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In vitro activity of the nitrogen fixation regulatory protein NIFA

(activation of transcription/σ^{54}/integration host factor/*Klebsiella pneumoniae*)

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ABSTRACT **We have detected activity of the nitrogen fixation regulatory protein NIFA of *Klebsiella pneumoniae in vitro*. To do so we directed synthesis of NIFA in a coupled transcription–translation system and detected its ability to activate expression of a translational fusion between the *nifH* and *lacZ* genes. We infer that NIFA stimulates initiation of transcription by σ^{54} holoenzyme from the *nifHDK* promoter. The activity of NIFA was lost rapidly under both aerobic and anaerobic conditions at 30°C and was lost somewhat less rapidly at 0°C. Loss of activity was not accompanied by degradation of NIFA polypeptide. Loss of activity was approximately exponential and was not affected by NIFA concentration over a 5-fold range. Therefore, NIFA inactivation does not appear to be due to self-association. We found that the factor in crude extracts previously demonstrated to bind to the *nifHDK* promoter-regulatory region [Beynon, J., Cannon, M., Buchanan-Wollaston, V., and Cannon, F. (1983) *Cell* 34, 665–671] is the integration host factor, which is known to bend DNA. Since the binding site for integration host factor lies between the upstream binding site for NIFA and the *nifHDK* promoter, integration host factor may bend the DNA between these two sites to facilitate productive interactions between NIFA and σ^{54} holoenzyme.**

Genetic evidence indicates that the NIFA protein is an activator of transcription for the nitrogen fixation (*nif*) operons of a number of nitrogen fixing bacteria (1–6). NIFA activates transcription by an alternative holoenzyme form of RNA polymerase that contains the product of the *ntrA* gene, σ^{54}, as σ factor. The NIFA protein apparently binds to sites more than 100 base pairs (bp) upstream of the transcriptional start sites for *nif* operons (7). This binding was elegantly demonstrated *in vivo* for the NIFA protein of *Klebsiella pneumoniae* in the promoter-regulatory region for the *nifHDK* operon, which encodes dinitrogenase and dinitrogenase reductase (8, 9). NIFA is of interest not only as a transcriptional activator for *nif* operons but also because its activity is regulated by oxygen (10–12), it is implicated in conferring unusual stability on *nif* mRNAs in *K. pneumoniae* (13), and it is required for the establishment of stable symbiotic relationships between members of the genera *Bradyrhizobium* (5) or *Rhizobium* (6) and their plant hosts.

Neither the transcriptional-activating function of NIFA nor its DNA-binding ability has been demonstrated *in vitro* because NIFA extracted from cells that overproduce it has been insoluble (14, 15). The insolubility of the protein and the consequent difficulty in working with it were in contrast to the behavior of the general nitrogen regulatory protein NTRC, a closely related transcriptional activator for σ^{54} holoenzyme (14, 16). NTRC has been purified and studied extensively at the promoter for *glnA*, the gene encoding glutamine synthetase; NTRC catalyzes isomerization of closed complexes between σ^{54} holoenzyme and the *glnA* promoter to open complexes in an ATP-dependent fashion (17).

We have now demonstrated activity of *K. pneumoniae* NIFA protein *in vitro* by directing its synthesis in a coupled transcription–translation system (S30 extract) and immediately detecting its ability to activate expression of a *nifH–lacZ* translational fusion product. In addition, we have found that the integration host factor (IHF), which is present in the S30 extracts, binds to the *nifHDK* promoter-regulatory region at a site located between the upstream binding site for NIFA and the promoter.

MATERIALS AND METHODS

Construction of Plasmids. All enzymes used to manipulate DNA were purchased from New England Biolabs. DNA isolation and cloning were carried out following standard procedures (18). The vectors used for expression of proteins were pT7-5 and pT7-7 (S. Tabor, personal communication), both of which allow transcription of inserted DNA fragments from the ϕ10 promoter of phage T7 (dependent on T7 RNA polymerase). Plasmid pT7-7 carries, in addition, the ribosome binding site for the ϕ10 gene and an open reading frame in its polylinker, which allows the construction of translational fusions to coding sequences present in inserted fragments.

