Disruption of *Escherichia coli* HepA, an RNA Polymerase-associated Protein, Causes UV Sensitivity*

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DNA-dependent RNA polymerase (RNAP) is the central enzyme of transcription and a major target for the regulation of gene expression. The association of a wide array of accessory proteins with RNAP is critical for the regulation of each phase of the transcription cycle: initiation, elongation, and termination. In addition to accessory factors that interact with RNAP to regulate the transcription process, protein-protein interactions couple RNAP to proteins participating in other cellular processes such as DNA repair (1).

During the development of purification procedures for *Escherichia coli* RNAP, we noticed the consistent co-purification of a 110-kDa polypeptide. The presence of this contaminant through the last step of varying purification procedures suggested that it was a previously unidentified factor specifically associated with the RNAP. Here, we identify the 110-kDa contaminant as the product of the *hepA* gene, a putative member of the SNF2 family of putative helicases. We have cloned the *hepA* gene and overexpressed and purified the HepA protein. We show in vitro that RNAP preparations have an ATPase activity only in the presence of HepA and that HepA binds core RNAP competitively with the promoter specificity of subunit 70 and a dissociation constant (*Kd*) of 75 nM. An *E. coli* strain with a disruption in the *hepA* gene shows sensitivity to the DNA damaging agent UV light.

**EXPERIMENTAL PROCEDURES**

**Cloning of the hepA Gene**—The *hepA* gene was PCR amplified from *E. coli* BL21 (DE3) genomic DNA using the following primers: HepAleft, 5′-GCCGAACACCCATGGCTTTTACACTTGGTC-3′; HepAright, 5′-CCATTTCGATCGTACTGTTGCTC-3′. NotI and BamHI sites were engineered into HepAleft and HepAright, respectively (underlined), to allow digestion of the amplified products and subsequent ligation into the corresponding sites of the T7-based expression vector pET15b (Novagen) to generate pET15b-HepA. The cloning created a mutation in the second amino acid of the protein (from Pro to Ala) as well as a Gin to Arg mutation of the terminal amino acid.

**Purification of Overexpressed HepA**—*E. coli* BL21 (DE3) cells were transformed with pET15b-HepA, grown to an *A*_{600} of 0.6, and induced by the addition of IPTG to a final concentration of 1 mM. Induction was allowed to proceed for 4 h. The cells were then harvested by centrifugation and stored at −80 °C. The cells were thawed, resuspended in 40 mM Tris–HCl, pH 7.9, 300 mM KCl, 10 mM EDTA, and lyzed by sonication. The lysate was spun to pellet cell debris. The supernatant was collected and subjected to TEGD and poly(ethyleneimine) (PEI) was slowly added to a final concentration of 0.8% (w/v). The PEI pellet was resuspended in TEGD and diluted with TEGD until the conductivity was less than TEGD

**Native Gel Shift Binding Assays**—50 pmol of HepA protein was applied to a 6% native polyacrylamide gel and electrophoresed at 20–30 mA until the xylene cyanol had migrated about three-fourths the length of the gel. Protein bands were visualized by Coomassie staining. To unambiguously identify the protein contents of the visualized bands, the protein bands of interest were excised from the gel with a scalpel, crushed, and we show in vitro that RNAP preparations have an ATPase activity only in the presence of HepA and that HepA binds core RNAP competitively with the promoter specificity of subunit 70 and a dissociation constant (*Kd*) of 75 nM. An *E. coli* strain with a disruption in the *hepA* gene shows sensitivity to the DNA damaging agent UV light.
left to soak for 10 min in SDS loading buffer supplemented with 5 mM β-mercaptoethanol. The samples were then analyzed by SDS-PAGE and Coomassie staining on a 4–12% Tris-glycine gradient gel (Novex).

For competition binding studies, 50 pmol of HepA protein was added to 25 pmol of core RNAP and incubated as above to form the core RNAP-HepA complex. 50 pmol of HepA was then added and incubated for another 15 min at 37 °C. Similarly, 50 pmol HepA was incubated with core RNAP to form holoenzyme, which was then incubated with 50 pmol HepA. The samples were then analyzed by the native gel shift assay described above, along with an excision of the bands and SDS-PAGE to determine the protein content of the bands.

