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Cloning and Nucleotide Sequence of the *firA* Gene and the *firA200*(Ts) Allele from *Escherichia coli*

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The *Escherichia coli* gene *firA*, previously reported to code for a small, histonelike DNA-binding protein, has been cloned and found to reside immediately downstream from *skp*, a gene previously identified as the *firA* locus. *firA* encodes a 36-kDa protein. The mutant *firA200*(Ts) allele was also cloned and shown to contain three mutations, each mutation giving rise to a single amino acid change. Partially purified wild-type FirA (from a *firA*$^+$ strain) and mutant FirA [from a *firA200*(Ts) strain] proteins have amino-terminal sequences predicted from their common DNA sequences. Both proteins lack an N-terminal methionine. Modest overexpression of wild-type or mutant FirA restored wild-type growth to *firA200*(Ts) strains at 43°C, whereas high-level expression of wild-type FirA was required for more complete suppression of the rifampin sensitivity of *firA200*(Ts) *rpoB* double mutants. High-level expression of mutant FirA did not suppress this rifampin sensitivity.

It is well documented that selection for rifampin resistance in *Escherichia coli* gives mutations in *rpoB*, the gene encoding the β subunit of RNA polymerase (13, 16, 27). Suppressors of the rifampin resistance phenotype are worth studying because of the insight they might provide into the components of the transcription machinery. Several loci which can suppress this rifampin resistance have been identified and have been termed *fir* mutants (*rif* spelled backwards): *firA* (19) in the 4-min region, *firB* (26) in the 90-min region, and *firC* (3). Among these, *firA* has been the most studied. The *firA 200*(Ts) allele has been shown to cause temperature sensitivity for growth and to reverse the rifampin resistance of rifampin-resistant *rpoB* mutants (21). *firA* was also reported to confer temperature sensitivity to in vitro RNA synthesis by RNA polymerase partially purified from a *firA200*(Ts) strain and has been hypothesized to interact with RNA polymerase in vivo (22). A recombinant lambda phage containing a 6.7-kb insert was shown to complement the *firA200*(Ts) phenotypes (20) and to encode a 17-kDa histonelike DNA-binding protein. Subsequently, researchers (15) localized this complementing region to a 2.3-kb fragment. DNA sequence analysis identified an open reading frame (ORF) encoding this 17-kDa protein. This ORF was called *skp* and was inferred to be identical to the *firA* gene (1).

In this study, we report the cloning of the *firA* and *firA200* genes and show that *firA* is located immediately downstream from the previously reported *skp* gene. We also discuss data which indicate that the FirA protein coimmunoprecipitates with RNA polymerase holoenzyme. This study is part of our larger interest in better defining the role, if any, of *firA* in transcription.

MATERIALS AND METHODS

Strains and media. All strains used are listed in Table 1. Strain RL25-1 was obtained from R. Menzel (Bristol-Myers-Squibb Co.), and KK2186 (31) was a gift from R. Zagursky (Du Pont Co.). JCR20, containing the authentic *firA200*(Ts) allele, was kindly supplied by J. Coleman, University of Louisiana, and was obtained by crossing a P1 lysate of the authentic RL25 into MF6R (Rif^r *dapD*) (7) and selecting for prototrophs. The library of Tn*10d*-Cam transposon insertions was made (A. Segall, National Institutes of Health) by the reference method (9). Cells were grown in LB medium alone or LB medium containing 150 μg of ampicillin per ml or 150 or 50 μg of rifampin per ml. For maintenance of pMS421, spectinomycin was used at 50 μg/ml. ID15 was isolated as a spontaneous rifampin-resistant colony on LB plates containing 150 μg of rifampin per ml. Plates were incubated at 42 and 43°C in water-jacketed incubators.

Strain construction. P1 transduction was performed as previously described (24) with P1 *vir* (R. Menzel; from RFM443).

Materials and chemicals. Restriction enzymes, plasmid pUC19, and T4 DNA ligase were purchased from New England BioLabs (Beverly, Mass.), Bethesda Research Laboratories (Gathersburg, Md.), or IBI. A *Hin*dIII library of *E. coli* K-12 DNA (MG1655), was prepared essentially as previously described (28) at the Cold Spring Harbor 1988 Advanced Bacterial Genetics Course on cosmid pLAFR2. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was done on 10 or 10 to 20% SDS gradient gels (84 by 90 by 1 mm) (Daiichi) run on an Enprotech Double Gel Device. [^{35}S]methionine (1,000 Ci/mmol) and [^{35}S]dATP (1,350 Ci/mmol) were obtained from NEN Research Products, Boston, Mass. Rabbit polyclonal antiserum to RNA polymerase holoenzyme was a kind gift from Richard Burgess, University of Wisconsin. *Staphylococcus aureus* cells were obtained from ICN Immunobiologicals (Lisle, Ill.) as 50-ml samples and were taken up in 50 ml of B buffer (0.12 M NaCl, 50 mM Tris hydrochloride [pH 8], 0.5% Nonidet P-40), once with 50 ml of B buffer–0.25 M LiCl, and again with B buffer.

DNA manipulations and transformations. All DNA manipulations and cell transformations were done as described by Sambrook et al. (28). DNA used for hybridization was purified by electroelution (28). After nick translation (28), the labeled DNA was purified by passage through a G-50 Sepharose column. DNA sequencing was performed by the dideoxy method with [^{35}S]dATP, using Sequenase and the sequencing kit from United States Biochemical Corp., Cleveland, Ohio, with primers prepared on an Applied Biosystems 380A DNA Synthesizer and purified on a Rainin Rabbit HPLC system.

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TABLE 1. *Escherichia coli* K-12 strains used in this study

Strain	Genotype	Reference or source
MG1655	Wild type	12a
RL25	*metB tonA firA200* HfrP4X	21
RL25-1[a]	*firA201*	R. Menzel
MF6R	Spontaneous rifampin-resistant mutant of MF6	7
JCR20	Same as MF6R but *dapD*$^+$ *firA200*	7
RFM443	*galK2 lac-74 rpsL200*	R. Menzel
ID15	Same as RFM443 but *rpoB*	Spontaneous Rif^r mutant
ID29	Same as RL25-1 but *firA*$^+$ *zae*::Tn*10d*-Cam	P1 (RFM443 pool Tn*10d*-Cam) × RL25-1
ID28	Same as RL25-1 but *zae*::Tn*10d*-Cam	
ID19	Same as RFM443 but *firA*$^+$ *zae*::Tn*10d*-Cam	P1 (ID28) × RFM443
ID23	Same as JCR20 but *zae*::Tn*10d*-Cam	P1 (ID19) × JCR20
ID18	Same as RFM443 but *firA200 zae*::Tn*10d*-Cam	P1 (ID23) × RFM443
ID20	Same as RFM443 but *rpoB firA200 zae*::Tn*10d*-Cam	P1 (ID18) × ID15
ID21	Same as RFM443 but *rpoB firA*$^+$ *zae*::Tn*10d*-Cam	P1 (ID18) × ID15
ID22	Same as ID20 but *recA*	
KK2186	*supE sbcB15 hsdR4 rpsL thi* Δ(*lac-proAB*) ([F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15])	31

[a] RL25-1 is our designation for the strain used in this study which was *firA*(Ts) but did not confer rifampin sensitivity to rifampin-resistant strains.

