JOURNAL OF BACTERIOLOGY, Jan. 1997, p. 41–45 Vol. 179, No. 1
0021-9193/97/$04.00+0

Characterization of *fhlA* Mutations Resulting in Ligand-Independent Transcriptional Activation and ATP Hydrolysis

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Received 19 August 1996/Accepted 21 October 1996

The FhlA protein belongs to the NtrC family of transcriptional regulators. It induces transcription from the −12/−24 promoters of the genes of the formate regulon by σ^{54} RNA polymerase. FhlA is activated by binding of the ligand formate and does not require phosphorylation. A mutational analysis of the *fhlA* gene portion coding for the A and C domains was conducted with the aim of gaining information on the interaction between formate binding and ATP hydrolysis plus transcription activation. Four mutations were identified, all located in the A domain; one of them rendered transcription completely independent from the presence of formate, and the others conferred a semiconstitutive phenotype. The FhlA protein of one of the semiconstitutive variants was purified. Catalytic efficiency of ATP hydrolysis of the mutant FhlA was increased in the absence of formate in the same manner as formate influences the activity of wild-type FhlA. Moreover, in vitro transcription occurred at much lower threshold concentrations of the mutant protein and of nucleoside triphosphates than with the wild-type FhlA.

The formate regulon (29) of *Escherichia coli* comprises four transcriptional units, namely, the *hyc* and *hyp* operons, the *fdhF* gene, and the *hydA* locus containing the *hydN* and the *hypF* genes (19, 24, 42). The *hyc* operon genes code for the structural components of hydrogenase 3 (*hycBCDEFGH*) plus a negative regulator (*hycA*) and a hydrogenase maturation protease (*hycI*) (6, 28, 32). The products of the *hyp* genes are involved in the maturation of hydrogenase 3 alone (*hypA* and *-C*) or of all three hydrogenases (*hypBDE* and *-F*) (23). *fdhF* codes for the selenopolypeptide of formate dehydrogenase H (26); the function of the *hydN* gene has not yet been resolved (24). The transcription of these units is dependent on the presence of formate and on the function of a transcriptional activator, FhlA (29, 35). *fhlA* is the promoter-distal gene of the *hyp* operon (22); *fhlA* is constitutively expressed at a low basal level under nonfermentative conditions and induced by formate, the product of the anaerobically active pyruvate formatelyase (29).

Transcription of the genes of the formate regulon occurs from −12/−24 promoters and therefore requires the function of σ^{54} plus the presence of a *cis* DNA element located between −100 and −140 bp upstream of the transcriptional start site, the upstream regulatory sequence (upstream activator sequence [UAS]) to which FhlA binds (4, 5, 14, 22, 36).

FhlA belongs to the large NtrC family of transcriptional regulators. Unlike NtrC, however, FhlA is activated not by phosphorylation but rather by direct binding of the effector formate (15). Binding of the ligand, which could be demonstrated in a purified system (15, 16), leads to a significant stimulation of the intrinsic ATPase activity of FhlA (15). Direct coupling of ligand coordination with stimulation of the catalytic efficiency was also observed in in vitro transcription assays employing purified components: the effect of formate could be substituted by a high concentration of nucleoside triphosphates in the reaction mixture (16).

Other members of the FhlA subclass are NifA (8), DmpR (37, 38), and XylR (18). Domain swapping experiments have shown that DmpR and XylR bind the respective phenolic ligands with an N-terminal domain (39); also, stimulation of the ATPase activity by phenols has been demonstrated with the aid of a partially purified DmpR preparation (40). A common concept for the action of these regulators is that inducer binding has the same consequence on ATPase activity and transcription activation as phosphorylation has in the case of the NtrC subclass (9, 14, 15, 40, 44). This contention is supported by the phenotype of mutants altered in other regulators of the NtrC family: DctD and some constitutive mutants of NtrC function in the absence of the N-terminal domain which is the site for phosphorylation, and XylR (10) and DmpR (40) become constitutively active in ATP hydrolysis and transcriptional activation when the binding domain has been removed (13, 17, 27).