Plasmid pJES238, which allows low levels of overproduction of the NIFA protein (data not shown), was constructed by inserting a 2.7-kilobase *Nae* I–*Sal* I fragment [from pSB2001 (19)] that contains the *K. pneumoniae nifA* gene and 574 bp of the upstream *nifL* gene into pT7-5 that had been digested with *Sma* I and *Sal* I. Plasmid pJES294, which allows the highest levels of NIFA overproduction that we have achieved (≈25% of total protein; data not shown), was constructed in the following several steps. (*i*) The initiating ATG of *nifA* was first joined to the T7 ribosome binding site. This was achieved by ligating a double-stranded synthetic oligonucleotide (39/37-mer) that begins with the *nifA* ATG and continues into *nifA* coding sequences to pT7-7 that had been digested with *Nde* I (to linearize it at the ATG of the open reading frame within the polylinker) and *Sma* I. (*ii*) The complete *nifA* gene was then reconstructed by joining to the oligonucleotide an 80-bp *Taq* I–*Pst* I fragment carrying adjacent *nifA* coding sequences and subsequently by inserting the remainder of the *nifA* gene. Plasmid pJES297 was constructed in the following two steps. (*i*) The *ntrC* gene was first transferred to pT7-7 by inserting a 1.9-kilobase *Eco*RI–*Sal* I fragment carrying *ntrC* into pT7-7 that had been digested with *Eco*RI and *Sal* I. (*ii*) The initiating ATG of *ntrC* was then joined to the T7 ribosome binding site by digesting with *Nde* I and *Pfl*MI (site within *ntrC*) and replacing the beginning of *ntrC* with a double-stranded synthetic oligonucleotide that extended from the initiating ATG to the *Pfl*MI site. Trans-

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Abbreviations: IHF, integration host factor; ONP, *o*-nitrophenol.

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formations were performed by the standard $CaCl_2$ procedure (18) using strain BNN45 (20).

***In Vitro* Transcription–Translation.** Plasmid templates for *in vitro* reactions were purified by gradient centrifugation in CsCl. Plasmids used to detect NIFA activity ("reporter plasmids") were pVSA2 (21) or p318 (22), both of which carry a translational fusion of the first codon of *nifH* to the *lacZ* gene. Plasmid pVSA2 carries the wild-type *nifH* promoter-regulatory region whereas p318 carries two mutations in this region, one or both of which appear to increase promoter efficiency (ref. 22; E.S., unpublished data). Plasmid p318 was used for the experiment of Fig. 3 to increase the sensitivity of detection of NIFA.

S30 extracts of *Salmonella typhimurium* strain SK419 [Δ*ntrA* Δ(*glnA ntrB ntrC*) *relA*$^-$ *hisT*$^-$ (23)] were prepared by the method of Artz and Broach (24) with slight modifications (25). Reactions were performed in a 50-μl total volume containing the following components (final concentrations), which were mixed in the order indicated: 60 mM Epps·KOH [*N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid] (pH 8), 120 mM potassium glutamate (pH 8), 27 mM ammonium acetate, 1 mM dithiothreitol, all common amino acids (each at 0.4 mM), 11 mM magnesium acetate, 2 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.5 mM UTP, 20 mM potassium phospho*enol*pyruvate, *Escherichia coli* tRNA (0.5 mg/ml; Boehringer Mannheim), M_r 6000–8000 polyethylene glycol (35 mg/ml; Sigma), 58 μM guanosine tetraphosphate, 4.2 mM polyamines (ratio of putrescine/spermidine is 3:1), and folinic acid at 20 μg/ml. When required, σ^{54} and NTRB (110 and 58 nM, respectively; purified from *S. typhimurium*) and T7 RNA polymerase (10–40 units as defined by New England Biolabs) were added. Unless otherwise indicated, reactions were allowed to proceed for 60 min at 30°C; at this time no further protein synthesis was occurring. Assays for β-galactosidase were performed at 30°C according to Miller (26) and activities, which reflect the amount of β-galactosidase synthesized in the coupled reaction, are expressed per 50 μl, the total volume of reaction mixtures.