Protein purification for antibody preparation and amino-terminal sequencing. The *firA200*(Ts) protein was partially purified from a culture of ID18 containing pID4 by making an aggregate protein preparation (29) as follows. Bacterial cells were harvested by centrifugation and resuspended in 1/20 the culture volume with lysis buffer (50 mM Tris hydrochloride [pH 7.9], 200 mM NaCl, 2 mM EDTA, 2 mM 2-mercaptoethanol). Lysozyme was dissolved in the preparation to give a final concentration of 200 μg/ml, and the mixture was incubated for 20 min at 0°C. Triton X-100 was added to a final concentration of 1%, and the mixture was incubated for 10 min at 0°C. Zwitterionic detergent 3-14 (Calbiochem) was added to a final concentration of 0.5%, and the mixture was incubated at 0°C for 10 min. The preparation was sonicated (three times for 10 s each time on ice), adjusted to concentrations of 5 mM $MgCl_2$ and 50 μg of DNase I per ml, and incubated at 37°C for 15 min. The mixture that had been treated with DNase was then overlaid on 40% sucrose in phosphate-buffered saline (PBS) and pelleted by centrifugation for 30 min. The pellet was resuspended in PBS. FirA was separated by preparative gel electrophoresis, and the gel slice was extracted with 0.1% SDS in 50 mM ammonium bicarbonate. Amino-terminal sequence analysis of mutant and wild-type FirA was done by an automated Edman degradation method, using an ABI 470A protein sequencer (gas/vapor) interfaced with an ABI 120A PTH analyzer. FirA (rabbit) polyclonal antiserum was raised against *firA200*(Ts) FirA protein (mutant) by Hazelton Research Products, Denver, Pa.

In vitro T/T. In vitro transcription/translation (T/T) was done with an S-30 extract (2, 23) from strain RFM443 supplied by R. Menzel and M. Carty and labeled with [^{35}S]methionine as previously described (23). The best results were obtained with plasmid DNA that had been purified by two equilibrium density gradient centrifugations, as previously described (28).

Sample preparation. Lysates for immunoprecipitation were prepared by labeling exponentially growing cells (1 ml, A_{600} = 0.4) for 10 min with 50 μCi of [^{35}S]methionine (1,000 Ci/mmol) in LB. The labeling was terminated by making the culture 10% in trichloroacetic acid and allowing it to stand at 0°C for 20 min. The trichloroacetic acid precipitate was pelleted by centrifugation for 5 min, washed twice with 80% acetone, and resuspended in 100 μl of sample buffer (1% SDS, 50 mM Tris hydrochloride [pH 8], 1 mM EDTA). The sample was boiled for 2 min and then diluted with 1 ml of AB buffer (2% Triton X-100, 50 mM Tris hydrochloride [pH 8], 150 mM NaCl, 0.1 mM EDTA). The sample was spun for 10 min at 4°C in a microcentrifuge, and the supernatant was carefully removed. The supernatant (2×10^6 cpm) was brought to a total volume of 500 μl with AB buffer, and 2.5 μl of FirA or RNA polymerase antibody was added. After sitting overnight at 4°C, a suspension of 50 μl of *S. aureus* cells was added. The mixture was vortexed and placed on ice for 10 min. After pelleting in the microcentrifuge, the supernatant was discarded and the pellet was washed once with B buffer (0.12 M NaCl, 50 mM Tris hydrochloride [pH 8], 0.5% Nonidet P-40), once with B buffer plus 0.25 M LiCl, and again with B buffer. The final pellet was resuspended in 25 μl of SDS loading buffer, boiled for 2 min, and loaded on SDS gels. Lysates for immunoblotting (Western blots) were prepared according to a published method (28). Western blots were performed by using a 1:1,500 dilution of FirA or RNA polymerase antiserum.

Computer programs. Computer programs used in analyzing nucleotide and amino acid sequences were provided by the University of Wisconsin Genetics Computer group (8) and were run on a VAX8800. Analysis for the presence of possible promoter sequences was done by using an algorithm from Mulligan et al. (25).

Nucleotide sequence accession number. The EMBL accession number for the *firA* sequence is X54797.

RESULTS

Genetics. Strain RL25-1, containing the *firA201*(Ts) allele, is our designation for a derivative of RL25 (21), known to contain the *firA200*(Ts) allele. *firA201*(Ts) can be distinguished from *firA200*(Ts) because it does not reverse the rifampin resistance of rifampin-resistant *rpoB* mutants. The *firA201*(Ts) allele was initially recognized by cotransduction with a linked chloramphenicol resistance marker. This was done by growing bacteriophage P1 *vir* on strain RFM443 containing a pool of random Tn*10d*-Cam insertions according to a published protocol (9). Next, a P1 lysate derived from this pool was used to infect RL25-1. Chloramphenicol-

resistant colonies growing at 43°C were selected. We were able to demonstrate that the wild-type *firA* allele was 55% cotransduced with the Cm^r marker. FirA-linked, chloramphenicol-resistant transductants that were temperature sensitive (ID3, *firA201*) and temperature resistant (ID1, *firA*$^+$) were used in further crosses. Any *fir* phenotype in ID3 was assessed by transfer of the temperature sensitivity phenotype to several spontaneous rifampin-resistant mutants derived from RFM443. Chloramphenicol-resistant, temperature-sensitive transductants showed no alteration in their rifampin resistance.

We established that *firA201*(Ts) and *firA200*(Ts) were different alleles as follows. JCR20 (*firA200 rpoB*) (7) was transduced to chloramphenicol resistance with a P1 lysate of ID1 (*firA*$^+$ Cm^r). Of the Cm^r transductants, 59% were temperature resistant and had regained a level of rifampin resistance equal to that of the control strain MF6R (*rpoB*). This established that the transposon and *firA* were linked. Phenotypic differences between the *firA200*(Ts) and *firA201*(Ts) alleles were clarified by crossing strain JCR20 with a P1 lysate of ID3 [*firA201*(Ts)]. Chloramphenicol-resistant transductants were selected and screened for temperature sensitivity. The key observation was that two classes of temperature-sensitive transductants were obtained: 56% were rifampin resistant [temperature sensitivity derived from the *firA201*(Ts) allele] and 38% were rifampin sensitive [temperature sensitivity derived from the *firA200*(Ts) allele]. The first class, containing the *firA200*(Ts) allele (rifampin sensitive), did not grow at all on LB plates at 42°C, whereas the class containing the *firA201*(Ts) allele grew only slightly at 42°C. Two other classes of transductants were isolated: 3% grew well at 42°C and were rifampin resistant and 3% were completely temperature-sensitive for growth at 42°C but nevertheless retained resistance to rifampin.

The relative order of the *firA201*(Ts) and *firA200*(Ts) alleles and the *dapD* and Cm^r markers was determined. By plating a P1 lysate of ID3 [*firA201*(Ts) *dapD*$^+$] on MF6R (rifampin resistant, *dapD* mutant) and selecting for *dapD*$^+$, followed by screening for both Cm^r and growth at 43°C, we established that the *firA201*(Ts) allele and Cm^r were 80 and 95% linked to *dapD*, respectively. The *firA200*(Ts) allele in the JCR20 background (ID23) was moved into our standard background (RFM443) by P1 transduction. Selection for chloramphenicol resistance and screening for temperature sensitivity gave ID18, and selection for chloramphenicol resistance gave ID19, the temperature-resistant isogenic strain. ID15 was isolated as a spontaneous rifampin-resistant mutant of RFM443 and was transduced to chloramphenicol resistance and screened for growth at 43°C, giving ID20 [*firA200*(Ts) *rpoB*] and ID21 (*rpoB*). The cotransduction frequencies between chloramphenicol resistance and the *firA201*(Ts) (55%) and the *firA200*(Ts) (59%) alleles and *dapD* are shown in Fig. 1; the *firA201*(Ts) and *firA200*(Ts) alleles are both downstream of the chloramphenicol resistance transposon.