For the quantitative binding assays to determine the dissociation constant and stoichiometry of the HepA-core RNAP interaction, calf heart protein kinase was used to phosphorylate HepA. HepA (20 μg) was incubated with [γ-32P]ATP and 100 units of calf heart protein kinase (Sigma) in 20 mM Tris-HCl, pH 7.9, 50 mM KCl, 5 mM MgCl2, 10 mM DTT. The phosphorylation reaction proceeded for 10 min at 30 °C. Unincorporated radioactivity was removed from the reaction mixture by buffer exchange using a Microcon 30 microconcentrator (Amicon) into 40 mM Tris-HCl, pH 7.9, 100 mM KCl, 10 mM MgCl2, 5% glycerol. Varying amounts of HepA protein (1–12 pmol) were added to 3 pmol core RNAP and incubated as above. The samples were then analyzed by the native gel shift assay. The bound and free HepA was quantified by a PhosphorImager (Molecular Dynamics Storm 860i).

Results

A 110-kDa Protein Co-purifies with E. coli RNAP—The initial observation that formed the impetus for this study is illustrated in Fig. 1. In attempting to purify RNAP by a standard procedure (12–14) from E. coli RL324 (harborin a C-terminal His6 tag in the chromosomal copy of rpoC, which codes for the RNAP β′ subunit, obtained from R. L. Landick), we noticed nearly stoichiometric amounts of a contaminating protein with a mobility by SDS-PAGE corresponding to about 110 kDa. The contaminating protein was present through the final step of purification in some core RNAP and holoenzyme fractions from an anion exchange column (Fig. 1, fractions 11, 12, 16, and 17). The contaminant was also present through the last step of a different purification procedure utilizing Ni2⁺ affinity chromatography. The presence of nearly stoichiometric amounts of the 110-kDa contaminant through the final steps of two different purification procedures suggested that this protein interacts with the RNAP itself. The 110-kDa protein was also present after purification of RNAP from E. coli JC7623 (10), the parent strain of RL324 containing the wild-type rpoC gene, eliminating the possibility that the 110-kDa contaminant adventitiously associated with the RNAP from RL324 through the His6 tag at the C terminus of the β′ subunit.

The 110-kDa Contaminant Is the Product of the HepA Gene—The N-terminal sequence of the 110-kDa contaminant was determined to be XPFPTLGQRWISDTESELG (2). A data base search revealed a single match to the product of an open reading frame denoted hepA (helicase putative; Refs. 15–17). The inferred amino acid sequence of HepA contained amino acid sequence similarity with motifs I, II, and III of the six “DEAD” box helicases (2). Later, a frameshift in the original DNA sequence was postulated that revealed that the downstream sequences also contained DEAD box helicase motifs V and VI (3). Based on extensive sequence similarity, HepA has already been grouped with the SNF2 family of putative helicases (3, 5).

We used PCR methods to clone the hepA gene into a T7-based overexpression vector (18). Because of ambiguity in the HepA sequence between helicase motifs II and V (where the frameshift was proposed to occur but its exact location could not be determined; Ref. 3), we sequenced this region of the gene. The sequencing confirmed the presence of a frameshift in the original sequence. The correct sequence gave rise to a peptide of 969 amino acids and a calculated molecular mass of 109,700 Da, consistent with the SDS-PAGE mobility. Between the time our sequencing was completed and this manuscript was written, an updated sequence of this region of the E. coli genome.
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was submitted to GenBank (accession number AE000116 U00096). This sequence exactly matched our sequence.