In the present study, we have examined the coupling between ligand binding and ATP hydrolysis by the isolation and characterization of constitutive or semiconstitutive mutants of FhlA. Several mutations in the N-terminal half of FhlA were obtained; we show that the mutations affect the kinetics of ATP hydrolysis and of transcription activation in the same manner as ligand binding does with wild-type FhlA (15, 16).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains of *E. coli* and the plasmids used in this study are listed in Table 1. A phosphate-buffered rich medium (TGYEP medium) (3) was employed for cultivating cells which were to be used in the mutagenesis procedure or for β-galactosidase assays. It was supplemented with 1 μM each sodium molybdate and selenite and with 5 μM nickel chloride. For anaerobic growth of RM2212, the medium had to be fortified with 0.1% sodium acetate. The screening for *fhlA* constitutive mutants was carried out with the following medium: 1% casein hydrolysate–0.5% glycerol–1 mM magnesium sulfate–0.1 mM calcium chloride–0.1 M potassium phosphate (pH 6.5)–1 μM sodium selenite and molybdate–5 μM nickel chloride–0.004% X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)–1 mM lactose. LB medium (25) was used to cultivate cells for immunoblotting analysis. Anaerobic growth of bacteria was achieved with established techniques (2). When required, the following antibiotics were added at the indicated final concentrations: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; kanamycin sulfate, 50 μg/ml; and tetracycline, 15 μg/ml.

Mutagenesis of the *fhlA* gene. *E. coli* RM2212 harboring plasmid pSH91 was grown in TGYEP medium to an optical density of 1 at 600 nm. Methanesulfonic acid ethyl ester was added to a concentration of 245 mM, and incubation of the culture was continued for 60 min at 37°C. The cells were then collected by

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TABLE 1. Bacterial strains and plasmids used in this study

E. coli strain, phage, or plasmid	Relevant genotype	Source or reference
JM109	F′ (*traD36 proAB*$^+$ *lacI*^{q}*ZΔ*M15) *recA1 endA1 gyrA96 thi-1 hsdR17 relA1 supE44* Δ(*lac-proAB*) λ$^-$	45
RM2212	MC4100 Δ*pfl act* Δ(*srl-recA*)*306*::Tn*10* λTS101	32a
SV83	Kan^r; MC4100 *fhlA*::λ p*lac*Mu53	34
M13mp19	*lacPOZ′*α	45
λTS101	λRS45 ϕ(*hypA′-hycA′-lacZ*$^+$)	14
pSH9	Cm^r; pACYC184 carrying *fhlA*	15
pSH91	pSH9 with *Alw44I* restriction site 35 bp upstream of the *fhlA* structural gene	This work
pF-E363Ka	pSH91 carrying FhlA-E363K	This work
pF-E363Kb	pSH91 carrying FhlA-E363K	This work
pF-E358K	pSH91 carrying FhlA-E358K	This work
pF-E183K	pSH91 carrying FhlA-E183K	This work
pSA36	Ap^r; *fhlA* in pUC19	36
pSA37-E363K	Ap^r; mutant *fhlA* in pSA36	This work
pBN208	Ap^r; ϕ(*fdhF′-′lacZ*)*hyb39* 240-bp 5′-flanking region of *fdhF*	5

centrifugation, washed, resuspended in the same volume of fresh TGYEP medium, and incubated for 2 h at 37°C. The cells were then collected by centrifugation, washed, resuspended in the same volume of fresh TGYEP medium, and incubated for 2 h at 37°C. The cells were sedimented, and the plasmid DNA was extracted and used to transform strain RM2212. Transformants which gave blue colonies on the indicator medium were chosen, and their plasmid DNA was isolated. A 701-bp *Alw44I-Eco*RV fragment and a 1,052-bp *Eco*RV-*Bss*HII fragment were isolated and separately cloned into identically digested plasmid pSH91.

Recombinant DNA techniques. Standard recombinant DNA techniques were adopted from the methods of Sambrook et al. (30). pSH91 was constructed from pSH9 by using phage M13mp19 (21). Plasmids were introduced into host cells by electroporation (11). The fragments of plasmid pSH91 which had been mutagenized were sequenced by the didesoxy chain termination method (31) using T7 DNA polymerase supplied by Pharmacia LKB Biotechnology (Freiburg, Germany) (41).

Determination of enzyme activities. For β-galactosidase assays, cells were grown under anaerobic conditions to an A_{600} of between 0.4 and 0.6. Determination of enzyme activity was conducted as described by Miller (25).

The ATPase activity catalyzed by FhlA was assayed as described previously (15). Wild-type or mutant FhlA was present at 471 nM (calculated for the monomer). Formate and the target DNA (the SL1-2 fragment of the UAS) were added at the concentrations indicated below. The $^{32}PO_4^{3-}$ product released was separated from the substrate by addition of activated charcoal and was quantitated via liquid scintillation counting in the ^{32}P channel of a Packard (Frankfurt, Germany) Tri-Carb 2100 TR liquid scintillation analyzer.

