The 5.5 Protein of Phage T7 Inhibits H-NS through Interactions with the Central Oligomerization Domain$^{\nabla}$†

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The 5.5 protein (T7p32) of coliphage T7 (5.5_{T7}) was shown to bind and inhibit gene silencing by the nucleoid-associated protein H-NS, but the mechanism by which it acts was not understood. The 5.5_{T7} protein is insoluble when expressed in *Escherichia coli*, but we find that 5.5_{T7} can be isolated in a soluble form when coexpressed with a truncated version of H-NS followed by subsequent disruption of the complex during anion-exchange chromatography. Association studies reveal that 5.5_{T7} binds a region of H-NS (residues 60 to 80) recently found to contain a distinct domain necessary for higher-order H-NS oligomerization. Accordingly, we find that purified 5.5_{T7} can disrupt higher-order H-NS-DNA complexes *in vitro* but does not abolish DNA binding by H-NS *per se*. Homologues of the 5.5_{T7} protein are found exclusively among members of the *Autographivirinae* that infect enteric bacteria, and despite fairly low sequence conservation, the H-NS binding properties of these proteins are largely conserved. Unexpectedly, we find that the 5.5_{T7} protein copurifies with heterogeneous low-molecular-weight RNA, likely tRNA, through several chromatography steps and that this interaction does not require the DNA binding domain of H-NS. The 5.5 proteins utilize a previously undescribed mechanism of H-NS antagonism that further highlights the critical importance that higher-order oligomerization plays in H-NS-mediated gene repression.

Bacteria employ an array of diverse strategies to control infection by phages, which are met by an equally diverse array of phage countermeasures. Frequent changes in the cell surface, restriction enzymes, spacer sequences within clustered regularly interspaced short palindromic repeat (CRISPR) elements, and toxin-antitoxin systems maintain immune diversity that prevent any phage lineages from gaining an overwhelming advantage over their bacterial hosts (26, 27, 32). One factor that could play a role in bacterial protection from phage infection is the nucleoid-associated protein H-NS. H-NS has been shown to play a major role in silencing transcription from sequences that are more AT-rich than the host genome, a characteristic of many genes that have been obtained via horizontal (lateral) gene transfer (15, 20, 45). The silencing of such sequences by H-NS, termed xenogeneic silencing, is thought to allow bacteria to safely acquire new genetic material without compromising their genomic and regulatory integrity (45). Although H-NS plays a clear role in mitigating the negative consequences presented by horizontally acquired genetic material, the role of H-NS in phage biology has remained largely unexplored.

A feature that is critical for H-NS function is its ability to multimerize to form higher-order nucleoprotein complexes (3, 60). The 80 N-terminal amino acids of H-NS contain two distinct dimerization domains that form an extended "superhelical scaffold" via head-to-head/tail-to-tail interactions (2). These H-NS scaffolds can bridge adjacent DNA segments *in vitro* that may organize the nucleoid into discrete loops and allow the molecule to constrain supercoiling within isolated domains (11, 12, 24, 48). Loops in DNA formed by H-NS may either trap RNA polymerase within the promoter region of a target gene or occlude the RNA polymerase from accessing the promoter sequence, preventing its active transcription (12, 56, 57). The ability of H-NS to constrain supercoils may also enable it to control promoters that are sensitive to supercoiling (24, 25, 43). Quite recently, studies have found that under certain conditions (e.g., low concentrations of magnesium *in vitro*), H-NS can coat DNA in a mode of binding distinct from bridging known as "stiffening," which could also lead to repression of target genes by a mechanism that is not yet understood (37, 67). Which of these two modes of binding predominates under physiologically relevant conditions is currently unclear.

Many antiphage systems are also encoded on mobile genetic elements, including plasmids and lysogenized phages, and presumably, by encoding such functions, these elements gain a territorial advantage by prolonging the survival of their hosts (18). Phage lambda, for example, encodes the *rexAB* genes that function to abort lytic growth by competing phages (53). Phage T4 *r*II mutants fail to infect *rexAB*⁺ lambda lysogens, a finding that was exploited to dissect fundamental gene structure, indicating that a subset of phages have effective countermeasures to lambda restriction (5). Growth of wild-type coliphage T7 in *Escherichia coli* is restricted by a number of mobile genetic elements, including Col1B and F-plasmids (17, 19, 44). It is notable that H-NS paralogs are frequently encoded on genomic islands and mobile genetic elements, including conjugative plasmids and, as recently identified, on phages (58). The plasmid-encoded H-NS paralog, Sfh, prevents disruption of regulatory networks likely caused by titration of endogenous H-NS by AT-rich plasmid sequences (14, 16). Whether H-NS paralogs play additional roles, such as protection against other foreign elements, including phages, has not been tested.

The *5.5* gene of coliphage T7 was originally identified during a

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TABLE 1. Oligonucleotides used in this study[a]

Name	Sequence
JMS bglGf	CTGCTGGCGGGGAAAGATAGCGACAAATAATTCACC
JMS bglGr	CGCGTTTTTGAAAGCCAATTCCGCGCCCCAT
blgG RT 5′	ACTGGCAATGGTCAGTTAGCGAGA
blgG RT 3′	TTCCTCTTGAGGTGATGGCAACCT
gyrB RT 5′	CACTTTCACGGAAACGACCGCAAT
gyrB RT 3′	TTACCAACAACATTCCGCAGCGTG
proV RT 5′	AATATTTGGCGAGCATCCACAGCG
proV RT 3′	TTTACCCGAGCCGGATAATCCCAT
SSA1	AAAA<u>CATATG</u>AGCGAAGCACTTAAAATTCTGAACAACATCC
SSA2	AAAA<u>CTCGAG</u>TTCCTTGATCAGGAAATCTTCCAGTTGCTTACC
SSA4	AAAA<u>CTCGAG</u>AGCGCTTTCTTCTTCACGACGCTCATTAACG
SSA5	AAAA<u>CTCGAG</u>CATTTCACGATACTGTTGCAGTTTACGAGTGCG
SSA6	AAAA<u>CATATG</u>CAGTATCGTGAAATGTTAATTGCCGACGGCA
SSA7	AAAA<u>CATATG</u>GCAGCTCGTCCGGCTAAATATAGCTATGTT
SSA24	AAAA<u>CCATGG</u>CTATGACAAAGAAATTTAAAGTGTCCTTCGACG
SSA26	AAAA<u>CCATGG</u>CTATCACTAAGAAATTTAAAGTGTCCTTTGATGTTACC
SSA27	AAAA<u>CTCGAG</u>AAGCAGTAATTTCCCAAGCGCCAC
SSA30A	GGCAATCTTAGAGAAAGATATGCCGCATCTATGTAAGCAGGTCGG
SSA30B	CCGACCTGCTTACATAGATGCGGCATATCTTTCTCTAAGATTGCC
SSA105	AAAA<u>CTCGAG</u>AGCCATGCTATTCAGCAGTTCATTCGGGT
SSA141	AAAA<u>CTCGAG</u>TCAGAACACCTCCCGTACTGTTGC
SSA151	AAAA<u>CCATGG</u>GTATTAACAAACAGTTTCGCG
SSA152	AAAA<u>CTCGAG</u>TCACTTAGTCACCCTCACGGTTG
SSA153	AAAA<u>CATGT</u>CTAAGATGACCGTTAACGTAAAAG
SSA154	TTTT<u>CTCGAG</u>TTAGCGGAATGTTACCAGAGCC
SSA158	AAAA<u>CTCGAG</u>TCATTTGAACACCTCTCGCACAGT
SSA159	AAAA<u>CCATGG</u>CTATTACTAAACGTTTTAAAGTATCATTC
SSA160	AAAA<u>CTCGAG</u>TTACTTCACCTCACGAATAGTTGCTGG
SSA162	AAAA<u>CCATGG</u>CTATTACTAAACGTTTTAAAGTATCATTTG
SSA163	AAAA<u>CTCGAG</u>TTACTTCACCTCACGGATAGTTGCTG
SSA165	AAAACAC<u>CCATGG</u>CAATGACCAAACAC

[a] Sequences are oriented from 5′ to 3′. Restriction sites are underlined.

random screen of highly mutagenized T7 phages for changes in protein expression (62). The product of the *5.5* gene (the 5.5_{T7} protein or $gp5.5_{T7}$) contributes to T7 growth on *E. coli* lysogens of phage lambda, and a mutant allele of the *5.5* gene renders T7 unable to infect *E. coli* that is lysogenized with phage lambda (the restricted by lambda, or *rbl*, phenotype) (35, 52). The allele that leads to the *rbl* phenotype has been mapped to a change in a single nucleotide that converts a leucine at position 30 of $gp5.5_{T7}$ to a proline (35). Whether or not the primary function of $gp5.5_{T7}$ is to enable T7 to avoid restriction by lambda-like phages is less clear. The *rbl* phenotype has only thus far been demonstrated for the L30P allele, here referred to as $gp5.5_{T7(rbl)}$. T7 phages containing a mutation of L30 to threonine or a premature amber codon in the *5.5* gene display normal growth on lambda lysogens (35). In a separate study, Liu and Richardson reported that $gp5.5_{T7}$ but not $gp5.5_{T7(rbl)}$ tightly associates with H-NS and demonstrated that it could antagonize silencing of the *proU* operon and T7 promoters by H-NS (36). The relationships between $gp5.5_{T7}$, H-NS, and genes carried on the lambda lysogen that restrict T7 mutants remain entirely unclear.