The absolute relationship between the two *firA* alleles was established by genomic sequencing of *firA* from *firA200* and *firA201* strains. As will be described below, the *firA200*(Ts) allele has three mutations compared with the wild-type sequence. The *firA201*(Ts) allele shares two of these three mutations in common. Therefore, both alleles very likely evolved from a common progenitor.

Cloning of the *firA* gene. A library of cosmid clones derived from a *Hin*dIII digestion of MG1655 was used to transform RL25-1 [*firA201*(Ts)]. Complementing clones which were

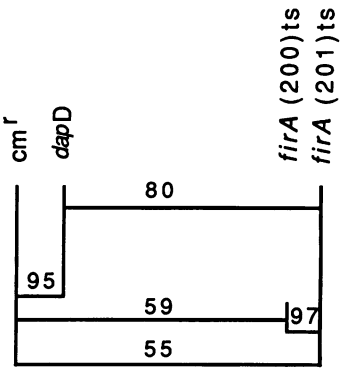


FIG. 1. Cotransduction frequencies. Positions of markers in the region of *firA* as determined by P1 transduction data. The anomalously high cotransduction frequency between *dapD* and *firA201*(Ts) (in the RM443 background) is reproducible.

tetracycline resistant and thermoresistant were obtained by double selection on LB medium plus tetracycline at 43°C. One candidate, pID1, was digested with *Pst*I and ligated into pUC19. The ligation mixture was used to transform RL25-1 to ampicillin resistance, and a complementing subclone, pID3, was isolated after screening on LB medium plus ampicillin at 43°C. Plasmid DNA from several clones contained a 1.4-kb insert, as determined by digestion with *Pst*I, *Hin*dIII, and *Eco*RV (data not shown). The orientation of this insert was opposite to *lacZ* and was reversed by cloning the insert into pUC18 (pID2). Subsequent experiments demonstrated that both the original cosmid clone, pID1, and the subclone, pID3, also complement the phenotypes of the *firA200*(Ts) allele (growth curves for strains with and without pID3 are shown in Fig. 2).

The *firA* gene is distinct from the *skp* gene. According to high-resolution restriction maps (1), pID3 covers a previously identified ORF (22a) coding for a 36-kDa protein (ORF36). The 5′ end of this clone overlaps the 3′ end of the *skp* gene. The *skp* gene was thought to encode a 17-kDa histonelike DNA-binding protein (HLPI) and was previously

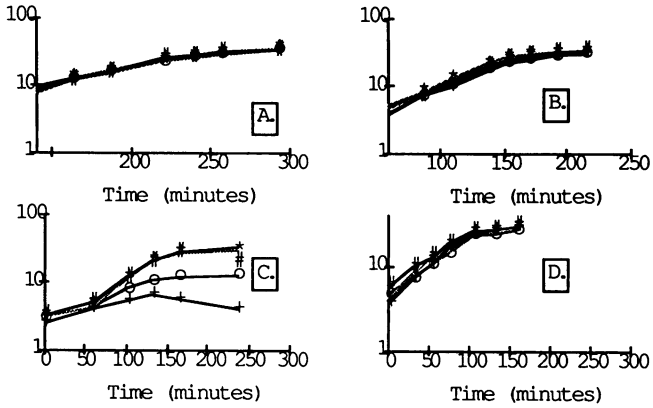
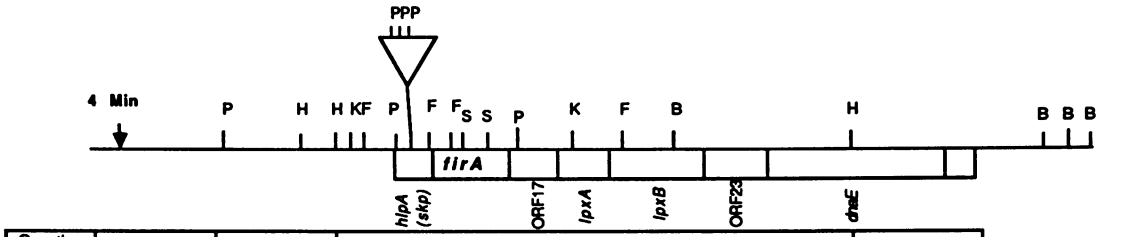


FIG. 2. Growth of strains in liquid medium. Overnight cultures of strains ID18 *firA200*: (○), ID19 *firA*$^+$ (*), ID20 *firA200 rpoB* (+), and ID21 *rpoB* (#) were diluted 1:100 into LB and shaken at 250 rpm. Growth was determined at 30°C (A), 37°C (B), and 40°C (C and D) in Klett units, converted to cell density, and plotted as 10^7 cells per ml. Strains containing the *firA* plasmid, pID3, were grown in LB medium containing 150 μg of ampicillin per ml (D).



Growth on LB @43 °C	Growth on LB/rif @32°C	Zone of inh. (mm)	Plasmid
+++	+++	ND	pID1
++	+++	12.3	pID2 >>
+++	++	14.5	pID3 ><
+	+/-	15.7	pID4 >>
++	+/-	15.7	pID5 ><
-	-	ND	pID6 >>
-	-	ND	pID7 ><
-	-	20.5	pID8 ><
-	-	ND	pID9 >>
+++	+++	13.0	pGAH317
-	-	26.0	pID14
-	-	24.3	pID15
-	-	22.0	-

B= *Bam*HI
F= *Eco* RV
H= *Hin* DIII
K= *Kpn* I
P= *Pst* I
S= *Bss* HII

FIG. 3. Genetic organization of the 4-min region of *E. coli* (adapted from reference 7), including complementation data. Shown above and below the map are defined regions used for complementation of the *fir* phenotypes. The designation >> indicates the orientation of *firA* is the same as *lacZ*; >< indicates the orientation is opposite to *lacZ*. By restriction analysis, cosmid pID1 contains the same 6.7-kb insert as the original *firA* lambda transducing phage (data not shown). The small insert size in the cosmid was apparently accommodated by insertion of the fragment into a dimer of the cosmid vector. Growth of transformants of ID22 (*firA200 rpoB recA*) containing various plasmids was assayed as follows. Fresh single colonies were streaked onto LB plates plus ampicillin at 43°C or onto LB plates with ampicillin and 50 μg of rifampin per ml at 32°C. The main streak was cross streaked with a second toothpick, and the cross streak was again crossed with a third toothpick. After incubation for 2 days, growth was scored as follows: +++, full growth on main, cross, and third streak; ++, growth on main and cross streak; +/−, growth on main streak only; −, no growth. Zones of inhibition were assayed by mixing 2 ml of overnight culture with 3 ml of soft agar, layering onto LB plates, and placing a 0.25-in. (0.64-cm)-diameter paper disk (31039; BBL Microbiology Systems) in the center of the agar. Ten microliters of rifampin (103 mg/ml in dimethyl formamide) was adsorbed onto the disk, and the plates were incubated overnight. The zone of inhibition is defined as the diameter of the circle within which rifampin had inhibited growth. ND, Not determined; inh., inhibition.

identified as the *firA* gene by complementation with pGAH317 (15), a plasmid covering both *skp* and ORF36. However, the *skp* gene has recently been shown to encode a protein involved in the export of bacterial proteins (14, 28a), and an analogous open reading frame in *Salmonella typhimurium* is thought to code for an outer membrane protein which binds to lipopolysaccharide (18).