Anaerobic transcription–translation reactions were performed in stoppered 0.5-ml serum vials under a nitrogen atmosphere, which was achieved by repeated cycles of flushing and evacuation using a manifold. Reactions were initiated by injecting S30 extract that was also under a nitrogen atmosphere. We confirmed that conditions remained anaerobic until reactions were completed by adding methylene blue to control vials and checking to see that it was reduced to the colorless form.

DNA-Binding Assays. DNA-binding assays were performed as described (27, 28) with minor modifications. A DNA probe of 294 bp carrying the *nifH* promoter-regulatory region was prepared by 5′ labeling the *Bam*HI site of pVSA2 [label at position +34 on the template (bottom) strand of *nifH*], digesting with *Eco*RI, and purifying the resultant fragment on a nondenaturing polyacrylamide gel. For gel-mobility-shift assays, this probe (≈1.3 nM) was incubated with S30 extracts or other crude cell extracts from *S. typhimurium* or *E. coli* or with purified IHF for 5 min at 30°C as described (17) except that sonicated calf thymus DNA was present in the buffer (63 μg/ml); total volume was 8 μl. After addition of sucrose (to 6%), reaction mixtures were loaded on a 5% nondenaturing polyacrylamide gel and subjected to electrophoresis at 10 V/cm for 2 hr at 30°C. The electrophoresis buffer contained 45 mM Tris borate (pH 8.3) and 1 mM Na_2EDTA. The gel was exposed to Kodak XAR film overnight at room temperature.

For footprinting studies the DNA probe described above (≈17 nM) was incubated with crude cell extracts including S30 extract, purified IHF, or bovine serum albumin, as described for gel-mobility-shift assays except that the total volume was 25 μl. After 5 min at 30°C, $CaCl_2$ and DNase I were added to 2 mM and 6.7 ng/ml, respectively, and incubation was continued for an additional 30 sec at 30°C (29). Reaction mixtures were then loaded on nondenaturing gels as described above. After electrophoresis and autoradiography of the gel, the appropriate bands were excised, DNA was electroeluted, and protected bases were analyzed on an 8% sequencing gel as described (17).

RESULTS

***In Vitro* Synthesis of NIFA and Detection of Its Activity.** To demonstrate NIFA activity *in vitro*, we directed synthesis of the protein from an expression vector (pJES294, the "driver plasmid") in a coupled transcription–translation system (S30 extract) and simultaneously detected its ability to activate expression of a *nifH–lacZ* fusion product (plasmid pVSA2, the "reporter plasmid"; Fig. 1). To the coupled system, we added RNA polymerase from phage T7 to drive NIFA synthesis and the alternative σ factor σ^{54} to allow transcription from the *nifH* promoter. We obtained high levels of