Phage T7 therefore serves as an attractive system to study the interaction of a phage with H-NS. The 5.5_{T7} protein bears no sequence homology to any other known protein, and the mechanism by which it interacts with H-NS to inhibit its function has not been explored. Here, we describe our initial work to characterize the $gp5.5_{T7}$/H-NS interaction and the mechanism by which $gp5.5_{T7}$ blocks H-NS-mediated gene repression. Phylogenetic analysis reveals that proteins related to 5.5_{T7} are

found exclusively in T7-like viruses (*Autographivirinae*) that infect enterobacteria. Although $gp5.5_{T7}$ is insoluble when expressed by itself, we have developed a method of purifying stable and soluble 5.5_{T7} protein through coexpression with a truncated H-NS fragment that is subsequently removed. We find that the 5.5_{T7} protein interacts with a recently identified central dimerization domain within H-NS to disrupt higher-order nucleoprotein complexes without perturbing DNA binding *per se*. We also unexpectedly find that $gp5.5_{T7}$ copurifies a heterogeneous mix of tRNA. H-NS antagonism through interference with the second oligomerization domain supports the role of this domain in formation of a proper nucleoprotein structure that is essential for H-NS-mediated gene silencing.

MATERIALS AND METHODS

Plasmid construction. Information about oligonucleotides, strains, and plasmids used in this study is summarized in Tables 1 and 2. The *Salmonella hns* locus was amplified from genomic DNA of strain LT2 using primers SSA1 and SSA2. The *hns* fragment was digested with NdeI and XhoI and ligated to the corresponding sites of pET-21b. The resulting plasmid, pSSA2, was used for production of the H-NS protein with a histidine tag at its C terminus. The H-NS truncation mutants were constructed by amplifying fragments from plasmid pSSA2 with the following primers: SSA1 and SSA4 for hns_{1-46}, SSA1 and SSA5 for hns_{1-64}, SSA1 and SSA105 for hns_{1-80}, SSA6 and SSA2 for hns_{60-137}, and SSA7 and SSA2 for hns_{90-137}. All the H-NS truncation fragments were cloned into pET-21b using the restriction sites NdeI and XhoI to incorporate a histidine tag at the C termini of the mutants.

To clone the *5.5* gene from phage T7, its coding sequence was amplified from a T7 lysate using primers SSA24 and SSA141. The resulting product was digested with NcoI and XhoI and ligated into the corresponding sites of vector pCDF-1b.

TABLE 2. Plasmids used in this study

Plasmid	Vector	Description or use	Reference
pSSA2	pET21b	Expression of H-NS$_{6His}$	This study
pSSA5	pET21b	Expression of H-NS1-46$_{6His}$	This study
pSSA6	pET21b	Expression of H-NS1-64$_{6His}$	This study
pSSA7	pET21b	Expression of H-NS60-137$_{6His}$	This study
pSSA8	pET21b	Expression of H-NS91-137$_{6His}$	This study
pSSA20	pCDF1b	Expression of T3 5.5	This study
pSSA22	pET21b	Expression of H-NS1-80$_{6His}$	This study
pSSA23	pCDF1b	Expression of T7 5.5	This study
pSSA26	pCDF1b	Expression of T7 5.5$_{rbl}$	This study
pSSA27	pCDF1b	Expression of K11 5.5	This study
pSSA29	pCDF1b	Expression of Mmp1 5.5	This study
pSSA30	pCDF1b	Expression of *Yersinia* phage Berlin 5.5	This study
pSSA31	pCDF1b	Expression of Kvp1 5.5	This study
pSSA32	pCDF1b	Expression of φSGJL2 5.5	This study
pWN426	pHSG576	Complementation with HA-tagged H-NS	47

Restriction digest with NcoI and XhoI eliminates the histidine tag from the cloning site of pCDF-1b. The plasmid generated, pSSA23, encoded an untagged version of the *5.5* gene, allowing for overexpression of native 5.5$_{T7}$ protein. The pCDF-1b vector is compatible with pET-expression vectors, facilitating coexpression of 5.5$_{T7}$ with the histidine-tagged H-NS constructs. Likewise, the *5.5* gene from a bacteriophage T3 lysate was cloned into pCDF-1b vector with primers SSA26 and SSA27. The coding sequences of *5.5* gene homologues from phages φSGJl2 (accession no. gi:189085873), Kvp1 (gi:212671404), *Yersinia* Berlin (gi: 119637767), K11 (gi:194100440), and Mmp1 (gi:194473825) were ordered from GenScript in the vector pUC57 and subcloned into pCDF-1b using the primer pairs SSA158 and SSA165, SSA162 and SSA163, SSA159 and SSA160, SSA151 and SSA152, and SSA153 and SSA154, respectively (Table 1).

Site-directed mutagenesis. To recreate the previously described (36) 5.5 *rbl* mutation (leucine at position 30 to proline), site-directed mutagenesis was performed for plasmid pSSA23 using oligonucleotides SSA30A and SSA30B. Plasmid pSSA23 was amplified by PCR with *Pfu* Ultra Fusion II polymerase (Stratagene) and primers SSA30A and SSA30B. The PCR product was treated with DpnI endonuclease in New England BioLabs buffer 4 for 1 h at 37°C to eliminate the PCR template. The restriction digest was purified using a Qiagen PCR cleanup column, and the resulting plasmid encoding 5.5 with the *rbl* mutation was transformed into *E. coli* DH5α. The presence of the *rbl* mutation was confirmed through DNA sequencing.

ChIP assay. For chromatin immunoprecipitation (ChIP) assays, a BL21(DE3) *hns* mutant strain was generated by P1 transduction from the previously constructed *E. coli* WN582 strain. Cultures of the BL21(DE3) Δ*hns* mutant harboring plasmids pWN426 (hemagglutinin [HA]-tagged H-NS) and pSSA23 (5.5$_{T7}$) and the BL21(DE3) Δ*hns* mutant harboring plasmids pWN426 and pSSA26 [5.5$_{T7(rbl)}$] were grown to mid-logarithmic phase (optical density at 600 nm [OD$_{600}$], 0.4 to 0.6) and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 h. Before and after IPTG induction, samples were treated with 1% formaldehyde for 15 min at room temperature. A small sample was also taken for RNA analysis prior to formaldehyde treatment (see below). The cross-linking reaction was then quenched with 1.25 mM glycine for 10 min. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and sonicated to generate DNA fragments of ~500 bp. Cell lysates were precipitated with an anti-HA antibody (Sigma catalog number H3663) using agarose protein G beads (Calbiochem). Real-time quantitative PCR (Q-PCR) analyses of H-NS-interacting DNA samples were performed using Sybr green mix from Bio-Rad according to the manufacturer's instructions. Work with each primer set was done in triplicate.

Reverse transcriptase quantitative PCR. The induced bacterial cultures (0.5 ml) used for ChIP analysis described above were mixed with 1 ml of RNAprotect bacterial reagent (Qiagen) and incubated for 30 min at room temperature. Subsequent RNA preparations were performed using an Aurum Total RNA minikit (Bio-Rad). Reverse transcription was performed using an iScript cDNA synthesis kit (Bio-Rad) with random hexamer primers. The cDNA generated was used for quantitative real-time PCR analysis as described above. The transcripts of the *proV*, *bglG*, and *gyrB* genes were analyzed by using the primers listed in Table 1 according to gene name. The transcript of the *gyrB* gene, which is not regulated by H-NS, was used as an internal standard for normalization.

