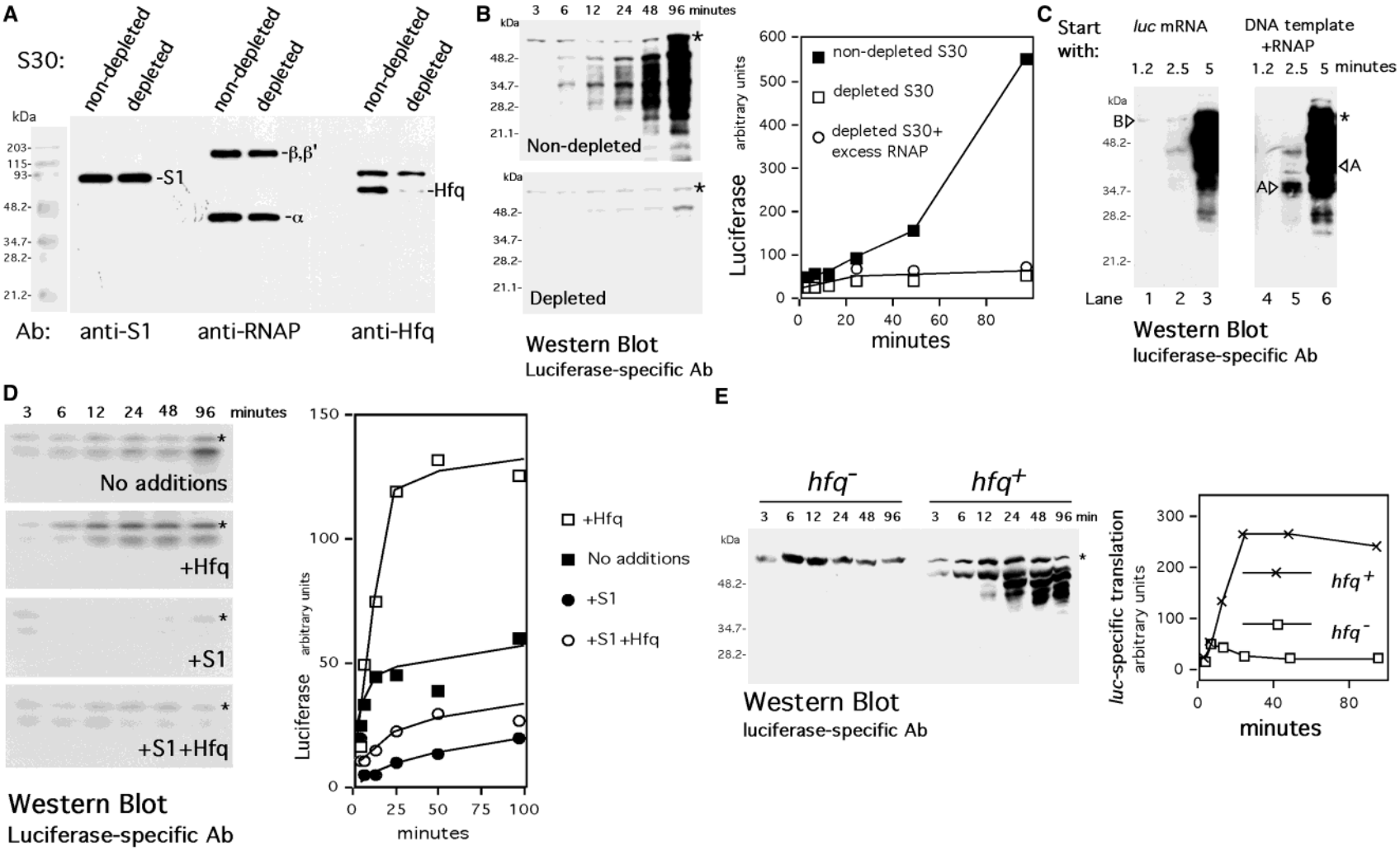
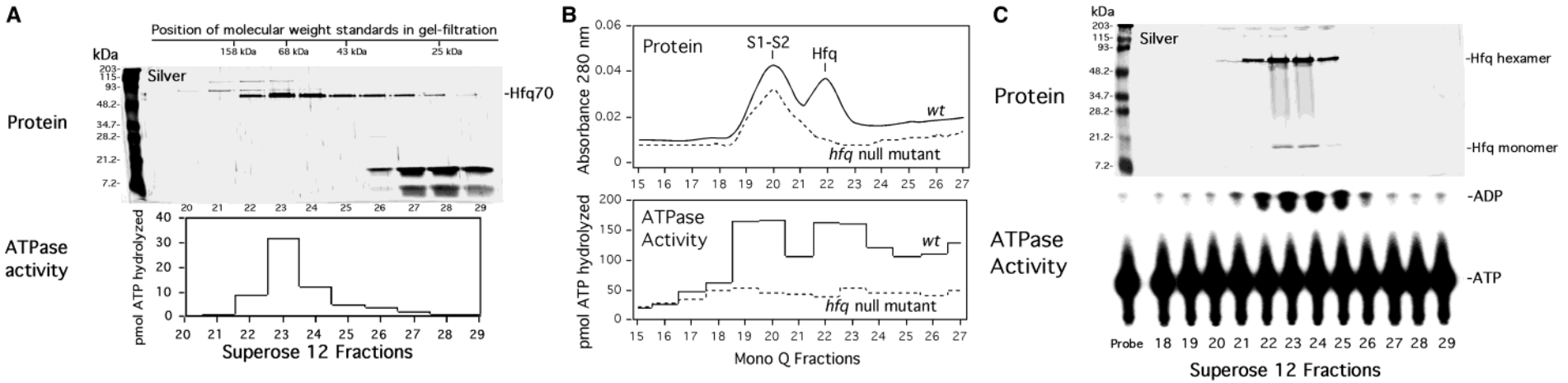