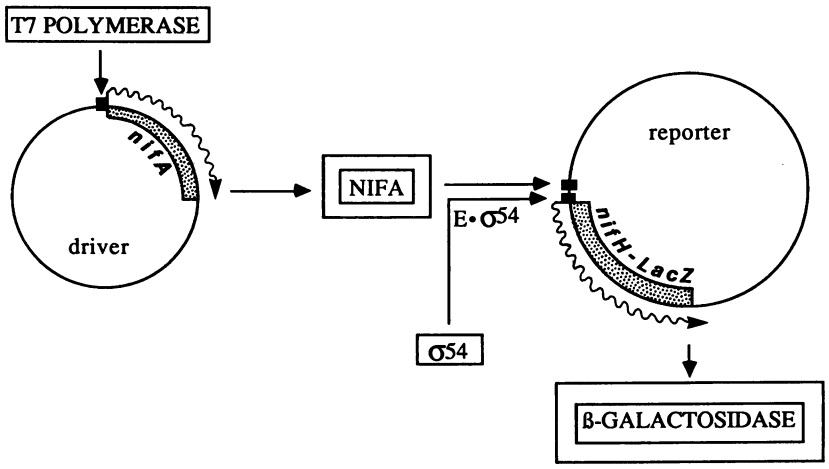

FIG. 1. Strategy for synthesizing NIFA in a coupled transcription–translation system and simultaneously detecting its activity (two-plasmid reaction). NIFA was synthesized from the expression vector pJES294 (driver plasmid) under the control of RNA polymerase from phage T7. The ability of NIFA to activate expression of a *nifH–lacZ* fusion product was detected by measuring β-galactosidase activity from plasmid pVSA2 (ref. 21; reporter plasmid). Purified proteins added to the coupled system are shown in single-edged boxes whereas those synthesized during the reaction are in double-edged boxes. E, core form of bacterial RNA polymerase; σ^{54}, alternative σ factor required for transcription from the *nifH* promoter.

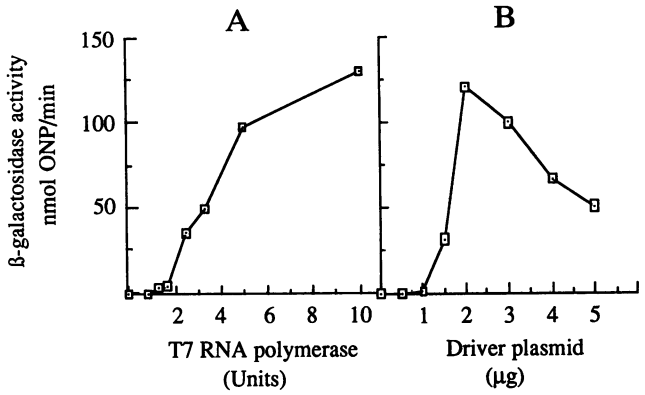

FIG. 2. Levels of NIFA-mediated expression of a *nifH–lacZ* fusion product in the coupled transcription–translation system. The reporter plasmid pVSA2 was present at 5 μg per reaction mixture. (*A*) The amount of driver plasmid pJES294 was maintained constant at 2.6 μg per reaction mixture and the amount of NIFA synthesized was varied by increasing the amount of T7 RNA polymerase as indicated on the *x* axis. (*B*) The amount of T7 RNA polymerase was held constant at 10 units per reaction mixture and the amount of NIFA synthesized was varied by increasing the amount of pJES294, as indicated on the *x* axis. When plasmid templates were further purified on Bio-Gel A-50m sieving columns (Bio-Rad), β-galactosidase activities were higher at lower template concentrations and the responses to T7 RNA polymerase and driver plasmid tended to be linear through the origin (data not shown). ONP, *o*-nitrophenol.

β-galactosidase activity that were strictly dependent on the addition of T7 RNA polymerase (Fig. 2*A*) and on the nifA driver plasmid (Fig. 2*B* and Table 1). Very low levels of activity were obtained when a driver plasmid that yielded lower levels of NIFA, pJES238, was used as a NIFA source (data not shown).

Table 1. σ^{54} dependence and heat sensitivity of NIFA activity

		β-Galactosidase activity, nmol of ONP per min		
		30°C		37°C
Activator	Reporter	$-\sigma^{54}$	$+\sigma^{54}$	$+\sigma^{54}$
None	*nifH-lacZ*	0.1	0.2	0.3
NIFA driver	*nifH-lacZ*	0.2	115	0.5
NTRC driver	*glnA-lacZ*	NT*	184	281
None	*lac* UV5	NT	94	167