Microarray. BL21(DE3) harboring a plasmid for expression of gp5.5$_{T7}$ or a plasmid for expression of H-NS$_{1-64}$ and BL21(DE3) cultures harboring empty vector controls pCDF-1b and pET21b were grown to mid-logarithmic phase (optical density at 600 nm [OD$_{600}$], 0.4 to 0.6) and induced with 0.1 mM IPTG for 1 h. Two microliters of induced culture was added to 4 ml of RNAprotect bacterial reagent (Qiagen) and incubated for 30 min at room temperature. Production of labeled cDNA was performed essentially as described previously (10). Briefly, RNA preparations were performed using an Aurum Total RNA minikit (Bio-Rad), and cDNA was synthesized using 25 μg total RNA, 25 μg random nonamer primers, and 400 units of Superscript II reverse transcriptase (Invitrogen). Aminoallyl-dUTP was incorporated into the cDNA by including a 3:2 ratio of aminoallyl-dUTP to dTTP in the reverse transcription reaction. The RNA was hydrolyzed by treatment with NaOH, and the cDNA was purified using Qiagen PCR purification columns. Purified cDNA was reduced to a volume of 3.5 μl in a centrifugal evaporator and labeled with Cy3 and Cy5 monoreactive dye packs from Amersham in a 7-μl reaction mixture. cDNA (2 μg) from BL21(DE3) cultures expressing gp5.5$_{T7}$ and H-NS$_{1-64}$ was labeled with Cy5, and cDNA from BL21(DE3) harboring the empty vector controls pCDF-1b and pET-21b was labeled with Cy3. Labeling reactions were quenched with 3.5 μl of 4 M hydroxylamine, and cDNA was purified with a Qiagen PCR purification kit. Cy3- and Cy5-labeled cDNA was reduced to ~5 μl, and samples were mixed in the following combinations: cDNA from BL21(DE3) cultures expressing gp5.5$_{T7}$ (Cy5 labeled) with cDNA from BL21(DE3) harboring the pCDF-1b plasmid (Cy3-labeled), and cDNA from BL21(DE3) cultures expressing H-NS$_{1-64}$ (Cy5 labeled) with cDNA from BL21(DE3) harboring pET21b plasmid (Cy3 labeled). Thirty microliters of 2× GEx Hyb buffer and 4 μl of blocking buffer from the Agilent gene expression hybridization kit were added to the combined cDNA samples. A total of 40 μl of labeled probe/hybridization mixture was hybridized to Agilent's *E. coli* 8x15k microarrays (Agilent design identification number 020097). The arrays were scanned using a Genepix Professional 4200A scanner. Intensity ratios were acquired using Imagene version 7.5 (Biodiscovery) and Lowess normalized using the R software package from Bioconductor (http://www.bioconductor.org). Significance analysis of microarrays (SAM) (65) was performed for results of three independent experiments using a 1% false-discovery rate (FDR).

Coexpression and purification of gp5.5 homologues with H-NS and H-NS truncation mutants. All coexpression studies were performed as follows. Plasmids encoding the T7 5.5 protein (pSSA23) and H-NS (pSSA2) were cotransformed into *E. coli* BL21(DE3) (63). Transformants were selected on Luria-Bertani (LB) agar plates supplemented with 100 μg/ml ampicillin and 50 μg/ml streptomycin. The resulting BL21(DE3) strain carrying plasmids pSSA23 and pSSA2 was cultured at 37°C until an optical density at 600 nm of 0.6 was reached. H-NS and 5.5 expression was induced with 0.1 mM IPTG overnight at 18°C. Cells were harvested by centrifugation, resuspended in cell lysis buffer (50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole, 10 μM β-mercaptoethanol, 5% glycerol [pH 7.0]) and sonicated. The cell lysate was centrifuged at 8,900 rpm in a FiberLite F-13 rotor for 45 min. The supernatant was incubated with Qiagen Ni^{2+} resin (pre-equilibrated in lysis buffer) for 15 min at 4°C. The Ni^{2+} resin/cell lysate mixture was applied to a gravity flow column and washed twice with 25 ml ice-cold wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 30 mM imidazole, 10 μM β-mercaptoethanol, 5% glycerol [pH 7.0]). H-NS and associated 5.5 proteins were then eluted from the column with 5 ml of elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 250 mM imidazole, 10 μM β-mercaptoethanol, 5% glycerol [pH 7.0]). Fractions were analyzed by SDS-polyacrylamide gel electrophoresis.

Overexpression and purification of H-NS. H-NS$_{6His}$ was expressed and nickel column purified from *E. coli* BL21(DE3) as described above for the coexpression studies, with the exception that the following buffers were used: cell lysis buffer (50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole, 10 μM β-mercaptoethanol, 5% glycerol [pH 8.0]), wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 30 mM imidazole, 10 μM β-mercaptoethanol, 5% glycerol [pH 8.0]), and elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 250 mM imidazole, 10 μM β-mercaptoethanol, 5% glycerol [pH 7.0]). The nickel column eluate was treated with DNase I for 30 min at room temperature, diluted 5-fold, and loaded onto a Hi-trapQ (GE Healthcare) anion-exchange column for a secondary purification step. The anion-exchange column was initially equilibrated with buffer A (10 mM Tris [pH 8.0], 1 mM dithiothreitol [DTT]), and H-NS was eluted by applying a gradient of 0 to 100% buffer B (10 mM Tris [pH 8.0], 1 M NaCl, 1 mM DTT). H-NS-containing fractions were pooled and buffer exchanged to 10 mM Tris, 50 mM NaCl, 2 mM DTT, 5% glycerol, and 2.5 mM EDTA (pH 8.0). The concentration of H-NS was determined by the Bradford method relative to a standard curve for bovine serum albumin (BSA).

Separating 5.5 from H-NS$_{60-137}$. Plasmids encoding H-NS$_{60-137}$ (pSSA7) and gp5.5$_{T7}$ (pSSA23) were coexpressed and purified by nickel chromatography as

described above. SDS-PAGE analysis of the nickel resin purification confirmed that H-NS$_{60-137(6His)}$ copurified with untagged 5.5$_{T7}$ protein. The elution fraction (containing both H-NS$_{60-137}$ and gp5.5$_{T7}$) was concentrated in a centrifugal filter unit (Millipore) to a final volume of 1 ml. The concentrated protein solution was loaded on a Superdex S200 gel filtration column pre-equilibrated with 10 mM HEPES, 100 mM NaCl, 1 mM DTT, and 5% glycerol (pH 7.0). H-NS$_{60-137}$ and 5.5 coeluted in a single peak at an estimated molecular mass of 90 kDa. Protein-containing fractions from the S200 column were pooled for a third purification step of anion-exchange chromatography. The H-NS$_{60-137}$/5.5$_{T7}$ complex was loaded onto a HiTrapQ column in the presence of binding buffer (10 mM HEPES [pH 7.0], 1 mM DTT), and the concentration of the 5.5$_{T7}$ protein eluted in the absence of H-NS$_{60-137}$ when the sodium chloride concentration was increased to 700 to 800 mM.

Electrophoretic mobility shift assays. The promoter region of the *bglG* gene was amplified by PCR from *E. coli* BL21(DE3) chromosomal DNA with the primer pair JMS bglGf and JMS bglGr. Purified H-NS and 5.5 proteins were combined with 40 ng of gel-purified *bglG* promoter DNA and binding buffer (15 mM HEPES [pH 8.0], 40 mM KCl, 1 mM EDTA, 0.5 mM DTT, 5% glycerol) in a 20-μl total reaction volume. The mobility shift reaction mixtures were incubated at room temperature for 30 min and combined with 4 μl of Fermentas 6× DNA loading dye. The DNA-protein complexes were separated on a 6% native polyacrylamide gel in the presence of 1× TAE (40 mM Tris acetate, 1 mM EDTA). Electrophoresis was carried out at 70 V for 2 h and 45 min at 4°C. The gels were stained with Sybr green for 20 min at room temperature and visualized with a Typhoon imager.

Gel filtration chromatography. Purified H-NS, gp5.5$_{T7}$, and H-NS/gp5.5$_{T7}$ complex were applied to a Tricorn Superdex 200 10/300 GL column at concentrations of 100 μM, 60 μM, and 100 μM, respectively. All proteins were buffer exchanged into running buffer (20 mM HEPES [pH 7.0], 150 mM NaCl, 1 mM DTT, 5% glycerol) using a HiTrap desalting column (GE Healthcare) prior to loading. The column was equilibrated in running buffer and calibrated by running molecular mass standards that covered the range of 18.2 to 440 kDa.

End-labeling RNA. To isolate the RNA that copurified with gp5.5$_{T7}$, a sample of purified 5.5$_{T7}$ protein was phenol-chloroform extracted, and the aqueous phase (contains nucleic acids) was ethanol precipitated. [^{32}P]pCp 3′ end labeling was carried out in a 20-μl reaction volume with 50 μCi [^{32}P]pCp, 5 μM ethanol-precipitated RNA, 40 units of T4 ligase, and 2 μl of 10× ligase buffer (New England BioLabs). [γ-^{32}P]ATP 5′ end labeling was also carried out in a 20 μl reaction volume with 50 μCi [γ-^{32}P]ATP, 5 μM ethanol-precipitated RNA, 20 units of T4 polynucleotide kinase, and 2 μl of 10× kinase buffer (New England BioLabs). The labeled RNA was separated on an 8% polyacrylamide–8 M urea sequencing gel and exposed to a phosphorimaging screen.