Transformants of ID22 [*firA200*(Ts) *rpoB recA*] with the clones pID2 or pID3 are thermoresistant and rifampin resistant, thereby strongly suggesting that ORF36 is the *firA* gene. To better define the region involved in complementation, a *Bss*HII fragment spanning the middle of ORF36 was deleted from pID2 and pID3, giving plasmids pID9 and pID8, respectively. These plasmids fail to complement ID22 for both phenotypes. Additionally, plasmids pID14 (pGAH317 with an internal deletion of the *Bss*HII fragment) and pID15 (*skp* ORF only) also fail to complement the phenotypes. In a marker rescue experiment, transformants of the *rpoB* *firA200*(Ts) *recA*$^+$ strain ID20 containing pID7 (this construct lacks sequences coding for the 30 C-terminal amino acids of HLPI, any putative *firA* promoter, and the translational start and first 21 amino acids of FirA) are temperature sensitive but give temperature-resistant recombinants at a frequency 100 times that of the simple reversion frequency. Of 16 recombinants tested, all regained a level of rifampin resistance at 32°C equal to the rifampin resistance of the *firA*$^+$ strain ID21. Thus, we conclude that *firA* restores growth at the nonpermissive temperature as well as rifampin resistance at 32°C by true complementation and that the *firA* gene is encoded by ORF36. The genetic organization of the 4-min region of *E. coli* (Fig. 3) shows the regions of DNA used in this and other studies for complementation analysis (4, 7, 15).

Cloning of the *firA* gene containing the *firA200*(Ts) allele. In order to clone the mutant *firA200*(Ts) allele, chromosomal DNA from JCR20 was digested with *Pst*I, and 1- to 2-kb

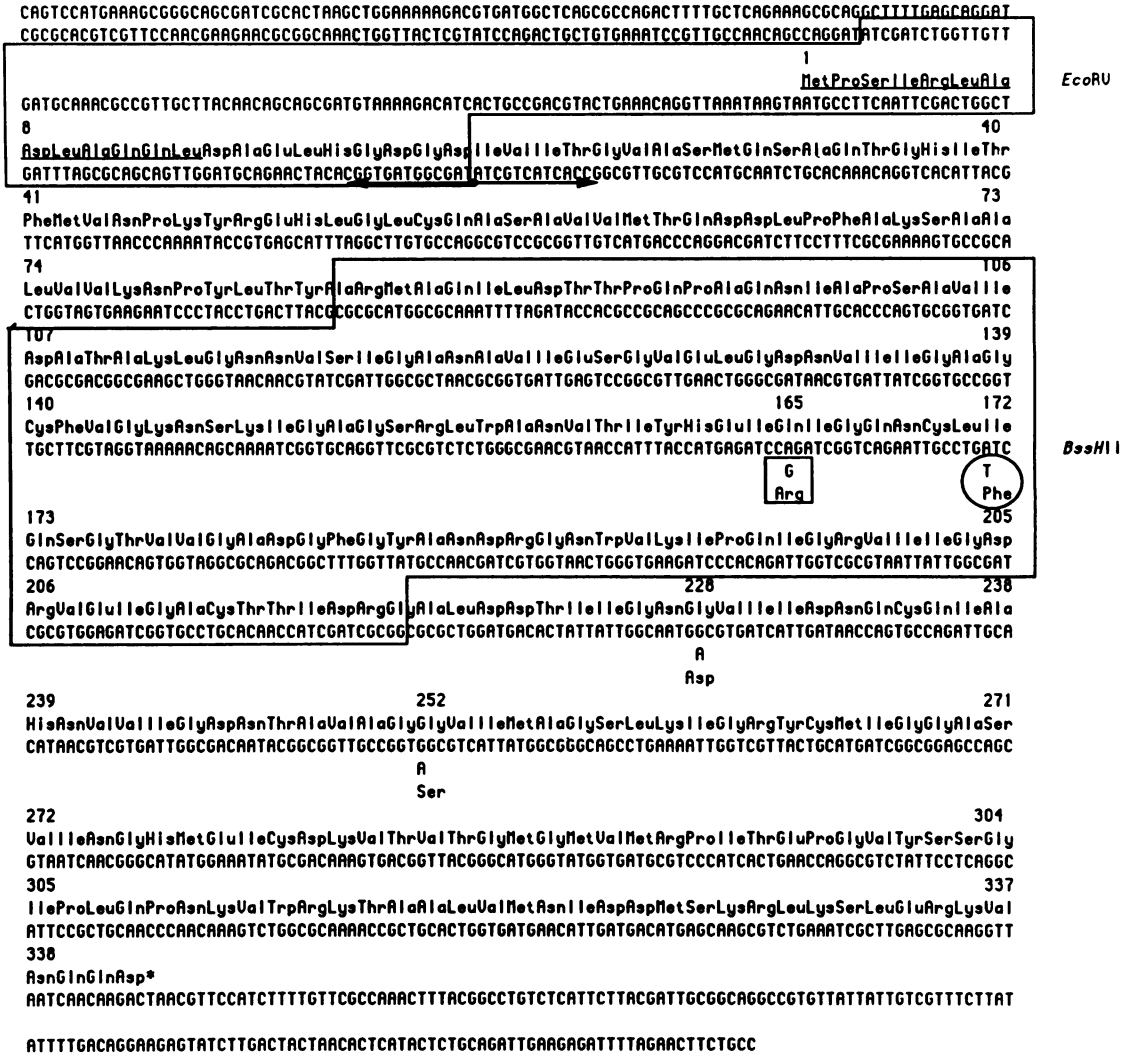


FIG. 4. Nucleotide and predicted protein sequence from wild-type, *firA200* and *firA201* DNA. Regions bounded by *Eco*RV and *Bss*HII sites are boxed. Arrows indicate inverted repeats which may serve as a transcriptional attenuator for transcripts initiated upstream of *hlpA* or a binding site for a regulatory protein. The predicted amino acid sequence of the wild-type sequence is shown above its nucleotide sequence. Nucleotide changes in the *firA200* and *firA201* mutants are shown below the wild-type sequence. Changes common to both mutants are unboxed and uncircled, the unique mutation in *firA200* is boxed, and the unique mutation in *firA201* is circled. Peptide sequences derived from amino-terminal sequencing of the wild-type and *firA200* FirA proteins are underlined.

fragments were selected. These fragments were ligated into a pUC19 vector and used to transform KK2186, selecting for ampicillin resistance. Clones containing the mutant *firA* gene were identified by colony hybridization with a ^{32}P-labeled probe prepared from the wild-type *firA* clone. Several clones were isolated which contained the mutant *firA* gene in both orientations on 1.4-kb *Pst*I fragments analogous to the wild-type fragment used as the hybridization probe. Restriction digestions identified pID4 as having the insert in the same orientation as wild-type pID2. Plasmid pID5 was analogous to pID3 in that the gene is oriented opposite to the *lacZ* transcriptional start.

Sequences of the cloned *firA* genes. Cloned DNA containing the wild-type and *firA200*(Ts) alleles was sequenced (Fig. 4). The wild-type sequence of *firA* is identical to that provided by C. McHenry (22a). The *firA200*(Ts) mutant contains three mutations giving rise to three amino acid changes: Gln-165 → Arg-165, Gly-228 → Asp-228 and Gly-252 → Ser-252. These changes result in an increase of two in the total charge on the protein, with no net change in the overall charge balance. For comparison, the genomic copy of *firA* from RL25-1 [*firA201*(Ts)] was determined by genomic sequencing. *firA201* also has three mutations: Ile-172 → Phe-172, Gly-228 → Asp-228, and Gly-252 → Ser-252, two of which are identical to the mutations in the *firA200* strain. The sequence of the *firA* gene of *S. typhimurium* has recently been determined (13a). The sequences are 99% similar and share 96% identical amino acid residues, as determined by the gapping algorithm provided by the University of Wisconsin Genetics Computer group (8). Such very high sequence homology is unusual even between *E. coli* and *S. typhimurium*. This degree of conservation suggests that *firA* has an important role in cellular function.