When induced with IPTG, HepA was expressed to very high levels comprising nearly 50% of total cellular protein. Overexpression of the protein had no obvious toxic effects. Upon cell lysis, the bulk of the overexpressed HepA was found in the soluble fraction. We purified the overexpressed HepA with a procedure similar to that used to purify RNAP (14). In the initial step of purification by anion exchange chromatography, the RNAP and HepA bound to the column in buffer containing 0.2 m NaCl. The excess HepA that was not associated with RNAP eluted from the column during a NaCl gradient at about 0.3 m NaCl. The resulting HepA was >95% homogeneous (based on overloaded, Coomassie-stained SDS gels), but in subsequent experiments it became clear that a substantial DNase activity either was associated with HepA or was contaminating it. We therefore performed an additional step of purification, gel filtration over a Superose 6 (Amersham Pharmacia Biotech) column. This effectively removed the DNase activity from the HepA protein.

HepA Binds Core RNAP but Not Holoenzyme—With the highly purified HepA in hand, we asked whether HepA formed a stable complex with RNAP in vitro, as suggested above. For this purpose, we used a native gel shift assay (Fig. 2A). When RNAP holoenzyme was mixed with a 2-fold molar excess of purified HepA and analyzed by polyacrylamide gel electrophoresis under nondenaturing conditions, bands corresponding to free HepA and free RNAP holoenzyme were observed (Fig. 2A, lane 1). Because mobility on the native gel is determined by both molecular weight and charge, the band in Fig. 2A corresponding to RNAP holoenzyme could conceivably contain a holoenzyme-HepA complex with the same mobility as holoenzyme. Therefore, we confirmed the protein components of the bands labeled in Fig. 2A by exciting them and analyzing their contents by SDS-PAGE (Fig. 2B). By this method, the band labeled A in Fig. 2A contains only β', β, σ70, α, and ω, the components of holoenzyme (Fig. 2B, lane A). Core RNAP alone gave rise to two bands on the native gel (Fig. 2A, lane 5), likely because of the presence of core RNAP monomers and dimers in equilibrium (14). A mixture of HepA and core RNAP (2:1 molar ratio) yielded a band corresponding to free HepA and a band distinct from the bands observed for core RNAP alone (Fig. 2A, lane 4, band C). This distinct band contained β', β, α, and ω (the components of core RNAP) and an apparently stoichiometric amount of HepA, based on the intensity of the Coomassie stain (Fig. 2B, lane C). Thus, we conclude that HepA forms a stable complex with core RNAP but not holoenzyme.

Additional experiments were conducted in which preformed complexes of core RNAP/HepA were challenged with an equimolar amount of σ70 or RNAP holoenzyme (core RNAP/σ70) was challenged with an equimolar amount of HepA. σ70 effectively displaced HepA from core RNAP, whereas HepA was unable to displace σ70 (data not shown).

We adventitiously found that treatment of purified HepA with [γ-32P]ATP and calf heart protein kinase resulted in covalent labeling of HepA with [32P]. We took advantage of this ability to radioactively label HepA to quantify its interaction with core RNAP. Increasing amounts of labeled HepA were mixed with a constant amount of core RNAP, incubated for 15 min at 37 °C to form a complex, and then analyzed by the native gel shift assay (Fig. 2C). The amounts of free HepA and HepA associated with RNAP (and therefore shifted to the lower mobility band) were quantitated by PhosphorImager analysis of the gels. A Scatchard analysis of the results (Fig. 2D) revealed that HepA interacts with core RNAP in a 1:1 complex with a dissociation constant \(K_d\) of 75 nM.