FIGURE 4: Effects of S1 and Hfq on the coupled transcription−translation reaction. Coupled transcription−translation reactions were performed as described in Materials and Methods. (A) Western blots showing the S1, RNAP core enzyme, and Hfq levels in the S30 extract before and after poly(A)−Sepharose treatment. 10 μL of the indicated S30 extract (diluted 1/40 with Laemmli sample buffer) was analyzed by immunoblotting using the S1-, core RNAP-, or Hfq-specific polyclonal antibodies as described in Materials and Methods. (B) Synthesis of luciferase in the S-30 extract. The position of the full-length reaction product is indicated. A graph illustrating the kinetics of luciferase production in the nondepleted (solid symbols) and the poly(A)−Sepharose-treated S-30 extracts (open symbols) is shown at the right. Where indicated, purified RNAP holoenzyme was added to the reactions to a final concentration of ~0.12 mg/mL. (C) Kinetics of *luc* translation in the coupled transcription−translation system (lanes 1−3) vs the translation reaction alone initiated by purified *luc* mRNA (lanes 4−6). *Luc* mRNA was obtained after in vitro transcription (using purified *E. coli* RNAP holoenzyme and pBEST*luc* supercoiled DNA template), nuclease S1 digestion of DNA, and phenol extraction and ethanol precipitation of the resulting RNA transcript. Purified *luc* mRNA was used in reactions at a concentration of ~0.2 mg/mL. S30 extract (Promega) and other reaction ingredients were as described in Materials and Methods. (D) Western blots showing the effects of S1 and Hfq on coupled transcription−translation in the Hfq-depleted S30 extract. When indicated, Hfq was added to a final concentration of 0.7 μM (hexamer) and S1 to 0.12 μM. The quantitated results of the experiment are shown at the right. (E) Western blot illustrating the kinetics of luciferase synthesis in S30 extracts derived from MC4100 and MC4100*hfq*⁻ (*21*) bacterial strains. The preparation of S30 extracts and coupled transcription−translation reactions were as described in Materials and Methods. The quantitated overall levels of *luc*-specific translation products at different time points are shown at the right.