RESULTS

Effects of 5.5 on H-NS/DNA binding and host gene expression *in vivo*. H-NS binds more than 400 regions in *E. coli* to control the expression of more than 500 operons (23, 28, 51). The previous study by Liu and Richardson demonstrated that overexpression of gp5.5$_{T7}$ tagged with maltose binding protein (MBP) increased expression of the H-NS-repressed osmoregulated *proU* promoter *in vivo*, although to levels significantly lower than those observed when wild-type *E. coli* is placed under conditions of high osmolarity.

To expand upon Liu and Richardson's findings and to determine if gp5.5$_{T7}$ can displace H-NS from the *E. coli* chromosome *in vivo*, we performed ChIP assays followed by quantitative PCR. Genes encoding 5.5$_{T7}$ or 5.5$_{T7(rbl)}$ were cloned into a pCDF-1b expression vector under the control of a T7 RNA polymerase promoter. The 5.5 proteins encoded on these plasmids were expressed by IPTG induction in an *E. coli* BL21(DE3) *hns* mutant harboring a low-copy-number plasmid expressing C-terminally HA epitope-tagged H-NS under the control of its native promoter. Cells were fixed with formaldehyde and sonicated to shear DNA into 500-bp fragments. DNA associated with H-NS was precipitated with an anti-HA antibody. Immunoprecipitation efficiencies (percent recovery after immunoprecipitation compared
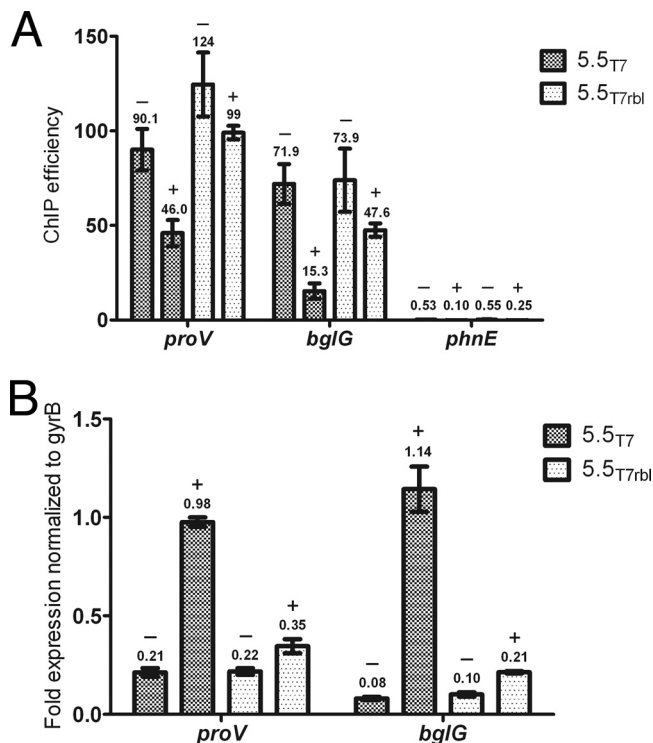

FIG. 1. Expression of gp5.5$_{T7}$ depletes H-NS binding *in vivo* and induces expression of H-NS-regulated genes. (A) ChIP experiments were performed with the BL21(DE3) Δ*hns* mutant harboring pWN426 (HA-tagged H-NS) and plasmids encoding either gp5.5$_{T7}$ (dark gray) or gp5.5$_{T7(rbl)}$ (light gray) under the control of an IPTG-inducible promoter. Enrichment of genes previously shown to be regulated by H-NS (the *proV* and *bglG* genes) and not regulated by H-NS (the *phnE* gene) were analyzed by quantitative RT-PCR before (−) and 1 h after IPTG induction of gp5.5$_{T7}$ expression (+). (B) Transcript levels of the same cultures used for ChIP analysis were determined by reverse transcriptase Q-PCR and normalized to a control transcript (the *gyrB* gene; see Materials and Methods). Error bars indicate standard deviations, and the numbers above each bar correspond to the values on the y axis (ChIP efficiency or RNA transcript levels).

to initial input) of the *bglG* and *proV* genes, which were previously shown to be H-NS regulated, were measured by Q-PCR (Fig. 1A). As a negative control, the ChIP efficiencies were also measured for the *phnE* gene, which was previously shown to not bind or be regulated by H-NS.

The ChIP Q-PCR experiments reveal that expression of gp5.5$_{T7}$ results in the partial but not complete dissociation of H-NS from the *bglG* and *proV* promoter sequences. Immunoprecipitation efficiencies for the *proV* and *bglG* genes were reduced approximately 2-fold and 3-fold, respectively, in strains expressing gp5.5$_{T7}$ compared to that from the gp5.5$_{T7(rbl)}$ strain. The immunoprecipitation efficiency of the *phnE* gene was over 150-fold less than that observed for the *proV* gene, indicating this sequence was largely not bound by H-NS. It is notable that the enrichment of *proV* and *bglG* DNA after expression of gp5.5$_{T7}$ exceeds that of the *phnE* gene 200-fold and 50-fold, respectively. We conclude that expression of gp5.5$_{T7}$ leads to a significant (2- to 3-fold), but not complete, loss of H-NS from the *bglG* and *proV* promoter sequences.

Samples from the same cultures used in the ChIP assay were

also analyzed for the effects of 5.5 on gene expression using reverse transcriptase Q-PCR (Fig. 1B). Total RNA was purified from strains expressing 5.5_{T7} and $5.5_{T7(rbl)}$ and reverse transcribed using random hexamer primers. The cDNA generated was used for Q-PCR analysis with *proV*- and *bglG*-specific primers. The *gyrB* gene, which is unaffected by H-NS, served as an internal control for normalization. *proV* and *bglG* transcript levels increased over 2-fold and 5-fold, respectively, in strains expressing $gp5.5_{T7}$ compared to that for the $gp5.5_{T7(rbl)}$ negative-control strain. These findings are consistent with the ChIP results and indicate that induction of $gp5.5_{T7}$ expression leads to decreased H-NS binding and increased expression of the H-NS-regulated *proV* and *bglG* genes.

It is possible that the effects of $gp5.5_{T7}$ on the host cell would extend beyond its ability to interact with H-NS and such effects would be missed by a biased sampling of a limited number of genes. To determine the global effects of $gp5.5_{T7}$ expression on the *E. coli* transcriptome in an unbiased manner, we employed microarray analysis of *E. coli* strain BL21(DE3) overexpressing either $gp5.5_{T7}$ or the H-NS$_{1-64}$ protein. The latter construct, which contains the N-terminal dimerization domain of H-NS, has been shown to act in a dominant-negative fashion to interfere with H-NS-mediated silencing when overexpressed (66, 68). As such, this construct served as a positive control for antagonism of H-NS activity. RNA isolated 1 h after IPTG induction was labeled and hybridized to a commercial *E. coli* microarray containing oligonucleotides corresponding to most open reading frames (ORFs). Data were analyzed using a set false-discovery rate (FDR) of 1%, and transcripts were considered affected if they displayed 3-fold or greater changes compared to the plasmid-only control (see Table S1 in the supplemental material).

The data indicate that the effects observed by overexpression of the 5.5_{T7} protein largely parallel the effects observed by overexpression of H-NS$_{1-64}$. Expression of $gp5.5_{T7}$ increased steady-state levels of transcripts corresponding to 239 ORFs, including many previously characterized H-NS-repressed loci, such as the *gadX* (38), *bglG* (56), *csgAB* (50), *ompF* (64), *hlyE* (70), *hdeA* and *hdeD* (71), *cadCBA* (31), and *leuO* (30) loci. Two-thirds (159) of the ORFs upregulated by $gp5.5_{T7}$ were also upregulated in cells overexpressing H-NS$_{1-64}$. Several of the transcripts that were not categorized as also upregulated by H-NS$_{1-64}$ were excluded only because of the stringency of our threshold for inclusion. Surprisingly, only seven transcripts were downregulated by expression of $gp5.5_{T7}$, most of which had relatively mild changes in expression (less than 4-fold). Some of these, including DnaK, DnaJ, and GroES, play a role in protein folding. The reason these genes were expressed is unclear and may be indirect rather than due to the effect of $gp5.5_{T7}$ on H-NS. Overexpression of H-NS$_{1-64}$ triggered a broader set of transcriptional changes than that observed with $gp5.5_{T7}$, with increased levels of transcripts corresponding to 310 ORFs. There were also a larger set of genes (39 total) that displayed lower levels of expression after H-NS$_{1-64}$ induction, including previously known genes involved in motility. None of these genes corresponded to the genes showing lower expression in the $gp5.5_{T7}$ data set.