In vitro transcription assay. In vitro transcription assays were carried out as described previously (16). CsCl gradient-purified plasmid pBN208 DNA was added at a final concentration of 7.4 nM. Core RNA polymerase from Biozym (Hessisch Oldendorf, Germany) was present at 3.3 nM (0.1 U/20 μl), and σ^{54} was present at 245 nM. FhlA and ribonucleoside triphosphates were added as indicated in the figure legends. Subsequent primer extension analysis of the transcripts was also performed as described by Hopper et al. (16). A total of 150,000 cpm of radioactively labelled oligodesoxyribonucleotide SH11 was added to the transcripts, precipitated, and hybridized. Primer extension analysis was performed with 6.5 U of avian myeloblastosis virus reverse transcriptase. After electrophoresis, the dried gels were exposed to a CEA X-ray 90 film.

Protein purification. Plasmid pSA36 was used for overproduction of wild-type FhlA protein, and plasmid pSA37-E363K was used for overproduction of FhlA-E363K. The latter plasmid was constructed by isolation of a 2,468-bp *Hin*dIII-*Bam*HI fragment containing the structural gene for FhlA-E363K and insertion into identically restricted plasmid pSA36. The overexpression of the two genes and the purification of their products were carried out as described elsewhere (36).

Immunoblotting analysis. Polyclonal antibodies directed against FhlA (33) were used in a 1:1,000 dilution in immunoblotting experiments employing the Amersham (Braunschweig, Germany) enhanced chemiluminescence system.

RESULTS

Isolation and characterization of *fhlA* constitutive mutants. The *fhlA* gene (Fig. 1) has been mutagenized in two parts to facilitate subsequent localization of mutations, namely, as an *Alw44I-Eco*RV fragment and as an *Eco*RV-*Bss*HII fragment. Each mutagenized fragment was cloned into the appropriate position of the unmutagenized *fhlA* gene carried by plasmid pSH91. The recombinant plasmids were transformed into strain RM2212, which is *pfl* and therefore cannot produce formate under anaerobic conditions. RM2212 also carries a ϕ(*hypA′-hycA′-lacZ*$^+$) fusion in the λ attachment site. Formation of blue colonies (blue phenotype) by the transformants, therefore, can be used as an indication of the induction of the *hyc* operon in the absence of formate.

A total of 136 mutants with a blue phenotype were isolated, representing 0.17% of the population. Similar clones from the same set were pooled, and the plasmids were isolated and retransformed into strain RM2212. From the pool in which the *Eco*RV-*Bss*HII fragment had been mutagenized, clones with a blue phenotype were recovered from each set. From the

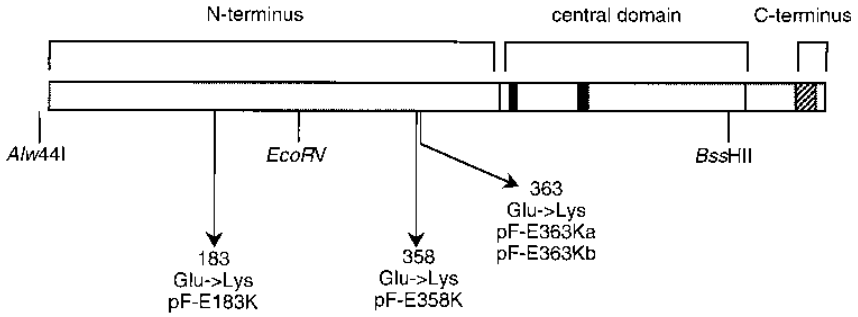

FIG. 1. Scheme of the *fhlA* gene and the relevant domains of the protein. The restriction sites used in this study and the location of the respective mutations are given. The nucleoside triphosphate binding motifs (black bars) and the helix-turn-helix motif at the C terminus (hatched bar) are indicated.

TABLE 2. β-Galactosidase activity of strain RM2212 [ϕ(*hypA′-hycA′-lacZ*$^+$)] carrying wild-type or mutant *fhlA* genes on a plasmid[a]

Plasmid	β-Galactosidase activity (Miller units)	
	Without formate	With formate (30 mM)
pSH91	338	5,362
pF-E183K	10,330	9,292
pF-E358K	6,684	13,757
pF-E363Ka	11,646	16,180
pF-E363Kb	8,805	13,307

[a] Cells were grown anaerobically at 37°C. Values are the averages of two experiments assayed in triplicate.