In vitro expression of FirA. To identify the FirA protein, we used an S-30 in vitro T/T system (2, 17, 23). T/T of pID2, pID3, pID4, and pID5 shows that a 36-kDa protein is

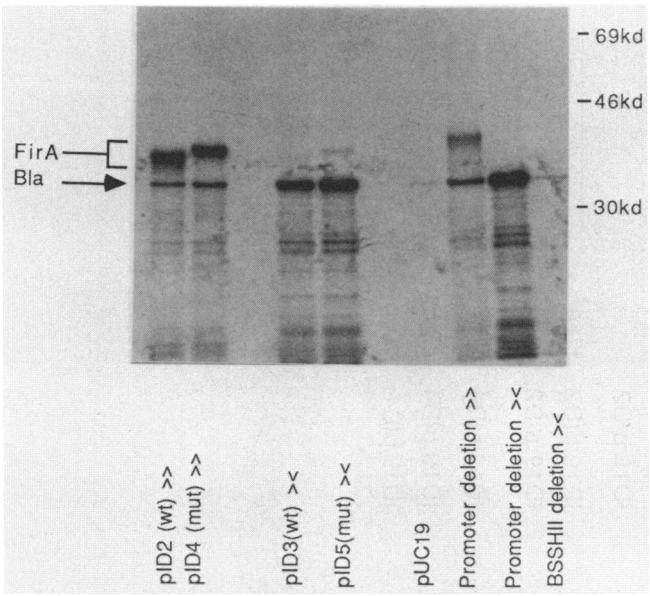


FIG. 5. In vitro synthesis of wild-type and mutant FirA protein by T/T from *firA* and *firA200* plasmid DNA. S-30 extracts from RFM443 (23) were incubated at 37°C for 1 h with 2 to 5 μg of plasmid DNA which had been purified twice by equilibrium density gradient centrifugation. The reactions were stopped by adding 1 ml of 10% cold TCA and incubating on ice for 20 min. Samples were spun at 14,000 for 10 min, washed twice with 80% acetone buffer (50 mM Tris hydrochloride [pH 8], 1 mM EDTA), resuspended in SDS sample buffer, and run on a 10 to 20% SDS gradient polyacrylamide gel after boiling for 5 min. The designation >> indicates the orientation of *firA* is the same as *lacZ*; >< indicates the orientation is opposite to *lacZ*. Bla, β-lactamase. Lanes: 1, pID2 (wild type driven by *lacZ* promoter); 2, pID4 (*firA200*) mutant, driven by *lacZ* promoter; 3, blank; 4, pID3 (wild type); 5, pID5 (*firA200* mutant); 6, blank; 7, pUC19; 8, pID6 (*Eco*RV deletion from pID2); 9, pID7 (*Eco*RV deletion from pID3); 10, pID8 (*BSS*HII deletion from pID3).

expressed (Fig. 5). Plasmids pID2 and pID4 were much more highly expressed than pID3 and pID5, thereby indicating that the *lacZ* promoter was stronger than any internal *firA* promoter. The wild-type protein migrated at a slightly lower molecular mass than the mutant on SDS-polyacrylamide gels. This lower apparent molecular mass is possibly a reflection of the difference in overall charge on the proteins. As predicted on the basis of the deletion of the putative promoter region, plasmids pID6 and pID7 did not express a 36-kDa protein. However, T/T of pID6 did result in the expression of a larger 38-kDa protein which is most likely a fusion of upstream *skp* sequences to *firA*, using Met-74 from *skp* as the start codon and incorporating the next 58 amino acids from *skp* while lacking the first 21 amino acids of *firA* (Fig. 5).

FirA is a 36-kDa protein missing its terminal methionine. An aggregate preparation (29) of cell proteins was made from a strain overproducing the mutant FirA protein. The proteins were separated by gel electrophoresis on a 10 to 20% gradient SDS-polyacrylamide gel, and the mutant protein was identified by comparison to the mobility of a sample of [^{35}S]methionine-labeled mutant FirA prepared in vitro. The FirA-containing band was excised, and the protein was recovered by elution in 0.1% SDS in 50 mM ammonium bicarbonate. Amino-terminal sequencing gave the first 14 amino acids predicted by the DNA sequence. The amino-terminal methionine was missing, indicating in vivo processing. Sequencing of aggregate preparations of protein from strains overproducing wild-type FirA was more difficult (because of the smaller amount of wild-type FirA). However, careful separation of the wild-type protein from adjacent bands gave sufficiently pure protein for sequencing. An identical amino acid sequence (14 cycles) was obtained that was also lacking the N-terminal methionine.

In vivo FirA expression is determined by *firA* orientation within the vector. In order to study in vivo expression of FirA, polyclonal antiserum was raised against the mutant FirA protein. The mutant [*firA200*(Ts)] FirA protein was used because of its ease of isolation compared with that for the wild type. This polyclonal antiserum recognizes the *firA200*(Ts) FirA protein and the wild-type FirA protein, as determined by Western blot analysis of lysates from control and overproducing strains.

As discussed above, the in vitro expression level of both wild-type and mutant FirA depends on the orientation of the *Pst*I fragment in the pUC19 vector. When the *firA* gene was in the same orientation as the upstream *lacZ* promoter (from the pUC19 vector), the FirA protein was highly expressed (Fig. 5). We find that in vivo expression of FirA is similar with high-level expression resulting from having the *firA* gene downstream of the *lacZ* promoter. When the orientation of the *firA* gene is opposite to that of the *lacZ* transcriptional start, its expression in vivo is approximately 50-fold lower. Overexpression from the first construct is presumably a reflection of the strong *lacZ* promoter driving increased transcription, whereas the relatively low expression from the other construct is probably an indication of the relative weakness of whatever promoter sequences RNA polymerase recruits within the 3′ end of the *skp* gene.

As originally indicated by Coomassie blue-stained gels (data not shown) and also seen by Western blot analysis (Fig. 6), the relative amount of the mutant FirA protein appears to be greater than that for the wild type when the proteins are overexpressed in their respective backgrounds [ID18(pID4) versus ID19(pID2)], despite having the same strong *lacZ* promoter. We are considering the possibility that differences in the efficiency of translation or stability of the message cause this difference.

Complementation analysis with the *firA* and *firA200*(Ts) genes. The extent to which the wild-type *firA* or *firA200*(Ts) genes (present on multicopy plasmids) restore temperature resistance to the *firA200*(Ts) *rpoB recA* strain ID22 depends upon their orientation. When the *firA* gene was oriented opposite to the *lacZ* transcriptional start, complementation for growth at high temperature was better; i.e., transformants of ID22 containing pID3 grew better on LB plates at 43°C than did those containing pID2 (Fig. 3). The *firA200*(Ts) mutant plasmid pID5 gave poor growth relative to the wild-type pID3. Plasmid pID4 gave the poorest complementation. As discussed, in vivo expression of wild-type or mutant forms of FirA encoded by pID3 and pID5, respectively, was far lower than the high-level expression driven by pID2 and pID4. Thus, a moderate level of wild-type FirA expression was responsible for better *ts* complementation. If the phenotypes associated with the *firA200* allele were due to a total loss of function, overproduction of the mutant would not be expected to restore this function. The partial complementation by mutant FirA protein suggests that the *firA* phenotypes are a result of a loss of function which is partly restored by an increase in concentration, although just as for the wild type, this level is important since overexpression of