Of the subset of transcripts that were upregulated by $gp5.5_{T7}$ and that were not affected by H-NS$_{1-64}$, several were involved in metabolism, including subunits of the F_1F_0 ATPase (*atpEFGH*) and NADH dehydrogenase (*nuoAB*) genes. The mechanism by which several of these genes could be affected by $gp5.5_{T7}$ expres-

sion is unclear and may be indirect. There is some indication that $gp5.5_{T7}$ expression causes a mild degree of cell stress that is not observed with H-NS$_{1-64}$. Our microarray analysis revealed that $gp5.5_{T7}$ induced the upregulated expression of the *sulA*, *minD*, and *minE* genes, which inhibit cell division, and IS1 and IS30, whereas H-NS$_{1-64}$ did not. There was also mild induction of genes encoded on the DE3 prophage, including the cII protein. Upregulation of the *sulE* gene is usually driven by the SOS response; however, few other SOS-induced loci were upregulated, indicating that DNA damage is likely not the source of stress caused by $gp5.5_{T7}$ overexpression. In summary, we conclude that the 5.5_{T7} protein indeed acts to counter H-NS-mediated repression on a global scale.

5.5 is soluble when coexpressed with H-NS. Liu and Richardson reported that purification of gp5.5 from coliphage T7 as a soluble protein was difficult and that adequate concentrations of pure protein were only achievable by fusing maltose binding protein (MBP) to the N terminus of the $gp5.5_{T7}$ coding sequence. In our attempts to isolate pure and soluble 5.5_{T7} protein, we also found that neither native nor hexahistidine-tagged $gp5.5_{T7}$ could be expressed as a soluble protein in *E. coli* under standard expression conditions, including growing the producing strain at a lower temperature. Small quantities of soluble 5.5_{T7} protein could be obtained when the coding sequence was fused to large N-terminal tags, including SUMO and MBP (data not shown).

Difficult-to-express proteins are often stabilized when coexpressed with interacting partner proteins. This is true, for example, of the Hha protein, which is largely insoluble unless coexpressed with H-NS (55). We employed a coexpression strategy by cloning the 5.5_{T7} gene and *Salmonella hns* genes into the compatible expression vectors pCDF-1b and pET-21b, respectively. The C-terminal end of H-NS was tagged with six-histidyl residues for purification by chromatography over nickel resin, a location for the tag that we have found does not affect the ability of H-NS to bind DNA or functionally complement an *hns* mutation *in vivo* (22, 47). Using this approach, we were able to obtain high yields of a soluble $gp5.5_{T7}$/H-NS complex with an approximate stoichiometry of 1:1, as indicated by Coomassie staining (Fig. 2). Coexpression of the untagged $5.5_{T7(rbl)}$ protein with H-NS confirmed that no association between the two proteins could be detected and, further, that the $5.5_{T7(rbl)}$ protein was insoluble while the H-NS protein remained soluble (data not shown).

5.5 proteins from several T7-like phages bind H-NS. Coliphage T7 is a member of the *Autographivirinae* family of viruses; short-tailed, double-stranded DNA (dsDNA) phages that encode an RNA polymerase necessary for the transcription of phage genes involved in DNA metabolism and phage morphogenesis (2). BLAST and PSI-BLAST (1) searches revealed that $gp5.5_{T7}$ homologues are found exclusively in the subset of *Autographivirinae* that infect bacteria of the family *Enterobacteriaceae* (i.e., the *5.5* gene is absent in those phages that infect *Vibrio* and *Pseudomonas*). In all cases, the genomic location of the gp5.5 homologue was highly conserved, residing between the *5* gene, encoding the phage DNA polymerase, and the *6* gene, encoding an exonuclease that liberates deoxynucleotides through degradation of host DNA and suppresses the accidental packaging of host DNA. All *5.5* genes are found immediately upstream of a T7 *5.7* gene homologue, often with overlapping start/stop codons. *Autographivirinae* that do not infect enterobacteria, such as vibriophages VP4 and N4 and
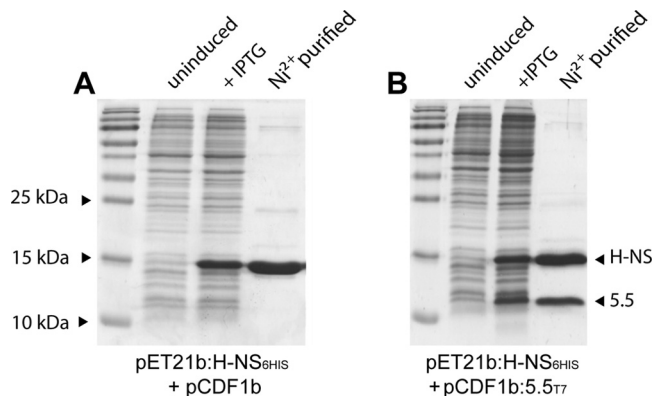
FIG. 2. The 5.5_{T7} protein forms a soluble complex when coexpressed with H-NS containing a C-terminal six-histidyl tag. Coomassie-stained SDS-PAGE gels of H-NS$_{6His}$ expressed from vector pET-21b purified over nickel resin after coexpression with a control pCDF-1b vector (A) or with gp5.5$_{T7}$ (B). The lanes include total bacterial protein prior to addition of IPTG (uninduced) and after addition of IPTG (+IPTG) and the eluate after passing cell extracts over nickel column and eluting in the presence of imidazole (Ni^{2+} purified).

Pseudomonas phage gh-1, encode a 5.7 protein without an associated *5.5* gene. The function of the *5.7* gene remains unknown. The 5.5 proteins show an unusually high degree of sequence divergence with only 7 of the approximately 100 residues that are absolutely conserved among the 16 orthologs analyzed. Pairwise alignments of each gp5.5 homologue to all of the others reveals that they share, on average, only 47% identity (range, 95% to 24%). In contrast, the products of the nearby *5*, *5.7*, and *6* genes are also highly conserved, with an average amino acid identity between homologues of 77%, 84%, and 69%, respectively, similar to the conservation among most proteins that are common to all *Autographivirinae*.