Reactions were performed in a coupled transcription–translation system. The NIFA and NTRC drivers were pJES294 (2 μg) and pJES297 (0.55 μg), respectively. The *nifH–lacZ* and *glnA–lacZ* reporters were pVSA2 (5 μg) and pJES40 (3.6 μg), respectively. The *lac*UV5 plasmid was pRS229 (2.5 μg; ref. 23). T7 RNA polymerase (10 units) was added to each reaction mixture containing a driver and NTRB was added to reactions containing the NTRC driver. The alternative σ factor σ^{54} (110 nM) was added as indicated and reaction mixtures were incubated at 30°C or 37°C as indicated.
*The *glnA–lacZ* plasmid, which is known to be σ^{54}-dependent (26), and the *lac* UV5 plasmid, which is σ^{70}-dependent, were not tested (NT) in this experiment.

To confirm that the β-galactosidase activity we detected was actually due to NIFA-mediated activation of expression of the *nifH–lacZ* fusion product, we demonstrated that this activation had two characteristics of NIFA activation *in vivo* (Table 1 and refs. 30–32). (*i*) It was dependent on σ^{54} (line 2) and (*ii*) it was not observed if the coupled system was incubated at 37°C rather than the usual 30°C (line 2). This pronounced heat sensitivity was specific for NIFA activation in that NTRC-mediated activation of transcription from the *glnA* promoter (also σ^{54}-dependent) did not show it (line 3) nor did transcription from the lacUV5 promoter (line 4). In these latter two cases, activity was higher at 37°C than at