Enzyme	$K_{m,\ ATP}$ (mM)	$V_{max,\ ATP}$ (pmol ATP hydrolyzed per µg Hfq/min)	$k_{cat\ Mn^{++}}/k_{cat\ Mg^{++}}$
Hfq_{wt}	0.28	5.7	3.2
Hfq_{Q35K}	0.3	5.6	3.0
Hfq_{K56W}	0.9	6.0	1.1

FIGURE 5: Hfq harbors an intrinsic ATPase activity. (A) Comigration of the peak of ATPase activity in chromatography on a Superose 12 column with the peak of native Hfq. 4 μL from each Superose 12 fraction was assayed for ATPase activity as described in Materials and Methods for high specific activity ATPase reactions. The silver-stained protein gel (16 μL from each Superose 12 fraction/lane) is shown at the top. (B) Absence of the ATPase activity peak in Mono Q fractions derived from an *hfq* null mutant. Protein purification from the wild-type (MG1655) cells and the *hfq* null mutant [MC4100*hfq::Cm*^{r} (*21*)] bacterial strain was carried out as described in Materials and Methods. 2 μL aliquots from Mono Q fractions were assayed for ATPase activity as described in Materials and Methods for low specific activity ATPase reactions. (C) Comigration of the peak of ATPase activity in chromatography with the peak of purified His-tagged Hfq. The ATPase reactions were performed as described in panel A. His-tagged Hfq was purified as described in Materials and Methods. (D) Homology between Hfq and the part of the heat-shock protein ClpB that has been identified as the ATP-binding site. (E) Alteration of the properties of the Hfq ATPase by site-specific mutagenesis. The kinetic parameters of the wild-type and mutant Hfq proteins were measured in the presence of either 2 mM $MgCl_2$ ($k_{cat,Mg}$) or 2 mM $MnCl_2$ ($k_{cat,Mn}$, K_m, V_{max}). The ATPase reactions were carried out in the presence of 0.02, 0.04, 0.08, 016, 0.32, 0.64, and 1.28 mM ATP, and the kinetic parameters were extracted from initial velocity vs ATP concentration isotherms. The $k_{cat,Mn}/k_{cat,Mg}$ ratios represent the average of three independent experiments.