Given the large sequence divergence observed for 5.5 proteins, it was of interest to determine if the ability to bind H-NS is a conserved feature of these proteins. Toward this end, the coexpression/interaction assay described previously was employed to measure the interactions between H-NS and the 5.5 proteins of a number of *Autographivirinae*, including coliphage T3, *Salmonella* phage φSG-JL2, *Kluyvera* phage Kvp1, *Yersinia* phage Berlin, *Klebsiella* phage K11, and *Morganella* phage Mmp1 (Fig. 3A). These gp5.5 orthologs were selected to obtain a diverse sample set of 5.5 proteins that each varied significantly in sequence from
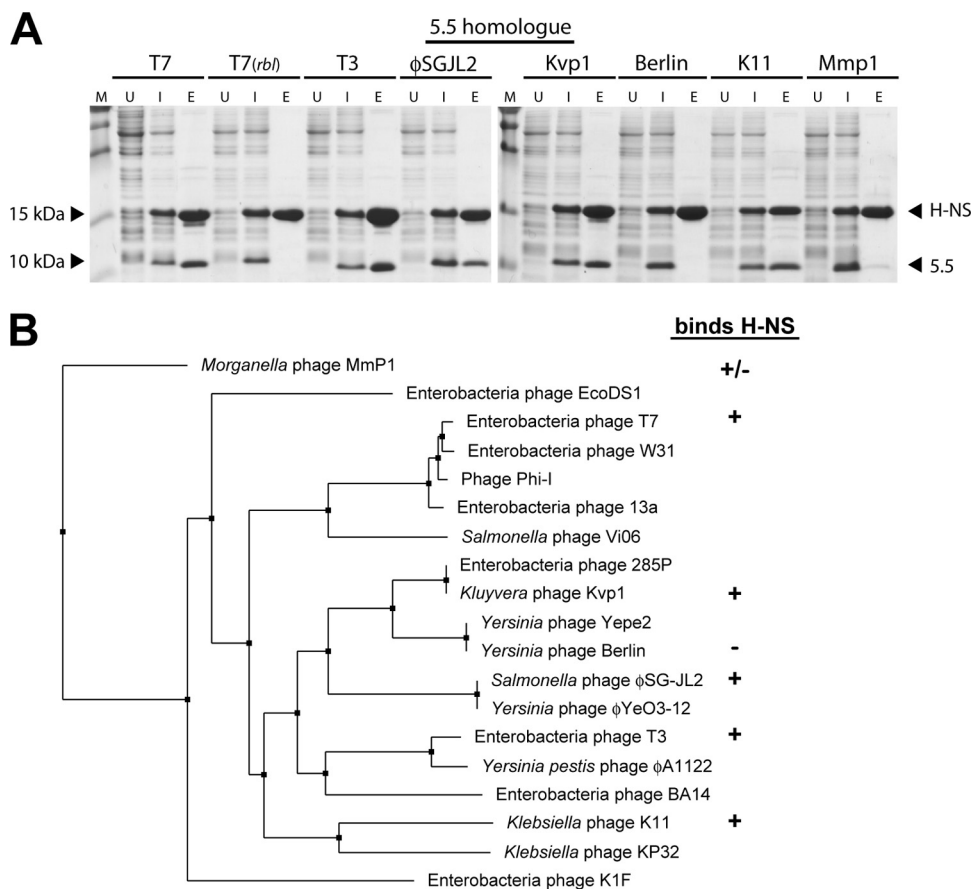
FIG. 3. Diverse 5.5 proteins from various *Autographivirinae* bind H-NS. (A) Full-length H-NS$_{6His}$ was coexpressed in strains of *E. coli* BL21(DE3), each containing a plasmid with a gp5.5 homologue as indicated on top. The ability of the two proteins to interact was assessed by SDS-PAGE and Coomassie after copurification using nickel chromatography. Lane labels: M, molecular mass marker; U, uninduced, cell lysate prior to induction with IPTG; I, induced, cell lysate after induction of protein synthesis by IPTG; E, eluate after nickel chromatography. (B) Results of the interaction assay in panel A are shown next to a nearest-neighbor tree of the various gp5.5 homologues. Symbols indicate whether the indicated gp5.5 homologue binds (+), does not bind (−), or displays strongly diminished binding (+/−) to H-NS.
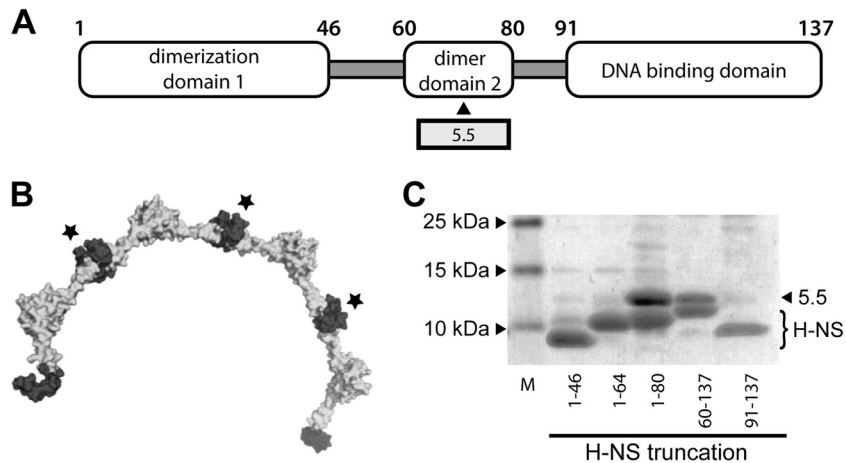
FIG. 4. 5.5_{T7} protein binds the central H-NS dimerization domain contained within residues 60 to 80. (A) Diagram of the H-NS molecule showing the approximate boundaries of each distinct domain within H-NS. (B) Structure of H-NS helical multimer (8 H-NS monomers) as recently solved by Arold et al. (2). The central dimerization domains that interact with $gp5.5_{T7}$ are shown in dark gray and indicated with a star. (C) Coomassie-stained SDS-PAGE of the interaction between $gp5.5_{T7}$ and various $H-NS_{6His}$ truncations after coexpression and purification over nickel resin. The positions of $gp5.5_{T7}$ and various H-NS truncations are indicated.

one another according to our phylogenetic analysis (Fig. 3B). With the exception of gp5.5 from *Yersinia* phage Berlin, all of the proteins tested copurified with *Salmonella* H-NS. The 5.5 protein from *Morganella* phage Mmp1 (Mmp1_gp28), which is highly divergent from 5.5_{T7}, interacted with H-NS only weakly. Control experiments in which the 5.5 homologues were expressed in the absence of H-NS demonstrated that these proteins have no affinity for nickel resin and, to various degrees, are insoluble in the absence of H-NS. These experiments indicate that despite the fairly low degree of conservation, the ability to interact with H-NS is a property widely conserved among the 5.5 proteins.

The 5.5_{T7} protein interacts with the central linker domain of H-NS. The H-NS protein is 137 amino acid residues long and contains three functional domains (59, 66). The N-terminal domain contains a large coiled-coil region responsible for dimerization and interaction with the Hha and YdgT corepressor proteins (Fig. 4A). The C-terminal domain from amino acids 91 to 137 contains the DNA binding activity. The central region, located between residues 46 and 90, was thought to be a "flexible linker" important for association of H-NS molecules into higher-order oligomeric states. A recent structural study has revealed that the linker region harbors a second dimerization domain contained within residues 60 and 80 that enables H-NS to multimerize in a head-to-head/tail-to-tail fashion to generate an extended helical scaffold for DNA binding (Fig. 4B) (2). To determine the region of H-NS that interacts with the 5.5_{T7} protein, $gp5.5_{T7}$ was coexpressed with a series of H-NS deletion mutants, each of which were tagged at the C terminus with six-histidine residues. $gp5.5_{T7}$/H-NS complex formation was assessed by the ability of the 5.5_{T7} protein to copurify with the various $H-NS_{6His}$ truncations during purification over nickel resin (Fig. 4C). No significant association of the 5.5_{T7} protein was observed with either the N-terminal dimerization domain (residues 1 to 46 or 1 to 64) or the C-terminal DNA binding domain (residues 91 to 137). In contrast, the 5.5_{T7} protein associated tightly with constructs containing residues 60 to 137 and 1 to 80 of H-NS. The results indicate that $gp5.5_{T7}$ associates with a region of H-NS contained between res-

idues 60 and 80, corresponding to the recently identified central dimerization domain.

Isolation of pure 5.5 as a soluble and functional protein from $H-NS_{60-137}$. Purification of $H-NS_{6His}$ alone or with $gp5.5_{T7}$ over nickel resin yields partially pure preparations that were contaminated with small amounts of other proteins and DNA. A multistep purification protocol was developed that would consistently yield substantial quantities of the $gp5.5_{T7}$/H-NS complex that were sufficiently pure to use in downstream studies. This procedure included DNase treatment of cleared cell lysates, purification of complex over nickel resin, a second round of chromatography over Q-Sepharose, and a final purification/buffer exchange by gel filtration. During the purification of $gp5.5_{T7}$ in complex with various truncation mutants, we serendipitously found that the 5.5_{T7} protein would dissociate from $H-NS_{60-137}$ in a soluble form during chromatography over Q-Sepharose when concentrations of NaCl reached approximately 700 mM (Fig. 5A). The identity of the purified protein band observed on SDS-PAGE as 5.5_{T7} was verified by N-terminal protein sequencing by Edman degradation.

To determine if the soluble 5.5_{T7} protein was functional and retained its ability to bind H-NS, we employed an on-column association assay (Fig. 5B). Pure 5.5_{T7} protein was mixed with purified $H-NS_{6His}$ prior to chromatography over nickel resin. Purified 5.5_{T7} protein alone was unable to associate with the nickel resin and eluted off the resin during the wash step. When $gp5.5_{T7}$ and tagged $H-NS_{6His}$ were mixed together, the 5.5_{T7} protein was retained on the column but coeluted with the H-NS protein upon the addition of imidazole. This result indicated that the purified 5.5_{T7} protein retained its ability to interact with the H-NS protein after isolation through this procedure.

The 5.5 protein disrupts higher-order H-NS-DNA complexes. The effects of the soluble 5.5_{T7} protein on the ability of H-NS to interact with DNA *in vitro* were assessed using an electrophoretic mobility shift assay (EMSA) (Fig. 6). Several independent laboratories have observed that H-NS exhibits
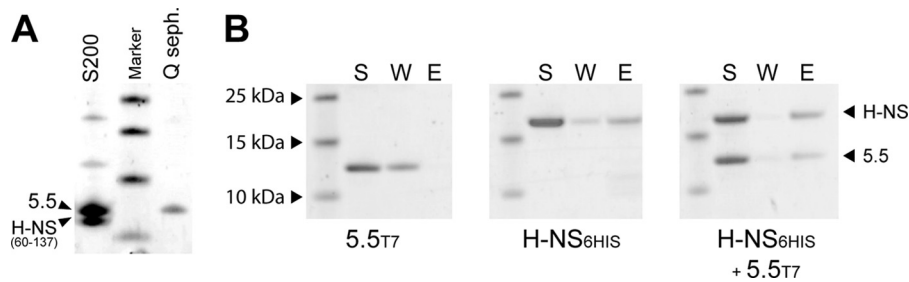
FIG. 5. Purification of gp5.5$_{T7}$ as a soluble and active protein by dissociation from H-NS$_{60-137}$. (A) SDS-PAGE of eluted fractions of coexpressed gp5.5$_{T7}$ after gel filtration chromatography on a Hiload 16-60 Superdex S200 prep grade column and after ion-exchange chromatography over a HitrapQ fast flow column (Q Sepharose.). (B) Purified gp5.5$_{T7}$ was applied to nickel resin either in the absence (left panel) or presence (right panel) of H-NS$_{6His}$ and association was assessed. The central panel shows H-NS$_{6His}$ in association with nickel resin in the absence of gp5.5$_{T7}$. Lanes: S, supernatant, sample prior to loading onto nickel resin; W, wash fraction; E, eluate after nickel chromatography.