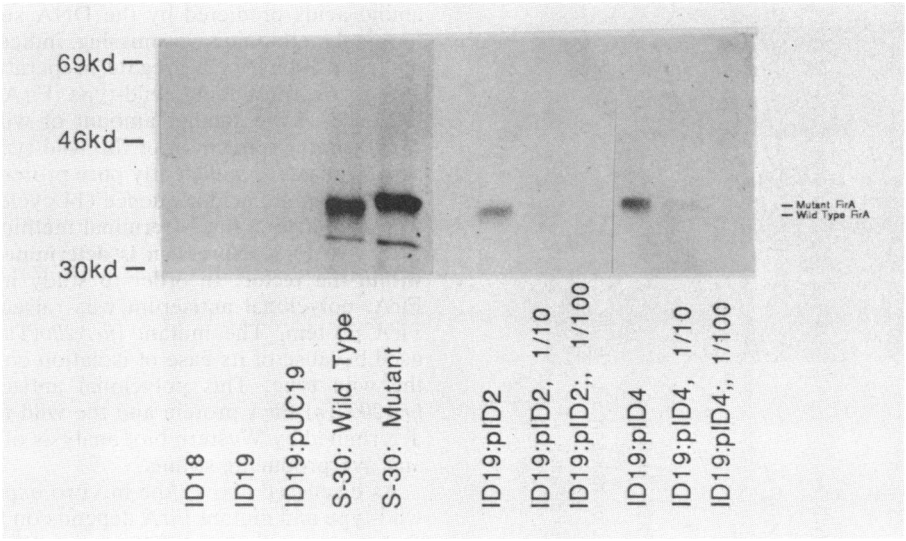


FIG. 6. Western blot analysis of lysates of strains with and without *firA* and *firA200* plasmids. Overnight cultures of strains were diluted 1:20 and grown in LB or LB plus ampicillin for 2 h. Samples were prepared as previously described (28) and subjected to SDS chromatography on 10 to 20% gradient polyacrylamide gels. The proteins were electroblotted onto nitrocellulose, and the blot was briefly soaked in 5% BSA before probing with a 1:1,500 dilution of FirA antiserum in 1× PBS. The FirA-antiserum complexes were labeled by reaction with ^{125}I-labeled protein A. Lanes (numbered from left to right): 1, ID18 (*firA200*); 2, ID19 (*firA*$^+$); 3, ID19(pUC19); 4, ^{35}S-labeled wild-type FirA from in vitro T/T of pID2; 5, ^{35}S-labeled *firA200* mutant FirA from in vitro T/T of pID4; 6, ID19(pID2) (wild type, driven by *lacZ* promoter); 7, ID19(pID2), 1/10 dilution; 8, ID19(pID2), 1/100 dilution; 9, ID19(pID4) (*firA200* mutant, driven by *lacZ* promoter; 10, ID19(pID4), 1/10 dilution; 11, ID19(pID4), 1/100 dilution.

the mutant by pID4 gives poorer complementation than with pID5.

In accord with this notion, it was reasoned that reducing expression from pID2 and pID4 might improve *ts* complementation to the level conferred by pID3 and pID5. This was evaluated by growing these strains in the presence of glucose or by cotransforming these strains with the compatible plasmid pMS421 containing the *lacI*^{q} gene (12). Under these conditions, we predicted that reduced transcription from the *lacZ* start (*lacOP*) because of the reduction of CAP protein and the presence of lac repressor, respectively, would increase *ts* complementation. This was observed (Table 2). *ts* complementation by plasmids in which the *lacZ* promoter was 5′ to the *firA* gene (pID2 and pID4) was increased in the presence of 0.3% glucose (although for some reason the presence of the *lacI*^{q} gene product eliminated growth of ID22 containing pID4 and pID5). Thus, it appears that excessive overexpression of FirA is not well tolerated at the nonpermissive temperature, although growth curves at permissive temperatures show no such adverse effects on growth versus controls (data not shown). This suggests that the FirA protein may have a latent activity which only manifests itself at higher temperatures.

Restoration of rifampin resistance is also determined by FirA levels. Restoration of rifampin resistance to ID22 (Table

TABLE 2. Relative growth at 43°C of transformants of ID22 *firA200 rpoB recA* containing wild-type and mutant *firA* clones[a]

Plasmid	Growth on:			
	LB	LB + glucose	LB (with *lacI*^{q})	LB + glucose (with *lacI*^{q})
pID3	5.0	5.0	5.0	5.00
pID2	2.5	3.5	4.0	5.00
pID5	1.5	1.0	0.0	0.05
pID4	2.0	3.0	0.0	0.05

[a] Transformants of ID22 containing pID2 through pID5 were plated as follows. Fresh single colonies were streaked onto LB plates plus ampicillin with and without 0.3% glucose; strains containing pMS421 (*lacI*^{q}) were plated onto LB plates plus ampicillin and spectinomycin and with or without 0.3% glucose. The main streak was cross streaked with a second toothpick, and the cross streak was again crossed with a third toothpick. After incubation for 1 day at 43°C, growth was scored as follows: 5, full growth on main, cross, and third streak; 3, growth on main and cross streak; 1, growth on main streak only.

TABLE 3. Relative growth on rifampin at 32°C of transformants of ID22 (*firA200 rpoB recA*) containing wild-type and mutant *firA* clones[a]

Plasmid	Growth on:			
	LB	LB + glucose	LB (with *lacI*^{q})	LB + glucose (with *lacI*^{q})
pID3	5.0	3.5	5.0	4.0
pID2	4.5	3.0	3.0	1.0
pID5	0.05	0.05	1.0	0.05
pID4	2.5	2.0	0.5	0.05

[a] Transformants of ID22 containing pID2 through pID5 were plated as follows: fresh single colonies were streaked onto LB plates plus ampicillin containing 50 μg of rifampin per ml with and without 0.3% glucose; strains containing pMS421 (*lacI*^{q}) were plated onto LB plates plus ampicillin and spectinomycin containing 50 μg of rifampin per ml with or without 0.3% glucose. Controls (data not shown) indicate that the *recA* mutant background itself reduced the rifampin resistance conferred by the *rpoB* allele. The main streak was cross streaked with a second toothpick, and the cross streak was again crossed with a third toothpick. After incubation for 2 days at 32°C, growth was scored as follows: 5, full growth on main, cross, and third streak; 3, growth on main and cross streak; 1, growth on main streak only.

TABLE 4. Rifampin resistance of strains assayed by zone-of-inhibition studies[a]

Plasmid	Ratio of zone of inhibition[b]			
	ID18	ID19	ID20	ID21
None	1.2	1.0	0.90	0.70
pID2	1.0	1.0	0.72	0.68
pID4	1.0	1.0	0.81	0.76

[a] Strains ID18 through ID21 with and without plasmids pID2 and pID4 were grown overnight in LB or LB plus ampicillin, respectively. An overnight culture (2 ml) was mixed with 3 ml of soft agar (with ampicillin for strains containing plasmids) and layered on top of LB plates. A 0.25-in. (ca. 0.64-cm)-diameter paper disk (31039; BBL Microbiology Systems) was placed in the center of the agar, and 10 μl of rifampin (103 mg/ml in dimethyl formamide) was adsorbed onto the disk. The plates were incubated at 32°C overnight with the disk side up. The diameter of the circle within which rifampin had inhibited growth was measured. The technique was reproducible to within 1 mm (triplicate).

[b] Values are expressed as the ratio of the diameter (12 to 24 mm) of the inhibited zone to that for ID19.

3) shows that pID2 or pID3 reverses the rifampin sensitivity induced by the *firA200*(Ts) allele. In contrast, overproduction of mutant FirA restores growth on rifampin-containing media to a negligible extent. However, in direct contrast to the results obtained for the temperature sensitivity phenotype, the use of conditions to reduce the expression of FirA from pID2 results in poorer growth on rifampin-containing media. Why rifampin resistance is greater with higher levels of FirA whereas temperature resistance is greater with lower levels of FirA is not presently understood.