*Alw*44*I-Eco*RV pool, only one clone could be obtained. The inserts of four mutant clones from independent mutagenesis sets (the single mutant from the 5′ segment and three from the 3′ segment of the mutagenized portion of the *fhlA* gene) were sequenced. Intriguingly, all four had a glutamic acid-to-lysine change, and three of them were clustered (Fig. 1).

The effect of the mutations on the expression of the ϕ(*hypA′-hycA′-lacZ*$^+$) fusion carried by strain RM2212 was assayed (Table 2). The mutation E183K conferred a completely constitutive phenotype. E358K and E363K, on the other hand, were only semiconstitutive in their ϕ(*hypA′-hycA′-lacZ*$^+$) expression; formate still could induce between 1.4- and 2-fold. The expression levels attained by the mutant *fhlA* alleles were two to three times that conferred by the wild-type gene. Immunoblotting analysis of cell lysates from the cultures carrying the plasmid with the different *fhlA* alleles was carried out to preclude that the altered expression of ϕ(*hypA′-hycA′-lacZ*$^+$) was caused by an alteration of the amount of FhlA. It was found that all strains possess comparable quantities of the protein (data not shown).

Purification of FhlA-E363K and analysis of its ATPase activity. To analyze the biochemical basis of the constitutive activation by mutant FhlA proteins, purification of the mutant variants FhlA-E183K and FhlA-E363K was attempted. FhlA-E363K could be purified to apparent homogeneity by employing the procedure developed for purification of wild-type FhlA (36). Unfortunately, the fully constitutive variant, despite extensive efforts, was refractory to purification because of its apparent sticking to any matrix employed and the consequent inability to desorb or elute it in concise fractions (data not shown). The formate dependence of ATP hydrolysis, therefore, could be determined only for the FhlA-E363K variant (Fig. 2). It is evident that the mutant protein has a significantly increased ATPase activity in the absence of formate which is stimulated to a lesser degree than wild-type FhlA. Addition of the target DNA (containing the UAS) increased the rate of ATP hydrolysis by both proteins.

Table 3 summarizes the kinetic parameters of wild-type and mutant FhlA for ATP hydrolysis. As has been shown previously, the addition of formate and the target DNA lowers the apparent K_m for ATP and increases the V_{max} of the reaction (15). Interestingly, the apparent K_m of FhlA-E363K for ATP in the absence of formate lies in the range of that for the wild-type protein in the presence of the ligands. The same holds true for V_{max}. The overall catalytic efficiency of the mutant protein (V_{max}/K_m) is affected by the mutation of FhlA-E363K in the same manner as that of the wild-type protein by binding of the ligands formate and target DNA.

In vitro transcription activation by wild-type and mutant FhlA. In previous experiments, it had been shown that there is a direct link between the binding of formate to wild-type FhlA and the stimulation of ATP hydrolysis. Transcription was activated in the absence of the inducer provided that the concentrations of nucleoside triphosphates were increased (16). Thus, at low nucleoside triphosphate levels, FhlA required formate, but at high concentrations it did not.

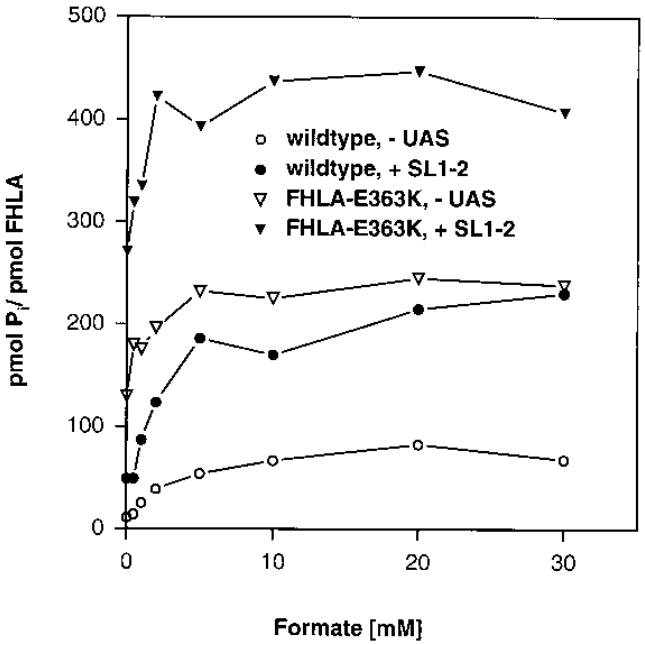

FIG. 2. Influence of the formate concentration on ATP hydrolysis by FhlA and FhlA-E363K (471 nM) in the absence (−) and the presence (+) of the SL1-2 desoxyribonucleotide (1 μM) containing the UAS (17).