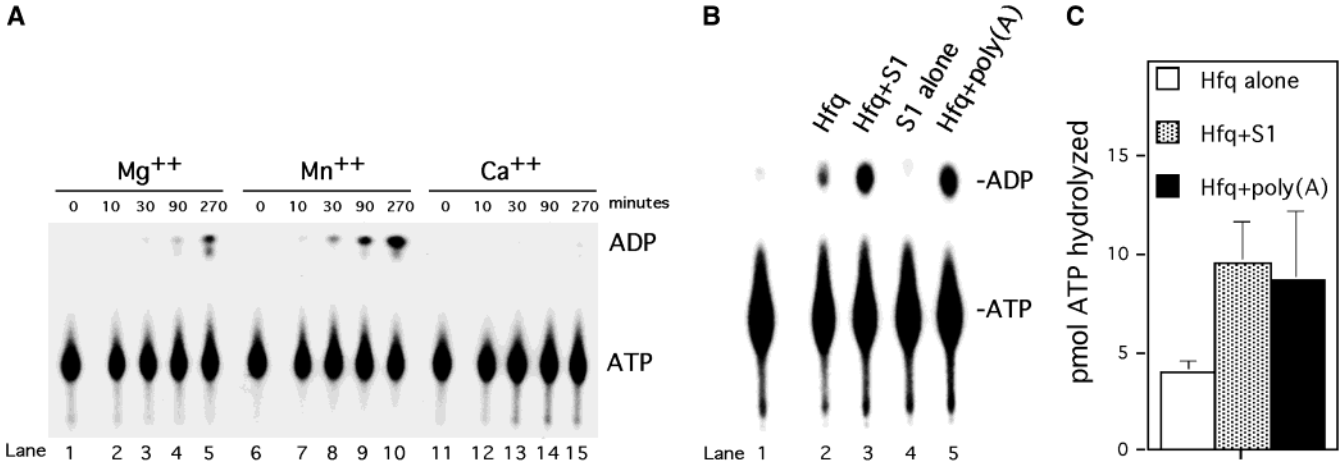


FIGURE 6: Properties of the Hfq ATPase. (A) Metal specificity of the Hfq ATPase. Kinetics of ATP hydrolysis in the presence of 2 mM $MgCl_2$ (lanes 1−5), 2 mM $MnCl_2$ (lanes 6−10), or 2 mM $CaCl_2$ (lanes 11−15). (B) Effects of S1 and poly(A) on Hfq-mediated ATP hydrolysis. The high specific activity ATPase reactions (see Materials and Methods) were carried out in the presence of 2 mM $MnCl_2$. When indicated, poly(A) was added to a final concentration of 6 A_{260} units and S1 to ~0.2 μM. (C) Quantitated data demonstrating effects of S1 and poly(A) on Hfq-mediated ATP hydrolysis. Data represent the average of two independent experiments.

of the purified proteins. Overall, the amounts of S1 and Hfq that copurified with RNAP throughout step 3 in Figure 1A represented a significant fraction of RNA polymerase: ~0.8 mol/mol of the free RNAP holoenzyme (peak 5 in Figure 1B) or ~0.5 mol of each protein, S1 and the Hfq hexamer, per total moles of the RNAP holoenzyme (taking into consideration the additional fraction of holoenzyme engaged in the holoenzyme−RapA complex; peak 7 in Figure 1B). This RNAP-associated fraction of the Hfq hexamer represented 5−9% of the total amount of Hfq in the cell (Figure 1E); therefore, if we assume the total amount of RNAP holoenzyme to be about 800 molecules/cell (*35*), the bacterial cell will harbor 4800−8000 molecules of the Hfq hexamer, a figure comparable to the estimate made by Kajitani et al. [30000−60000 of Hfq monomers/cell (*19*)]. A similar calculation would put the S1 level at 20000−40000 molecules/cell, the number, if correct, exceeding the number of bacterial ribosomes.

Interaction of S1 anf Hfd with RNAP and Effects of the Two Proteins on RNAP Transcriptional Activity. Using glycerol gradient ultracentrifugation binding assays, we have demonstrated that S1 associates with core RNAP in vitro, and purified Hfq associates with the S1−RNAP complex, presumably via S1 (Figure 2). Hfq−S1 complex formation is also supported by the stimulatory effect of S1 on the Hfq ATPase (Figure 6B,C). Complex formation between S1 and Hfq is not entirely surprising in light of the known requirement of both proteins for bacteriophage Qβ replication (*17*). It seems unlikely that protein−protein interactions were solely responsible for the copurification of stoichiometric amounts of S1, S2, and Hfq with RNAP during the initial purification steps, because the proteins separated from RNAP at 0.25−0.3 M NaCl at the Mono Q stage. More likely, ribosome-associated S1, S2, and Hfq were connected tightly to RNAP via mRNA (which was presumably hydrolyzed during the later purification stages) and were thus able to withstand the high-salt washes during the two initial purification steps. Thus, despite indirect evidence indicating that the S1−RNAP interaction is unlikely due entirely to protein-associated nucleic acids (data not shown), we cannot presently rule out the possibility that small fragments of protein-associated nucleic acids could participate in the interaction.