In order to better quantify the effects of background and plasmid type, restoration of rifampin resistance was also assayed by zone-of-inhibition studies (Table 4 and Fig. 3). Strains harboring pID2 regained rifampin resistance, indicating that the FirA protein complements the phenotype. Strains containing pID4 showed a marginal increase in rifampin resistance, perhaps indicating that the mutant protein, when overexpressed, is able to complement the phenotype to some small extent. The most important result from these experiments, however, is that the *firA200*(Ts) allele induces greater rifampin sensitivity to the already rifampin-sensitive wild-type control strain. This suggests that this phenotype is not allele specific since it is imparted by the *firA200* allele regardless of whether the polymerase is mutant or wild type.

Coimmunoprecipitation of FirA with RNA polymerase antiserum. To investigate the possible interaction of FirA with RNA polymerase in vivo, strains with and without plasmids overproducing FirA were labeled with [^{35}S]methionine. Immunoprecipitation of proteins with antiserum raised against RNA polymerase shows that the FirA protein coimmunoprecipitated along with the RNA polymerase subunits when FirA was overexpressed (Fig. 7). Wild-type FirA comigrated with the α subunit of RNA polymerase on 10 to 20% gradient SDS gels, whereas the mutant FirA migrated more slowly and was resolved. Both wild-type and mutant forms of FirA were resolved from the α subunit on 10% SDS gels. Control Western blots indicate that the RNA polymerase antiserum was not cross-reactive with the FirA protein (data not shown).

To determine the relative amount of FirA protein coimmunoprecipitating with RNA polymerase antiserum, we probed unlabeled RNA polymerase immunoprecipitates with FirA polyclonal antisera (Fig. 8A). FirA was clearly present in RNA polymerase immunoprecipitates from strains in

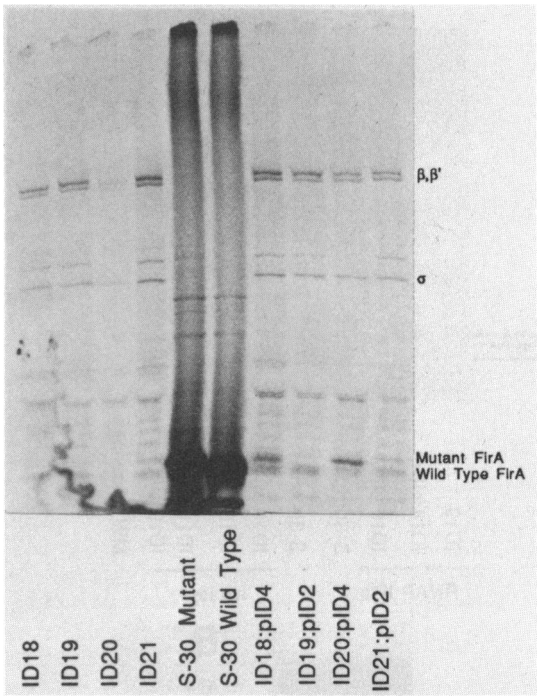


FIG. 7. RNA polymerase immunoprecipitates of [^{35}S]methionine-labeled proteins. Strains ID18 through ID21 with and without pID2 (overproducing wild-type FirA) and ID18 through ID21 with and without pID4 (overproducing *firA200* mutant FirA) were labeled with [^{35}S]methionine, and RNA polymerase holoenzyme was immunoprecipitated as described in Materials and Methods. Samples were run on a 10% polyacrylamide–SDS gel. Lanes: 1, ID18; 2, ID19; 3, ID20; 4, ID21; 5, ^{35}S-labeled wild-type FirA from in vitro T/T of pID2; 6, ^{35}S-labeled *firA200* mutant FirA from in vitro T/T of pID4; 7, ID18(pID4); 8, ID19(pID2); 9, ID20(pID4); 10, ID21(pID2).

which it is overproduced. A control (FirA preimmune serum washed with *S. aureus*) showed no comparable FirA coimmunoprecipitated with RNA polymerase (Fig. 8B). The intensity of the FirA band in the RNA polymerase immunoprecipitates (Fig. 7) was greater than that of the β and β′ bands. Since FirA is smaller than these subunits (36 versus 180 kDa), we conclude that the molar ratio of FirA to RNA polymerase within the RNA immunoprecipitates is likely to be greater than one.

On the basis of the relative intensity of the mutant FirA band, there is more mutant protein coimmunoprecipitating than wild-type FirA. This may simply reflect the higher level of the mutant protein versus the wild-type protein since FirA immunoprecipitates or Western blots done with FirA antiserum give the same result. As noted before, Coomassie blue-stained gels show more of the mutant being expressed than the wild type.

DISCUSSION

In 1977, Lathe (19) found that the *firA200* allele of *E. coli* renders RNA synthesis thermosensitive and eliminates the high-level resistance to rifampin associated with certain mutations in the β subunit of RNA polymerase. Because of this, *firA* was identified as being likely to code for an essential component of the transcription machinery. In a later report (21), a lambda transducing phage, 21*firA*19, was identified which restored growth at 42°C and rifampin resis-

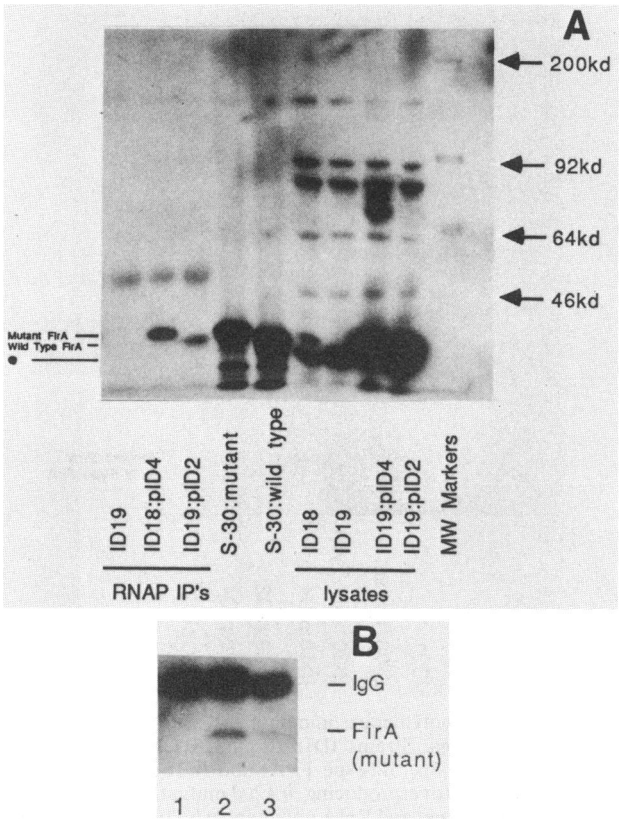


FIG. 8. (A) Probe of RNA polymerase immunoprecipitates with FirA antiserum. RNA polymerase immunoprecipitates from unlabeled strains were run on a 10% SDS–polyacrylamide gel. For comparison, lysates from the same strains were also run on the same gel. A protein recognized nonspecifically by the FirA antiserum which is visualized after long exposure times is indicated by the solid circle to the left of the gel. The proteins were electroblotted to nitrocellulose and probed with FirA antiserum as described in Materials and Methods. Lanes: 1, ID19; 2, ID18(pID4); 3, ID19(pID2); 4, ^{35}S-labeled *firA200* mutant FirA from in vitro T/T of pID4; 5, ^{35}S-labeled wild-type FirA from in vitro T/T of pID2; 6, ID18; 7, ID19; 8, ID19(pID4); 9, ID19(pID2); 10, standard molecular mass markers. (B) Same as panel A except the lysate from ID20(pID4) was prewashed with *S. aureus* cells before immunoprecipitation. Lanes: 1, FirA rabbit preimmune serum; 2, FirA antiserum; 3, RNA polymerase antiserum. IgG, Immunoglobulin G.

tance to *firA200*(Ts) *rpoB* double mutants. This phage contains a 6.7-kb *Hin*dIII insert and encodes a 17-kDa basic protein which binds to DNA and to DEAE and phosphocellulose columns and is present in large quantities in the cell. This protein was named HLPI (for histonelike protein I).