To gain information on the ATP concentration dependence of FhlA-E363K in the activation process, in vitro transcription was determined at different nucleoside triphosphate concentrations and in the presence and absence of formate (Fig. 3). The results show that the concentration of nucleoside triphosphates at which transcription can take place is lowered with the mutant protein. However, at the borderline concentration (12.5 μM for FhlA-E363K and 50 μM for wild-type FhlA), the formation of transcript is greatly dependent on the presence of formate in both instances.

Next, the threshold amounts of FhlA required for in vitro transcription activity in the absence of formate and at two different nucleoside triphosphate concentrations were determined (Fig. 4). This threshold value is drastically reduced for FhlA-E363K (Fig. 4B) in comparison to that for the wild-type protein (Fig. 4A). It should be emphasized that the lowest concentration tested and shown to be at least partially active represents a 1:1 ratio between FhlA-E363K and the target DNA.

DISCUSSION

FhlA, like XylR (7, 18, 27) and DmpR (37, 38, 40), is activated not by phosphorylation but by binding of a ligand, in this case formate. The availability of intact FhlA in a purified form allowed the detailed analysis of the effect of ligand binding on the kinetics of ATP hydrolysis and on transcription activation (15, 16). It was found that the binding of formate phenomenologically affects these activities of FhlA in the same way that phosphorylation affects NtrC activity (40, 44). In analogy to the action of NtrC-P (12, 20), a model suggesting that FhlA oli-

TABLE 3. Kinetics of ATP hydrolysis by wild-type FhlA and FhlA-E363K

Components added	Apparent K_m (mM ATP)		V_{max} (pmol of $P_i \cdot min^{-1} \cdot$ pmol of FhlA monomer^{-1})		V_{max}/K_m	
	Wild-type FhlA	FhlA-E363K	Wild-type FhlA	FhlA-E363K	Wild-type FhlA	FhlA-E363K
None	1.0	0.28	5.0	28.6	5.0	102.1
Formate + SL1-2	0.29	0.18	29.4	37.7	101.4	209.4

gomerizes at the DNA and that this oligomerization is favored by high nucleoside triphosphate concentrations and by formate (15) has been proposed.

The isolation of mutations in *fhlA* and the analysis of a mutant protein support this contention. The amino acid changes of the four mutants characterized are located within the large, N-terminal half of FhlA. It is intriguing that all four mutations create charge changes in the protein. The alteration in FhlA-E183K causes a fully constitutive phenotype and may affect formate binding or change the conformational state of the protein, as formate does with the wild type. The phenotype of the other three variants may reflect an altered conformational interaction between the formate binding and ATP hydrolysis functions; the binding of formate appears not to be affected, since activation of ATP hydrolysis by it occurs at the same concentrations as with the wild-type protein (Fig. 2).

The kinetic parameters of FhlA-E363K in ATP hydrolysis are qualitatively and also quantitatively similar to those conferred to wild-type FhlA by the binding of formate. In addition, identical interplays between the presence of formate and the concentration of nucleoside triphosphates in the transcription