Due to the critical role of S1 in translation initiation (see references cited in the introduction), there is little or no opportunity to study the functional significance of the RNAP−S1 interaction in vivo: S1 is essential for the bacterial cell (*36*; we also failed to select *rpsA* deletion recombinants, using a DNA construct containing an antibiotic cassette inserted at the *Bst*EII restriction site of the *rpsA* gene), and S1 mutagenesis without producing a general effect on S1-mediated translation initiation seems unlikely. Under such circumstances, analysis of the effect of S1 on RNAP transcriptional activity and regulation of the S1−RNAP interaction are alternative means of determining its physiological relevance. S1 had a stimulatory effect on RNAP transcriptional activity (Figure 3A,B,D,E). This effect has to be taken with the notion that the specific roles of potential S1−RNAP and S1−nucleic acid, either transcript RNA or DNA template, interactions during in vitro transcription have yet to be clarified. The fact that the stimulatory effect of S1 was apparent during single-round transcription reactions (Figure 3E, lanes 4−9) suggests that S1 is capable of altering the catalytic mechanism of RNAP, although the same data argue that the significant accumulation of transcript RNA in the presence of S1 was due at least in part to an increase in the number of completed rounds of transcription (Figure 3E, compare lanes 3, 6, and 9). Given the body of evidence suggesting that S1 interacts with RNA (*8*−*11*), including the presence of multiple RNA-binding motifs in S1, it seems natural to speculate that the transcription−stimulatory effect could be due to sequestering of the RNAP-associated RNA transcript by S1. The RNAP−S1 interaction was downregulated by the RNAP-associated homologue of SWI2/SNF2 RapA (Figure 2), the function of which was associated with posttermination events in transcription (*7*), perhaps a meaningful regulatory effect if the RNAP SWI/SNF subunit were to trigger the release of ribosome-associated S1.

The mild transcription−stimulatory effect observed in the presence of Hfq was likely due to its nucleic acid-binding properties, since Hfq showed little or no affinity to RNAP (Figure 2D,E). As noted above, a near-covalent stability of

Hfq−transcript RNA complexes prevented adequate analysis of the levels of promoter-specific transcripts in the presence of Hfq (the quantitation of a total transcript RNA in the presence and absence of Hfq was performed as described in Materials and Methods).

Effects of S1 and Hfq on the Coupled Transcription−Translation Reaction. The coupled transcription-translation system was an avenue that we explored in order to study the roles of S1 and Hfq in the bacterial cell under in vivo-like conditions. Our choice of poly(A)−Sepharose treatment for preparation of the Hfq-depleted S-30 extract gave us the benefit of a "purified" system: the extract thus treated efficiently synthesized initial copies of the target protein but was incapable of efficient multiple-round translation, essentially yielding the same advantages in interpretation of the experimental data that single-round transcription can give as opposed to multiple-round transcription. The inhibitory effect of excess S1 in the coupled transcription−translation system (Figure 4D) was surprising, particularly given the fact that the bacterial cell was able to tolerate overproduction of His-tagged recombinant S1. Perhaps the effect resulted from nonproductive competition of purified S1 with an intrinsic, ribosome-associated S1 under the specific in vitro conditions. There was a mild stimulatory effect of Hfq on the coupled system (Figure 4D); however, this 2−3-fold stimulatory effect was marginal in comparison with the activity of the extract not treated with poly(A)−Sepharose (Figure 4B); possibly, the treatment resulted in the removal of some factors besides Hfq that were required for efficient multiple-round translation. The addition of further excess Hfq to the nondepleted system had an inhibitory effect on translation of luciferase (data not shown).

Although experiments with S30 extracts obtained from the *hfq* null mutant and the parental MC4100 bacterial strain (*21*) produced consistent results, indicating an overall stimulation of translation in the hfq^+ strain compared to the *hfq* deletion mutant (Figure 4E), one of the major limitations of these experiments was our inability to transfer the *hfq* deletion mutation into the MG1655 background by phage-mediated transduction, suggesting that Hfq expression may be of much greater importance to the bacterial cell in a wild-type prototype background.