**Fig. 2.** HepA binds core RNAP (but not holoenzyme) with a 1:1 stoichiometry and a \(K_d\) of 75 nM. A, holoenzyme or core RNAP were analyzed by electrophoresis on a 5% polyacrylamide gel under non-denaturing conditions in the presence or absence of a 2-fold molar excess of HepA. Lane 1, holoenzyme + HepA; lane 2, holoenzyme; lane 3, HepA; lane 4, core + HepA; lane 5, core; lane 6, HepA. The boxed regions labeled A, B, or C were excised from the native gel and analyzed by SDS-PAGE as shown in panel B. B, the bands excised from the native gel (panel A) were analyzed by SDS-PAGE and Coomassie staining to determine the protein composition of each band. The labels above the lanes (A, B, and C) correspond to the bands from the native gel. The lane marked M contains markers (RNAP holoenzyme and purified HepA). C, increasing amounts of [32P]HepA (0.5–3.75 molar excess over core RNAP) were combined with a constant amount of core RNAP, incubated at 37 °C for 15 min, electrophoresed on a 5% polyacrylamide gel under non-denaturing conditions to separate bound (B) and free (F) HepA. The gel was visualized by a PhosphorImager. D, the RNAP-bound and free HepA in C were quantitated, and the results are presented as a Scatchard plot (21). \(v\) is the mol of bound HepA/mol of free HepA; \(l\) is the concentration of core RNAP in the binding reaction (μM).
The finding that HepA binds core RNAP and not holoenzyme is not in contradiction to our earlier finding that HepA contaminated purified fractions of both core RNAP and holoenzyme off the final MonoQ column fractionation (Fig. 1). During the purification of overexpressed, free HepA, we found that it eluted from the MonoQ column at about 0.3 M NaCl, about the same as core RNAP (13). Therefore, we believe the HepA found in core RNAP fractions (Fig. 1, lanes 11 and 12) corresponds to the unbound fraction of HepA, whereas the HepA found in holoenzyme fractions (Fig. 1, lanes 16 and 17) corresponds to HepA in the core RNAP-HepA complex, which co-elutes roughly with holoenzyme. This is possible because the molar amount of σ73 is roughly half that of the RNAP. Based on the Coomassie-stained bands, it appears that roughly 60% of the HepA is bound to RNAP, whereas about 40% is found in the unbound fraction.

Insertion Inactivation of HepA Results in Sensitivity to DNA Damage—To assess the role of HepA in cellular processes, we constructed an insertion of a Km r cassette at codon 304 (between helicase motifs II and III) in the genomic hepA gene of two separate strains of E. coli, NM522, and JC7623 (recBC sbcBC; Ref. 10). Insertion of the Km r cassette at this position of the hepA gene results in a predicted protein product less than one-third the length of full-length HepA and containing only two of the six helicase motifs (I and II). Thus, the normal function of HepA was undoubtedly disrupted.

The two E. coli strains with the disrupted hepA gene (JC7623ΔhepA and NM522ΔhepA) did not exhibit any obvious growth phenotypes over a temperature range of 25–45 °C. Because of the role, or suspected role, of many SNF2 family members in various DNA repair processes, we tested JC7623ΔhepA and NM522ΔhepA for their ability to survive exposure to UV light, which is known to cause DNA damage. An example of the results is shown in Fig. 3, where survival of JC7623 and JC7623ΔhepA to increasing times of UV exposure is compared. The results, tabulated in Table I, show clearly that the hepA gene disruption in JC7623ΔhepA and NM522ΔhepA results in significantly reduced survival to UV exposure compared with the parent strains JC7623 and NM522.

ATPase Activity Associated with HepA—Because several members of the SNF2 family have been shown to be DNA-dependent ATPases (5), we tested fractions of the highly purified HepA from the Superose 6 gel filtration column for ATPase activity. Ability to hydrolyze the γ-phosphate from [γ-32P]ATP was monitored by thin layer chromatography (11), revealing an ATPase activity above background (Fig. 4A). The ATPase activity in each fraction was nearly exactly proportional to the protein concentration in the fraction (Fig. 4B).

To rule out the possibility that this weak ATPase activity arose from a contaminant of the purified HepA preparation, we compared the ATPase activity of purified RNAP from JC7623ΔhepA and JC7623ΔhepA/pHepA (JC7623ΔhepA transformed with a plasmid expressing HepA under the control of the Trc promoter). As expected, the purified RNAP from JC7623ΔhepA/pHepA induced with IPTG contained HepA (Fig. 4C).
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4C, lane 2), whereas RNAP from JC7623ΔhepA did not (Fig. 4C, lane 3). An ATPase assay of these same samples indicated that RNAP from JC7623ΔhepA had ATPase activity similar to a background sample with no added protein (Fig. 4C, lanes 6 and 7), whereas RNAP from JC7623.hepA+pHepA had ATPase activity well above background (Fig. 4C, lane 5). Thus, we conclude that RNAP preparations have an ATPase activity only in the presence of HepA. This ATPase activity was not DNA- or RNA-dependent and was not stimulated by the addition of tRNA, double-stranded DNA, single-stranded DNA, or core RNAP (data not shown).