Other workers seeking candidates for histonelike DNA-binding proteins independently isolated a 17-kDa protein and prepared oligonucleotides on the basis of its N-terminal amino acid sequence. By hybridization to an *E. coli* library, the gene coding for this protein was localized to a 2.3-kb fragment (pGAH317) (15). From the DNA sequence, it was concluded that this fragment encoded the 17-kDa protein and they referred to the gene as *skp*. Subsequently, they reported (1) that this 17-kDa protein was identical to HLPI because of their identical N-terminal amino acid sequences. In addition, since the nucleotide sequence of a 155-bp *Pst*I fragment (from the original lambda *firA* phage which complements the *firA200* phenotypes) codes for the amino terminus of HLPI (7), it was concluded that the *skp* gene, which encodes the 17-kDa protein, was identical to the *firA* gene, which encodes HLPI. As discussed in this paper, this conclusion was erroneous due to the presence of multiple ORFs on the complementing phage.

In this report, we have provided evidence that the assignment of *skp* as the *firA* gene is incorrect. All of the published complementation experiments made use of clones which included the 1-kb open reading frame immediately downstream of *skp*. We now know that this downstream region is sufficient to complement the *firA* phenotypes of both the *firA200* and *firA201* alleles. Western blot analysis of transformants complemented by these clones have an elevated level of a 36-kDa protein. Deletions within plasmid pID3 that are predicted to remove either the putative *firA* promoter and the N terminus of the FirA protein (pID7) or the central portion of the open reading frame (pID8) fail to complement the *firA* phenotypes of the *firA200* allele and show no increased level of the 36-kDa protein versus controls. Similarly, in vitro T/T from small clones containing only the downstream ORF (pID2 and pID4) show only the expression of a 36-kDa protein. This protein is not expressed when there are deletions in these clones.

On this basis, we have concluded that the ORF immediately downstream of *skp* is the *firA* gene and that the 36-kDa protein, FirA, which is encoded by the *firA* gene, is responsible for the complementation of the *firA200* phenotypes. We suggest that *skp* gene be called *hlpA* since it encodes the putative histonelike (DNA-binding) protein I (HLPI). The gene order for the 4-min region of the *E. coli* chromosome is then *hlpA*-*firA*-ORF17-*lpxA*-*lpxB*-ORF23-*dnaE*-ORF37.

That the observed complementation is not caused by second-site suppression is demonstrated by the fact that transformants of the *rpoB firA200* strain ID20 containing the deletion construct pID7 show a 100-fold higher frequency of marker rescue of the temperature and rifampin sensitivity phenotypes. Since pID7 does not complement these phenotypes directly, the complementation must be due to recombination with regions of pID7. This plasmid contains wild-type sequence covering the region which has the three mutations.

The *firA* gene does not appear to have a normal promoter region since only 3 bases separate the *hlpA* (*skp*) stop and *firA* start sites. Application of an algorithm (25) used to rate promoter strength identifies two possible promoter sites within the 3′ end of the *hlpA* gene, so it is formally possible that these sites are used in vivo. We consider it more likely that the *firA* gene is cotranscribed with *hlpA* and that the low level of FirA relative to HLPI is due to either transcriptional or translational attenuation. In this regard, we speculate that the inverted repeats in the 5′ end of *firA* may play a role in this regulation. In either case, expression of FirA from the complementing plasmids lacking the *hlpA* promoter region is probably a consequence of RNA polymerase being able to recruit weak promoter sequences from the vector.

The growth of transformants of the *firA200* mutant containing the wild-type clone is comparable to that of the wild-type control strain at the restrictive temperature when *firA* is oriented such that transcription is driven by its own promoter. In this case, low levels of FirA are present in vivo as determined by Western blot analysis. This is in contrast to the high levels required for full complementation of the rifampin resistance phenotype of the *firA200 rpoB* double mutant. We do not understand these differences, but our thinking at this time is that *firA* is tightly regulated in the cell and its expression and activities are in balance with genes

upstream and downstream with itself with which it probably forms an operon. Evidence for cotranscriptional control of this region has been obtained by S1 mapping (22a) and has also been inferred for the genes downstream from *firA*, i.e., ORF17, *lpxA*, *lpxB*, and *dnaE*. That multiple copies of the mutant *firA* gene partially complement the temperature sensitivity phenotype of the *firA200* strain suggests that the mutant FirA protein retains a partial biological activity at the nonpermissive temperature. This contrasts with the inability of the mutant FirA protein to suppress the rifampin sensitivity phenotype of the *firA200 rpoB* double mutant, even when present in large amounts. We believe this indicates that the *ts* and *fir* phenotypes of the *firA200* allele are biologically different, although mediated by the same protein. This is also supported by the lack of the *fir* phenotype associated with the *firA201* allele. As described in this paper, these two alleles have two out of their three mutations in common and differ only in their 5'-most mutations. This might suggest that the Gln-165 → Arg-165 mutation in the *firA200* allele (the unique mutation in that allele) is particularly important in the rifampin sensitivity phenotype. Alternatively, the rifampin sensitivity phenotype might arise from the common mutations, and the Ile-172 → Phe-172 change in *firA201* might be compensatory for rifampin sensitivity while remaining temperature sensitive.

Genetic evidence suggests that the FirA protein is interacting with the transcription machinery, since the phenotypes associated with these mutations are reciprocally amplified; i.e., the *firA200*(Ts) allele increases the rifampin sensitivity of strains having a mutant (*rpoB*) polymerase and *rpoB* alleles increase the temperature sensitivity of a *firA200* strain. However, we find that even a wild-type strain which is already rifampin sensitive becomes even more rifampin sensitive on introduction of the *firA200*(Ts) allele. We find this interesting since certain *rho* mutants also have increased sensitivity to rifampin (11), which might indicate a common site of action. We conclude that the rifampin sensitivity phenotype is general to the interaction of the mutant FirA protein with the transcription machinery, but how is FirA interacting with the transcriptional machinery?

Until now, evidence of physical interaction between RNA polymerase and FirA has been lacking. The coimmunoprecipitation of FirA by RNA polymerase antiserum reported here is suggestive of such an interaction and strengthens the hypothesis that FirA is directly involved in transcription. However, numerous studies (5, 6, 10, 30) have shown that only the $\alpha_2\beta\beta\sigma$ holoenzyme complex is required for in vitro transcription. Possibly FirA is required catalytically for certain phases of growth or only under certain growth conditions. The low in vivo level of FirA is consistent with such a catalytic role.

The role which *firA* plays in *E. coli* is still unclear. However, the genetic and now physical data indicate that this gene is important in transcription and is probably essential for function. The high sequence homology to *S. typhimurium* is another indication of its importance. For these reasons, we are continuing to explore the role which the FirA protein plays in transcription. Currently, we are determining whether FirA modifies RNA polymerase. In particular, FirA has regions of limited homology to the *E. coli* acyltransferases encoded by *lpxA*, *lacA*, and *cysE* and to certain regions common to GTP-binding proteins. Therefore we are determining whether FirA has acetylation or phosphorylation activity. Finally, we are investigating whether there is some functional relationship between FirA and HLPI and whether *hlpA* and *firA* are part of the *dnaE* operon.

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