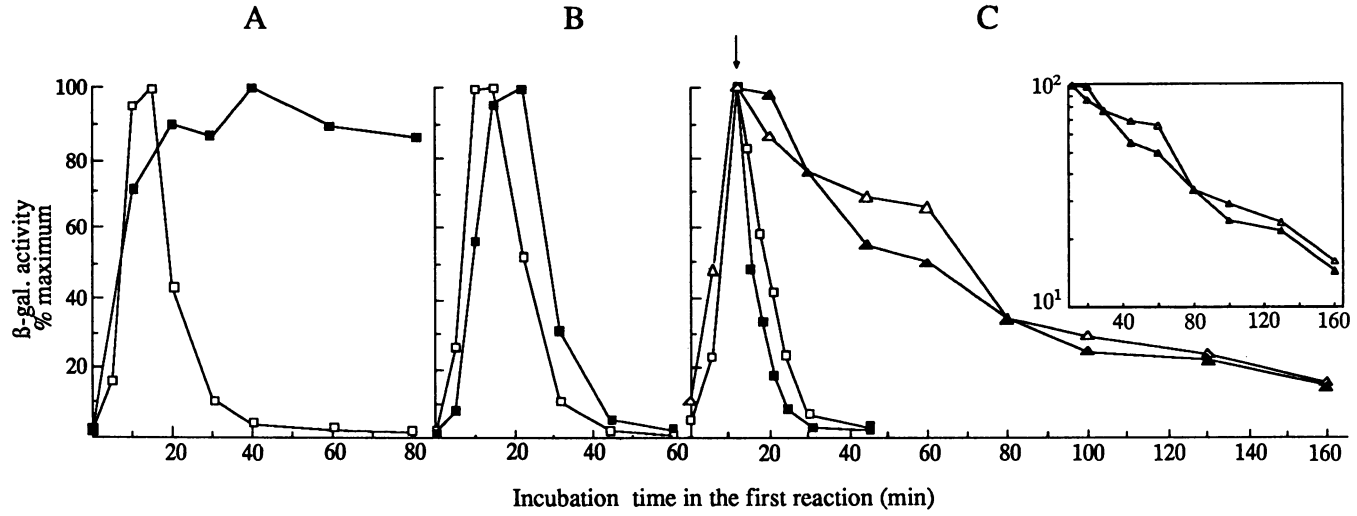

FIG. 3. Inactivation of NIFA synthesized *in vitro*. (*A*) Specificity. (*B*) Effect of anaerobiosis. (*C*) Effect of temperature and dilution on inactivation. Synthesis of NIFA was driven from plasmid pJES294 (5.2 μg per reaction mixture; 100 μl, total volume) with T7 RNA polymerase (80 units/100-μl reaction mixture). At the times indicated on the *x* axis, portions of the first reaction mixture were transferred to a second one containing the reporter plasmid p318 (22) and σ^{54} (110 nM) to allow detection of NIFA activity. After incubating the second reaction for 60 min, β-galactosidase activity was measured. (*A*) Synthesis of NTRC was driven from pJES297 (0.8 μg/100-μl reaction mixture) and NTRC activity was detected at the *glnA* promoter in a second reaction containing the *glnA–lacZ* fusion plasmid pJES40 (2 μg per reaction mixture; ref. 23) and σ^{54}. NTRB (58 nM), which phosphorylates NTRC to allow it to activate transcription, was present in both reactions. For NIFA (□) and NTRC (■), 6-μl portions of the first reaction mixture were transferred to the second. Maximal activities for NIFA and NTRC were 59 and 376 nmol of ONP per min, respectively. (*B*) Both reactions were incubated anaerobically (■) or aerobically (□) as for *A*. Portions (6 μl) of the first reaction mixture were transferred to the second. Maximal activities anaerobically and aerobically were 44 and 63 nmol of ONP per min, respectively. (*C*) The first reaction mixture was incubated continuously at 30°C (□) as in *A* and *B* or was shifted to 0°C (△) at 12 min as indicated by the arrow. At this time also, 30 μl of each first reaction mixture was diluted to 150 μl with buffer (20 mM Epps·KOH, pH 8/50 mM potassium glutamate/2 mM dithiothreitol) at the appropriate temperature and was incubated at 30°C (■) or 0°C (▲). Portions of the diluted first reaction mixtures (15 μl) or the undiluted reaction mixtures (3 μl) were transferred to the second. (In the latter case 12 μl of the dilution buffer was also added to the second reaction mixture.) Data at 30°C and 0°C were obtained in two separate experiments, for which the maximum activities were 47 and 25 nmol of ONP per min, respectively. (*Inset*) Semilogarithmic plot of the decay of NIFA activity at 0°C.

30°C. Moreover, labeling of proteins synthesized in the coupled system with [^{35}S]methionine indicated that more NIFA was synthesized at 37°C than at 30°C although the β-galactosidase band was not apparent at high temperature (data not shown); thus, heat sensitivity of NIFA-mediated activation of the *nifH–lacZ* fusion was not due to lack of efficient synthesis of NIFA at 37°C or to its proteolytic degradation.

The Activity of NIFA Synthesized *in Vitro* Is Unstable. When we attempted to synthesize NIFA in one reaction mixture and detect its activity subsequently in a second one, we found that the activity of the protein was not stable even at 30°C. For these experiments NIFA was first synthesized in a reaction mixture containing the driver plasmid pJES294 and T7 RNA polymerase but lacking a reporter plasmid and σ^{54}. NIFA activity was subsequently monitored by transferring portions of the first reaction mixture to a second one that contained σ^{54} and the reporter plasmid p318. (Similar results were obtained with reporter plasmid pVSA2.) T7 RNA polymerase was omitted from the second reaction mixture to prevent further synthesis of NIFA from residual driver plasmid that was transferred. As shown in Fig. 3*A*, NIFA activity increased with time for 15 min. Surprisingly, after that time it dropped rapidly (50% loss of activity within ≈5 min). This inactivation appeared specific for NIFA because the related transcriptional activator protein NTRC was active for at least 80 min (Fig. 3*A*). For this comparison NIFA and NTRC were synthesized from the same T7 promoter and the mRNA for each had the same 5′ untranslated region (plasmids pJES294 and pJES297); NTRC was converted to its active (phosphorylated) form by NTRB, which was present in both reaction mixtures.