strongly cooperative binding behavior, in which multiple sites on target DNA are occupied over a very narrow range, resulting in a low-mobility H-NS/DNA complex. Purified H-NS protein was mixed with PCR-generated DNA fragments containing the promoter of the *bglG* gene, previously demonstrated to be regulated by H-NS. Consistent with previous reports, the addition of 500 μM protein H-NS led to complete shifting of this DNA fragment into a low-mobility protein/DNA complex. Addition of purified gp5.5$_{T7}$ at increasing ratios with H-NS, ranging from 1:1 to 10:1, caused decreased shifting of the DNA-H-NS complex. However, at no concentration was complete displacement of H-NS from the DNA observed. No effect on DNA mobility was observed by the addition of gp5.5$_{T7}$ in the absence of H-NS.

H-NS is known to form higher-order oligomers spontaneously in solution at sufficiently high concentrations that can be observed by changes in the elution profile during gel filtration chromatography (3). The apparent masses of H-NS, gp5.5$_{T7}$, and the gp5.5$_{T7}$/H-NS complex were tested using a Sephacryl S200 column that was calibrated with a set of globular proteins of known sizes (Table 3). At a concentration of 100 μM, H-NS (monomer mass, ~15.5 kDa) elutes from the column with an apparent mass of 132 kDa, indicative of an octomer or nona-

mer. The average oligomer size is likely to be lower, however, given the elongated structure of H-NS oligomers. H-NS copurified with gp5.5$_{T7}$ as a complex, when adjusted to a concentration of 100 μM, displayed an apparent mass of 163 kDa, which was larger than that of H-NS alone. Isolated gp5.5$_{T7}$ protein, which has a predicted monomer mass of 11 kDa, elutes with an apparent mass of 146 kDa, which indicates that it may also exist as a higher-order multimer. The order of the oligomerization state of gp5.5$_{T7}$ is confounded by its association with RNA (see below).

The EMSA results are consistent with what we observed with ChIP analysis *in vivo* and support a model whereby gp5.5$_{T7}$ binding results in an alteration in the structure of oligomerized H-NS but does not directly interfere with the ability of H-NS to bind DNA. The results by gel filtration also suggest that the 5.5$_{T7}$ protein does not trigger dissociation of multimers into monomers or dimers, although these results are inconclusive. The observed smearing pattern of the protein-DNA complex is likely caused by decreased stability of the H-NS/DNA during electrophoresis due to a disruption in cooperative binding. A similar smearing pattern has been observed with EMSAs using oligomerization-defective mutants of a *Pseudomonas* H-NS analog, MvaT (8).

The 5.5 protein copurifies with tRNA. During the purification of gp5.5$_{T7}$ and subsequent attempts to characterize its stoichiometry, we noted that the molecule displayed very strong absorbance at 260 nm. During EMSA experiments, we noted the presence of a low-molecular-weight nucleic acid species, running near the dye front, that was present only in lanes to which the purified 5.5$_{T7}$ protein had been added.
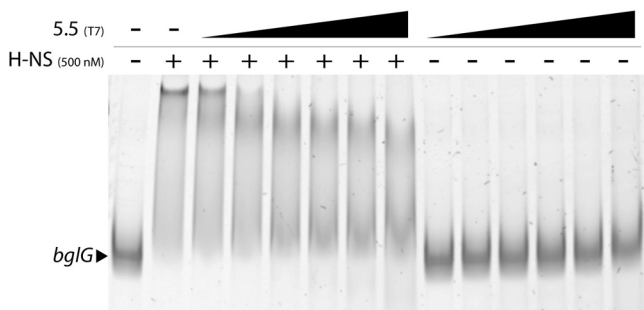
FIG. 6. Purified 5.5$_{T7}$ protein disrupts higher-order H-NS/DNA complexes *in vitro*. A 423-bp fragment of the *bglG* promoter region was incubated with 500 nM H-NS and increasing concentrations of gp5.5$_{T7}$ (500 nM, 1,000 nM, 1,500 nM, 2,000 nM, 2,500 nM, and 5,000 nM). The amount of *bglG* DNA in each reaction was 10 nM. 5.5$_{T7}$ was incubated with *bglG* DNA in the absence of H-NS at 500 nM, 1,000 nM, 1,500 nM, 2,000 nM, 2,500 nM, and 5,000 nM. Reactions were then separated on 6% acrylamide gels which were run at 4°C. + and − represent the presence and absence, respectively, of the indicated protein.

TABLE 3. Gel filtration of H-NS, gp5.5$_{T7}$, and the H-NS/gp5.5$_{T7}$ complex

Protein	Elution profile[a]		
	Concn (μM)	Monomer wt (kDa)	Calculated mass (kDa)
H-NS	100	15.5	132
H-NS/gp5.5	100		163
gp5.5	60	11	146

[a] Calculated mass from Tricorn Superdex 200 10/300 GL that was calibrated immediately prior to use with ribonuclease (13.7 kDa), ovalbumin (43 kDa), conalbumin (75 kDa), aldolase (158 kDa), and ferritin (440 kDa), a set of globular proteins with elution profiles that correlate well with mass.

FIG. 7. The 5.5 protein copurifies with tRNA. (A) After purification by gel filtration chromatography, associated nucleic acid was removed by phenol-choloroform extraction (5.5 preparation) and subsequently treated with DNase I or RNaseA as indicated. The plasmid control shown in the final two lanes demonstrates the activity of the DNase I used in the assay. (B) End labeling of isolated RNA reveals that it is heterogeneous and labels poorly at its 5′ end. Copurifying low-molecular-weight RNA was end labeled at the 3′ end with [^{32}P]pCp and RNA ligase or at the 5′ end with [γ-^{32}P]ATP using T4 polynucleotide kinase. The bar adjacent to the autoradiogram indicates the low-abundance (as determined by Sybr stain), presumably degraded, RNA that was efficiently labeled by PNK. (C) Diagram of tRNA^{Asp} showing, boxed and in bold, the segment obtained by cloning after reverse transcription of in vitro-polyadenylated RNA. Similar truncated clones were obtained from tRNA^{Ala} and tRNA^{Thr}. EthBr, ethidium bromide; nt, nucleotides.

Further analysis revealed that the small nucleic acid was co-purifying with gp5.5$_{T7}$ through multiple chromatographic steps, including nickel chromatography (in complex with H-NS), Q-Sepharose (at which point the H-NS protein dissociates from gp5.5$_{T7}$), and two rounds of gel filtration. The copurifying nucleic acid was also observed when gp5.5$_{T7}$ was coexpressed with the H-NS$_{1-80}$ construct that lacks the DNA binding domain, indicating that the nucleic acid was not copurifying due to an interaction with the DNA binding domain of H-NS (data not shown). The copurifying nucleic acid was extracted from gp5.5$_{T7}$ with phenol and subjected to digestion with DNase I or RNaseA (Fig. 7A). The results demonstrate that the nucleic acid copurifying with gp5.5$_{T7}$ is RNA and not DNA. Attempts to remove this RNA from the 5.5$_{T7}$ protein by treatment with RNase caused rapid aggregation of the 5.5$_{T7}$ protein, suggesting that the RNA was critical to maintain gp5.5$_{T7}$ solubility (data not shown).

A sequencing resolution acrylamide gel of the 3′-radiolabeled copurifying RNA indicated it was heterogeneous and composed of at least four major species with sizes ranging from approximately 75 to 95 nucleotides (Fig. 7B). The two most prominent species corresponded to approximately 77 and 82 nucleotides in length. Additional species present in smaller amounts were approximately 86 to 95 nucleotides in length. Multiple attempts using various linker-ligation cloning strategies were made to identify the sequences of the copurifying RNA, but no clones were obtained. Subsequent analysis of the copurifying RNA revealed that it was poorly amenable to radiolabeling at the 5′ end by T4 polynucleotide kinase (PNK) but that the 3′ ends could be labeled readily with [^{32}P]pCp using T4 RNA ligase. We correspondingly altered the cloning strategy by extending the 3′ end with a poly(A) tail using poly(A) polymerase followed by reverse transcription an oligo(dT) primer and ligation of the resulting product with an adaptor prior to cloning. By using this method, multiple clones containing fragments corresponding to 34 to 38 nucleotides of the 3′ ends of tRNA^{Ala} (encoded by the alaX gene), tRNA^{Thr} (thrW), and tRNA^{Asp} (aspT, aspU, or aspV) were isolated. Less abundant RNAs, presumably degradation products, were much more readily labeled with T4 PNK (Fig. 7B).