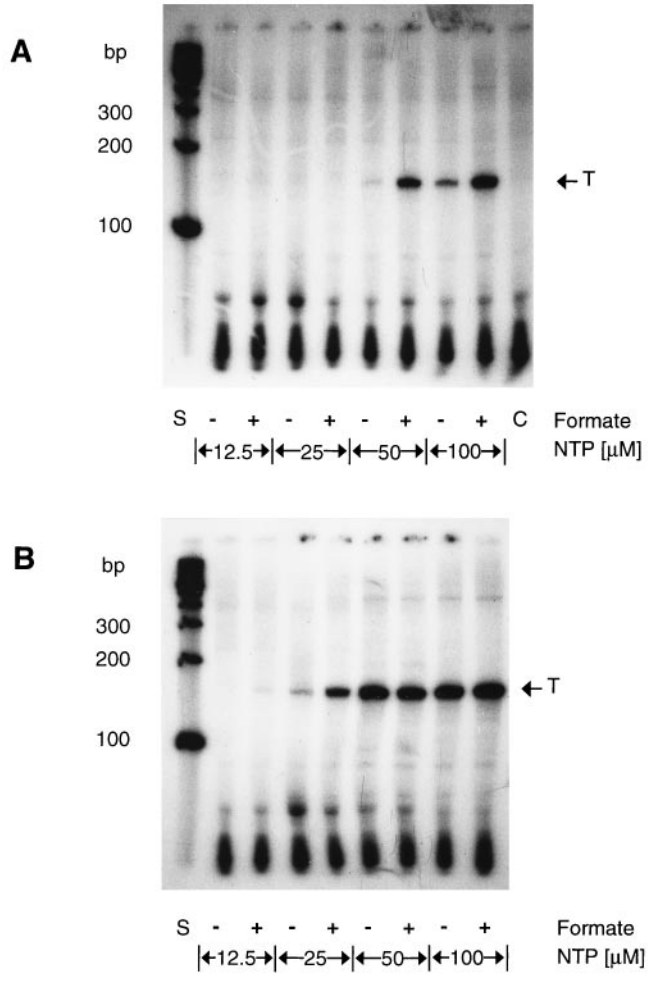

FIG. 3. Dependence of the in vitro transcription by wild-type FhlA (A) and FhlA-E363K (B) on the nucleoside triphosphate concentration. Each of the four nucleoside triphosphates (NTPs) was present at the concentration indicated. Formate, where indicated, was present at 10 mM. Lane C, control assay with 10 mM formate and 100 μM NTPs, lacking FhlA; lane S, size standard; T, transcript. +, presence; −, absence.

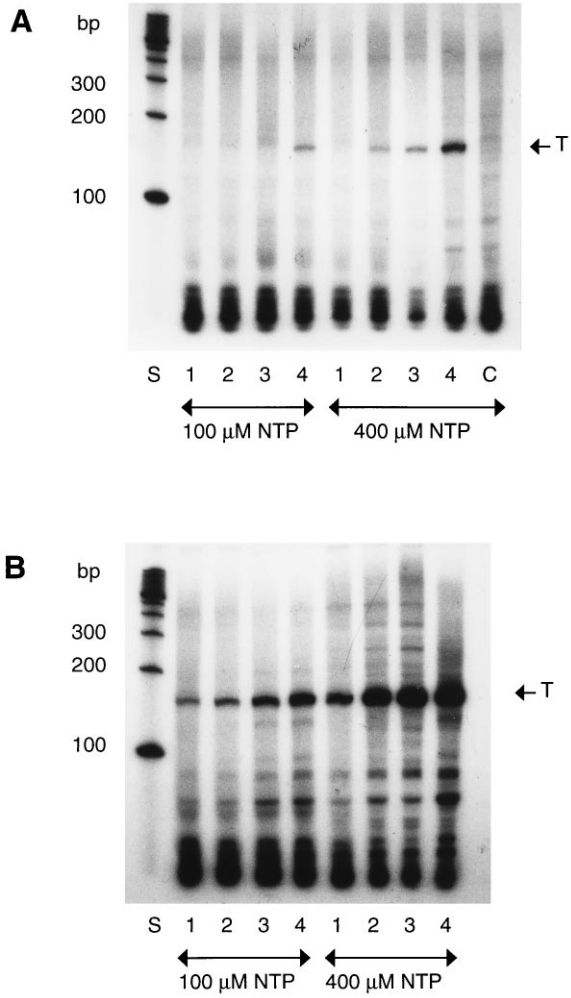

FIG. 4. In vitro transcription at different FhlA and nucleotide concentrations in the absence of formate. The following FhlA-to-DNA ratios were used: lanes 1, 4:1; lanes 2, 8:1; lanes 3, 16:1; and lanes 4, 32:1. Each of the four nucleotides is present at the final concentration indicated below the lanes. The reaction mixtures contain wild-type FhlA (A) or FhlA-E363K (B). Lane C, control assay lacking FhlA; lanes S, size standard. T, transcript; NTP, nucleoside triphosphate.

activation assays were observed. A direct coupling, such as in the case of NtrC-P, may be likely (43). This parallelism also indicates that FhlA-E363K is maintained in a state which the wild-type protein attains with bound formate; it is therefore a good candidate for physical analysis.

ACKNOWLEDGMENTS

We thank Eva Zehelein for help with the purification of FhlA and σ^{54}.

This work was supported by grants from the Deutsche Forschungsgemeinschaft "Regulationsnetzwerke in Bakterien" and from the Fonds der Chemischen Industrie.

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