The decrease in NIFA activity described above occurred after synthesis of NIFA in the first reaction mixture was essentially complete. As assessed by labeling with [^{35}S]-methionine, ≈70% of NIFA synthesis occurred within 15 min of initiation of the reactions (data not shown). Since the amount of labeled full-length NIFA did not decrease from this time until the end of the incubation period (80 min), inactivation of NIFA was not due to proteolytic degradation detectable by SDS/PAGE (data not shown). Performing both of the reactions under anaerobic conditions did not affect the rate of NIFA inactivation (Fig. 3*B*). Transferring the first reaction mixture to ice after 12 min slowed the rate of NIFA inactivation but did not prevent it (Fig. 3*C*; 50% loss of activity in ≈46 min). Diluting the first reaction mixture 1:5 after 12 min did not slow the rate of NIFA inactivation at either 30°C or 0°C and inactivation appeared to be exponential (Fig. 3*C*).

Binding of IHF to the *nifH* Promoter Region. We detected a binding factor in S30 extracts and other crude extracts of *S. typhimurium* and *E. coli* that provoked a dramatic decrease in the electrophoretic mobility of a DNA fragment carrying the complete *nifH* promoter-regulatory region (Fig. 4*A*). Since these extracts did not contain NIFA, we reasoned that the factor might be one such as IHF, the HU protein, or DNA gyrase that affected DNA conformation. When we tested purified preparations of these three proteins, we found that the factor appeared to be IHF: pure IHF produced the same decrease in mobility of the *nifH* promoter fragment as the factor in crude cell extracts (Fig. 4*A*) whereas the other proteins did not cause a mobility shift (data not shown). Further, IHF protected the same region (from approximately positions −74 to −39 on the template strand) from digestion by pancreatic DNase I as the factor in crude extracts (Fig. 4*B*). This region was the same as that demonstrated to be protected from DNase I digestion by crude extracts of *K. pneumoniae* (34).

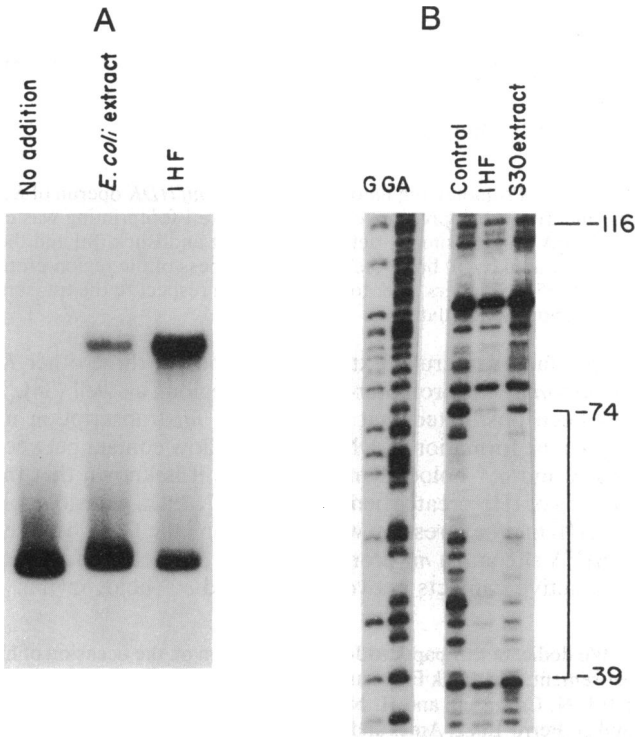

FIG. 4. Binding of IHF to the *nifH* promoter-regulatory region as indicated by gel-mobility shift of a ^{32}P-labeled DNA fragment (*A*) and footprinting with pancreatic DNase I (*B*). (*A*) A DNA fragment comprising the entire *nifH* promoter-regulatory region was incubated with no additions, a crude cell supernatant of *E. coli* strain NCM632 (1 μl), or purified IHF (175 nM) and was then subjected to electrophoresis in a nondenaturing gel. (*B*) *nifH* promoter fragment was incubated with bovine serum albumin (40 μg, control), purified IHF (250 nM), or S30 extract from *S. typhimurium* strain SK419 (1 μl) and was then subjected to digestion with DNase I. After isolation of complexes, protected bases were analyzed on a denaturing gel and the protected region is indicated by a bracket. Lanes labeled G and GA are Maxam–Gilbert sequencing lanes (38).