Taken together, the data indicate that the predominant co-purifying nucleic acids are a heterogeneous mix of mature tRNAs and less abundant larger precursor forms prior to processing by host RNAses. The sizes of the various RNA molecules are similar to those expected of tRNA (76 to 77 nucleotides) or tRNA precursors. The difficulties encountered in obtaining full-length clones are most likely due to strong secondary structure and the presence of modified nucleotides in tRNA that hindered ligation of linkers to the 5′ end and blocked primer extension steps by DNA/RNA polymerases. The poor radiolabeling of the 5′ end of the full-length RNA molecule by T4 PNK is entirely consistent with previous observations regarding the poor equilibrium constants observed when tRNA is labeled with this enzyme (33, 34). The clones that were obtained were likely derived from incomplete reverse transcription that was blocked by modified nucleotides contained within the anticodon loop (e.g., m^{2}A$_{37}$ in tRNA^{Asp}) (Fig. 7C).

DISCUSSION

Since the discovery in 1993 that the 5.5$_{T7}$ protein copurified with H-NS, the mechanism by which these two proteins interact and the effects of the interaction on phage infection have remained unexplored. Here, we extend the previous observations and find that 5.5 proteins of several T7-like phages in-

teract with H-NS despite their fairly large sequence divergence. We further find that gp5.5$_{T7}$ targets the recently described central dimerization domain of H-NS contained within residues 60 to 80. We have developed a method of purification of soluble 5.5 protein that retains H-NS binding activity that has enabled us to analyze its effects on H-NS DNA binding *in vitro*. The EMSA and gel filtration results support a role of 5.5 in disrupting proper nucleoprotein structure without perturbing DNA binding. This is entirely consistent with our finding that gp5.5$_{T7}$ interacts with the domain involved in higher-order structuring but not with the DNA binding or primary dimerization domain. The EMSA results are also in agreement with chromatin immunoprecipitation experiments showing limited depletion of H-NS caused by gp5.5$_{T7}$ overexpression at *bglG* and *proV* promoters *in vivo*. It is of note that our EMSA results differ from the previous observations of Liu and Richardson, who paradoxically observed increased shifting (i.e., supershifting) of H-NS/DNA complexes upon the addition of MBP-5.5$_{T7}$. This may be due to the fact that the protein used in those studies contained a very large N-terminal addition that altered mobility or failed to fully disrupt the H-NS/DNA complex *in vitro* under the conditions used.

H-NS silences gene expression primarily through promoter occlusion, polymerase trapping, and constraining supercoils, although the relative contributions of each can vary at different promoters (46). Accordingly, there are a large number of mechanisms described by which H-NS-mediated silencing can be relieved to enable gene expression (45, 61). These include direct competition for binding sites by sequence-specific DNA binding proteins and reorganization of the nucleoid structure in response to temperature such that H-NS fails to halt transcription. Apart from gp5.5$_{T7}$, only one other molecule, H-NST, found on pathogenicity islands encoded near the *serU* gene of some pathogenic strains of *E. coli*, is known to antagonize H-NS by interfering with multimerization (4, 69). H-NST is highly homologous to the first 80 amino acids of H-NS and acts in a dominant-negative fashion, presumably through interactions with both the N-terminal and central dimerization domains. The critical role that the fine higher-order structure of the H-NS-DNA complex plays in gene silencing is further highlighted by recent findings that Ler, an H-NS like protein that activates rather than represses gene expression, adopts a nucleoprotein structure distinct from the elongated filaments formed by H-NS (41, 42). One notable aspect of our microarray study is that expression of gp5.5$_{T7}$ and H-NS$_{1-64}$ triggered gene expression changes that correlated strongly but not perfectly. Differences observed in gene expression between the two constructs employed may be due to differences in levels of expression or activity (i.e., we did not obtain equivalent levels of H-NS inhibition, which is difficult to calibrate under the experimental setup used in these studies). It is also likely that the mechanism by which gp5.5$_{T7}$ and H-NS$_{1-64}$ antagonize H-NS are not identical and, therefore, that these proteins affect different promoters to different degrees. This notion is consistent with previous findings that alterations in higher-order structure have a greater effect on the expression of some promoters than others. For example, several mutations in H-NS that relieve silencing of the *proV* gene but that have no effect on the expression of the *bglG* gene have been identified (66). For most alleles, the underlying reason for the observed

differences remains unclear. For example, it was shown that a truncated H-NS molecule lacking a DNA binding domain required an association with the H-NS paralog StpA for repression of the *bglG* gene. However, this association was insufficient to repress the *proV* gene and was only relevant when the truncated H-NS was expressed at low levels (21).

The phylogenetic distribution of gp5.5$_{T7}$ orthologs revealed that these proteins are exclusively found in *Autographivirinae* that infect enterobacteria. Homologues of gp5.5$_{T7}$ are notably absent in the few members of this family that infect *Vibrio* and *Pseudomonas*. This may well correlate to the fact that members of the family *Pseudomonadaceae* harbor xenogeneic silencing proteins, MvaT and MvaU, that bear no similarity to H-NS (9). The H-NS molecules of *Vibrio* spp. are also quite distinct from those of the enteric bacteria, most notably with regard to their mechanism of oligomerization, and they possess almost no similarity to enteric H-NS in the region corresponding to the central oligomerization domain (49). The second dimerization domain of *Yersinia* H-NS differs by three residues from that of *Salmonella* and *E. coli*, and this may account for the inability of gp5.5 from *Yersinia* phage Berlin to bind H-NS from *Salmonella*. For these reasons, analogs of the 5.5 protein present in *Autographivirinae* from nonenteric bacteria, if they exist, might have diverged extensively from the 5.5 proteins to the point where they cannot be identified by sequence similarity.

Despite the fact that 5.5$_{T7}$ protein is believed to accumulate to very high levels during the course of infection, phage T7 5.5$_{rbl}$ mutants have no phenotype in most *E. coli* wild-type laboratory strains, and the role of this factor in the phage life cycle remains unclear (62). Genes present in phage genomes are usually clustered according to function, and one clue with regard to the 5.5 protein may be its invariant genetic linkage with the *5*, *5.7* and *6* genes. These genes are transcribed by the T7 RNA polymerase within 6 min of infection and are clustered with genes involved in replication and packaging of phage DNA. A speculative model proposed by Liu and Richardson, that release of H-NS during degradation of the host genome is detrimental to phage replication, seems unlikely, since T7 5.5$_{rbl}$ mutants grow normally in strains of *E. coli* that have intact H-NS (35). Furthermore, the T7 genome is not notably AT-rich except in the proximity of the late promoters that direct T7 RNAP to transcribe genes involved in phage morphogenesis. It is possible that a primary role of the gp5.5/H-NS complex is to enable T7 growth on lambda lysogens, but the fact that the 5.5$_{T7}$ amber mutant does not display an *rbl* phenotype indicates that the mechanism may be complex.

The association of gp5.5$_{T7}$ with heterogeneous tRNA after multiple chromatography steps was unexpected and points to a possible avenue of investigation regarding its role during phage infection. gp5.5$_{T7}$ carries an overall positive charge (pI, ~7.9) and it remains possible that the association of the protein with RNA is not specific. However, we have also observed that gp5.5$_{T3}$ also copurifies with low-molecular-weight RNA despite low conservation of sequence and a net negative charge (pI, ~5.4; data not shown). It has previously been demonstrated that during phage T4 infection, tRNA$_{Lys}$ is cleaved by a host-encoded anticodon nuclease, PrrC, in an effort to block phage replication by shutting down translation (29, 40, 54). Wild-type T4, however, encodes a polynucleotide kinase and tRNA ligase that repairs the cleaved tRNA, tipping control of

translation back in favor of the phage. In this light, it is possible that the interaction of gp5.5$_{T7}$ with tRNA is in some way related to gaining control of the host translational apparatus to the advantage of the phage and that this phenotype occurs only in certain strains of *E. coli*. It is also possible that tRNA is not the physiologically relevant RNA substrate during phage infection and that it adventitiously copurified with gp5.5$_{T7}$ due to its high abundance in the cell. Other potential RNA substrates that have strong secondary structure that would be relevant to phage infection include CRISPR-associated RNAs and recently identified antiviral RNA components of some toxin-antitoxin systems (6, 7, 13, 39). Determining the relevance of H-NS and, possibly, RNA in the function of the 5.5$_{T7}$ protein will require a better understanding of the conditions under which gp5.5$_{T7}$ is important for the phage life cycle. Our future efforts will be focused in that direction.

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