Current dogma postulates that in bacteria the purine-rich sequence just upstream from the initiator codon in mRNA is the carrier of the ribosome recognition signals (*37*, *38*; reviewed in ref *39*). However, early on it was noted that the Shine and Dalgarno domain and the initiator codon are not sufficiently information-rich to specify a ribosome-binding site (*40*, *41*; reviewed in ref *42*). In a coupled transcription−translation system, initial copies of luciferase were translated in ∼3 min (Figure 4B); this rate of synthesis virtually matched that of transcription: the time needed for complete transcription of the >1600 bp *luc* gene was 2−2.5 min under our experimental conditions. In addition, initiation of the coupled reaction by addition of a plasmid DNA/RNAP mixture (compared to purified *luc* mRNA) produced a noticeable increase in the overall level of translated products at early time points (Figure 4C, arrow A) that can be tentatively attributed to more efficient transcription−translation coupling, although the initiation of translation with mRNA resulted in an even faster synthesis of a small number of copies of full-length *luc* polypeptide (Figure 4C, arrow B), as expected. These results further fuel the speculation that additional (other than SD) recognition elements might exist between the RNAP−mRNA complex and the translation apparatus.

Hfq Is an ATPase. The following lines of evidence indicate that Hfq harbors an intrinsic ATPase activity. (a) Virtually homogeneous Hfq (the purified native protein did not reveal impurities on overexposed silver-stained gels; Figure 3H) showed ATPase activity in in vitro transcription reactions (Figure 3G). (b) The peak of ATPase activity comigrated in chromatography on a Superose 12 column with the peak of Hfq; both peaks ran with the molecular mass expected for the Hfq hexamer, ∼70 kDa (Figure 5A). (c) An *hfq* null mutant lacked the ATPase activity peak detected in the Mono Q fractions derived from hfq^+ cells (Figure 5B). (d) His-tagged Hfq, which was cloned, overexpressed, and purified using a dissimilar purification procedure, also showed ATPase activity, the profile of which co-aligned with that of the purified protein during chromatography (Figure 5C). (e) Hfq obtained from yet another independent purification procedure (*21*) also showed ATPase activity. (f) The N-terminal part of Hfq showed homology to the part of the heat-shock protein ClpB that had been identified as the ATP-binding site (*33*) and which includes the Walker A box, a benchmark ATP-binding motif (Figure 5D). (g) The properties of the Hfq ATPase could be altered by site-specific mutagenesis (Figure 5E). It is tempting to speculate that the K56 mutation that altered the $k_{cat,Mn}/k_{cat,Mg}$ ratio might point to a metal-binding site involved in Hfq−RNA interaction.

Given the general consensus that Hfq bears homology to Sm-like proteins (*20*−*22*), these results, taken together, predict that at least some representatives of the family are ATPases with yet to be defined properties; we are currently testing this hypothesis.

Hypothetical Roles of the RNAP−S1−Hfq Interaction and the Hfq-Associated ATPase Activity. The hypothetical implications of the RNAP−S1 interaction for transcription−translation coupling in vivo seem apparent; the data presented in this paper indirectly support this proposition. However, one of the unsolved issues that remains to be confirmed experimentally is the association of the ribosome-bound S1 with RNAP, particularly in light of the apparent abundance of S1 in the bacterial cell (see the estimates given above). The significant transcription−stimulatory effect of S1 also seems to support an independent role for this ribosomal protein.

It is unclear at the moment what biochemical reaction is coupled to Hfq-mediated ATP hydrolysis. It is possible to speculate that the Hfq ATPase could assist some sort of ATP-fueled translocation of mRNA at the ribosome or ribosome−RNAP complex, in a manner similar to the NTPase-coupled translocation on single-stranded nucleic acid proposed for hexameric helicases (reviewed in ref *43*). This role of Hfq could be distinct from its proposed role as a mediator of RNA−RNA interactions (*20*, *21*). Currently, we are performing more extensive mutagenesis of the Hfq nucleotide-binding domain in order to study the role of this hexameric ATPase in vitro and in vivo.

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