DISCUSSION

We have demonstrated *in vitro* activity of NIFA from *K. pneumoniae*; NIFA activates expression of a translational (gene) fusion of *nifH*, which encodes dinitrogenase reductase, to *lacZ* (Fig. 2). This activation is dependent on σ^{54} and is extremely heat labile (Table 1), two properties that characterize NIFA activity *in vivo* (30–32). Although our experiments do not address the mechanism of activation, we presume NIFA acts at the level of initiation of transcription because NIFA is very similar in function and sequence to NTRC (14, 16, 33). We have not ruled out the possibility that NIFA activity involves stabilization of *nifH–lacZ* mRNA.

By directing synthesis of NIFA *in vitro* we were able to ascertain that NIFA activity was rapidly lost under aerobic and anaerobic conditions at 30°C (Fig. 3 *A* and *B*) and that activity was lost somewhat more slowly at 0°C (Fig. 3*C*). Loss of NIFA activity did not appear to be due to degradation of NIFA polypeptide (data not shown). Since the loss of activity was approximately exponential and was not slowed by a 1:5 dilution of NIFA (Fig. 3*C*), NIFA inactivation does not appear to be due to aggregation; rather it may be due to an intramolecular reaction.

The IHF, which was present in the S30 extracts used to study NIFA activity, binds to the *nifH* promoter-regulatory region. The site for IHF is located between the *nifH* promoter, which is probably a binding site for σ^{54} holoenzyme (17), and the upstream site demonstrated *in vivo* to be a binding site for NIFA (ref. 8; Figs. 4 and 5). IHF is likely to

FIG. 5. Promoter-regulatory region of the *nifHDK* operon of *K. pneumoniae* indicating the region protected from DNase I digestion by IHF. [Boundaries are approximate because DNase I footprinting was carried out on the bottom (template) strand.] This region is located between the NIFA binding site [as defined by Morett and Buck (8)] and the Eσ^{54} promoter, which is characterized by the conserved doublets GG and GC separated by 10 bp. Note the A+T-richness of the region protected by IHF, which is characteristic of other IHF binding sites (for review, see ref. 35). Numbers refer to positions with respect to the transcriptional start site determined by 5′ labeling of the transcript (D. Popham and S.K., unpublished data).

be the factor in crude extracts that binds to three other *K. pneumoniae nif* promoter-regulatory regions as well (34). It has been postulated that activation of *nif* transcription involves the formation of DNA loops to allow contact between NIFA and σ^{54} holoenzyme (36). Since it is known that the binding of IHF creates bends in DNA (37, 39), a plausible role for IHF in *nif* expression would be to bend the DNA between a NIFA site and a *nif* promoter in such a way as to facilitate productive contacts between NIFA and σ^{54} holoenzyme.

We dedicate this paper to John L. Ingraham on the occasion of his retirement. We thank F. Ausubel and D. Ow for plasmids pVSA2 and p318, N. Cozzarelli and H. Nash for generous gifts of purified IHF, and G. Ferro-Luzzi Ames and Young Yang for purified HU and DNA gyrase. We thank G. Roberts for plasmid pSB2001 and for generously advising us and providing us with many *Klebsiella* strains. We are grateful to A. Glazer for advice on studying NIFA inactivation and to D. Weiss for helpful criticism of the manuscript. This work was supported by European Molecular Biology Organization Fellowship ALTF 266-1987 to E.S. and by National Science Foundation Grant 8714761 to S.K.

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