DISCUSSION

We have identified HepA, an E. coli protein that shares extensive sequence homology with the SNF2 family of putative helicases (3, 5, 17), as an RNAP-associated factor. We cloned and purified HepA and showed that RNAP preparations have an ATPase activity only in the presence of HepA and that HepA associates with core RNAP in vitro (but not holoenzyme). We disrupted the hepA gene in E. coli, resulting in a phenotype displaying sensitivity to UV exposure.

The SNF2 family includes proteins from viral, prokaryotic, and eukaryotic species with roles in cellular processes such as cell cycle control (STH1), transcriptional regulation and chromatin remodeling (ATR-X, BRM, hBRM, MOT1, ISW1, and SNF2), nucleotide excision repair (RAD16 and ERCC6), mitotic recombination (RAD54), and other types of DNA repair (RAD5). The family also includes many proteins with no known function.

HepA is predicted to be an ATPase based on its extensive sequence similarity with other ATPases, and thus the ATPase activity associated with RNAP preparations only in the presence of HepA is likely to belong to HepA itself. The results of our experiments, however, do not rule out the possibility that the ATPase activity is associated with another protein (perhaps the RNAP itself) and that this activity is greatly stimulated by HepA. In contrast to SNF2 family members that have been shown to have ATPase activity (Saccharomyces cerevisiae SNF2 and MOT1, human HIP116A); however, this ATPase activity does not appear to be DNA-dependent.

The close relationship between the SNF2 family of proteins and known helicases (2) led us to test the purified HepA protein for helicase activity on various DNA and RNA substrates. Helicase activity has not been demonstrated for any SNF2 family member, and we were unable to detect any activity for HepA (data not shown).

Because of the observed association between HepA and core RNAP, we also tested the effect of purified HepA protein on various in vitro transcription assays. We tested the effect of HepA on abortive initiation (19) by γ200 holoenzyme at the T7 A1 promoter. We also formed ternary elongation complexes containing a 20-mer transcript on the T7 A1 tr2 transcription unit (20), added HepA, and then added nucleotides to initiate transcription elongation. We then examined the effect of HepA on transcription pausing, on the overall transcription elongation rate, and on termination at tr2. Finally, we formed the 20-mer ternary complexes on the T7 A1 tr2 transcription unit and then added ATP and HepA and tested for displacement of the ternary complexes. In all of these investigations, we could not observe any effect of the purified HepA protein on abortive initiation, transcription elongation, or termination on the stability of the stalled ternary complexes (data not shown).

We constructed insertion inactivation mutants of hepA to obtain clues to the role HepA plays in cellular processes. The hepA disruption mutants were sensitive to UV exposure, suggesting that they were defective in some DNA repair process. The finding that HepA associates with RNAP links HepA with transcription, whereas the data from the hepA gene disruptions link HepA with DNA repair.

It is interesting to note that the original observation of HepA co-purification with RNAP came from E. coli RL324 (harboring a C-terminal His6 tag in the chromosomal copy of rpoC, which codes for the RNAP β′ subunit, obtained from R. L. Landick), and subsequently from E. coli JC7623 (10), the parent strain of RL324 containing the wild-type rpoC gene, eliminating the possibility that HepA adventitiously associated with the RNAP from RL324 through the His6 tag at the C terminus of the β′ subunit. Although we have found HepA co-purification with RNAP from other E. coli strains, the amounts of HepA associated with RNAP in these two strains is always substantially greater, suggesting that expression of HepA is up-regulated in these strains. Both RL324 and its parent JC7623 are recBC sbcBC mutants. These mutations in the RecBCD enzyme complex are necessary for the efficient transformation of linear DNA into E. coli and were thus used for the construction of RL324. The RecBCD enzyme complex plays important roles in both recombination and DNA repair pathways. Thus, it is interesting to speculate that the possible disruption of RecBCD function may be compensated by increased expression of HepA, which we have linked to DNA repair.

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