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Transcriptional Activation of the Nitrogenase Promoter In Vitro: Adenosine Nucleotides Are Required for Inhibition of NIFA Activity by NIFL

TREVOR EYDMANN, ERIK SÖDERBÄCK, TAMERA JONES, SUSAN HILL, SARA AUSTIN, AND RAY DIXON*

Nitrogen Fixation Laboratory, University of Sussex, Brighton, BN1 9RQ, United Kingdom

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The enhancer-binding protein NIFA is required for transcriptional activation of *nif* promoters by the alternative holoenzyme form of RNA polymerase, which contains the sigma factor σ^{54} (σ^{N}). NIFA hydrolyzes nucleoside triphosphates to catalyze the isomerization of closed promoter complexes to transcriptionally competent open complexes. The activity of NIFA is antagonized by the regulatory protein NIFL in response to oxygen and fixed nitrogen in vivo. We have investigated the requirement for nucleotides in the formation and stability of open promoter complexes by NIFA and inhibition of its activity by NIFL at the *Klebsiella pneumoniae nifH* promoter. Open complexes formed by σ^{54}-containing RNA polymerase are considerably more stable to heparin challenge in the presence of GTP than in the presence of ATP. This differential stability is most probably a consequence of GTP being the initiating nucleotide at this promoter. Adenosine nucleosides are specifically required for *Azotobacter vinelandii* NIFL to inhibit open complex formation by native NIFA, and the nucleoside triphosphatase activity of NIFA is strongly inhibited by NIFL under these conditions. We propose a model in which NIFL modulates the activity of NIFA via an adenosine nucleotide switch.

A distinct mechanism of transcriptional activation is observed among the family of prokaryotic enhancer-binding proteins which interact with the holoenzyme form of RNA polymerase containing the alternative sigma factor σ^{54} ($E\sigma^{54}$) (19, 26). The nitrogen fixation regulatory protein NIFA is a member of this family which binds to upstream activator sequences (UAS) and catalyzes the isomerization of closed promoter complexes to the open complex in a reaction which requires hydrolysis of a nucleoside triphosphate (25). Productive interactions between NIFA and $E\sigma^{54}$ are enabled by DNA loop formation, which is facilitated by the binding of integration host factor (IHF) (18, 30). The amino acid sequence of NIFA conforms to the three-domain model for the structure of σ^{54} (σ^{N})-dependent transcriptional activators (12), comprising (i) an amino-terminal domain with potential regulatory properties, (ii) a central domain possessing nucleoside triphosphatase activity, which interacts with $E\sigma^{54}$, and (iii) a C-terminal domain which recognizes the UAS. Biochemical analyses of the properties of NIFA from *Klebsiella pneumoniae* have been hampered by the insoluble nature of the native protein, although a maltose-binding protein fusion to NIFA has been used to demonstrate specific DNA binding and transcriptional activation in vitro (20). The purified central domain of *K. pneumoniae* NIFA activates transcription in the absence of specific DNA binding and possesses nucleoside triphosphatase activity (6). In contrast to its *K. pneumoniae* counterpart, the native *Azotobacter vinelandii* NIFA protein has been purified in a soluble form, and its properties with respect to DNA binding and catalysis of open complex formation have been characterized in vitro (2).

In both *K. pneumoniae* and *A. vinelandii*, the activity of NIFA is controlled by a second regulatory protein, NIFL, in response to the environmental effectors oxygen and fixed nitrogen (7, 22). Although NIFL proteins show homology in their C-terminal domains to the histidine protein kinase family of two-component regulatory proteins (14), NIFL and NIFA appear to interact at stoichiometric levels (5, 15), and phosphotransfer between the two proteins has not been detected in vitro (2, 21). Moreover, although *A. vinelandii* NIFL shows greater homology to the canonical histidine protein kinases than does *K. pneumoniae* NIFL and contains a conserved histidine residue known to be phosphorylated in other systems, mutagenesis of this residue does not impair NIFL function (40).

Members of the σ^{N}-dependent family of transcriptional activators have a nucleoside triphosphatase activity which is required for the catalysis of open complex formation, and in the case of NTRC this is regulated by phosphorylation (3, 27, 29, 37). We have previously demonstrated that ATP, GTP, or UTP can be utilized by *A. vinelandii* NIFA to promote the formation of open promoter complexes by $E\sigma^{54}$, although UTP is not as effective as the other nucleotides when the template DNA is linear (2). In this report we show that open complexes formed by NIFA at the *nifH* promoter in the presence of ATP are less stable than those formed in the presence of GTP and that this differential stability of open complexes is most probably a consequence of GTP being the initiating nucleotide at this promoter.

We also demonstrate that adenosine nucleotides are specifically required for *A. vinelandii* NIFL to inhibit open complex formation by NIFA. This finding correlates with our observation that the nucleoside triphosphatase activity of native *A. vinelandii* NIFA is strongly inhibited by NIFL when adenosine nucleotides are present. It would thus appear that adenosine nucleotide ratios may provide a switch to regulate the activity of *A. vinelandii* NIFA in response to NIFL.

MATERIALS AND METHODS

DNA templates. Plasmid pNH8 (2) carries a 240-bp *Eco*RI-*Bam*HI fragment containing the *nifH* UAS, the IHF binding site, and the *nifH* promoter from *K. pneumoniae* cloned into the transcription vector pTE103. Plasmid pJES409 (30)

* Corresponding author. Phone: (0273) 678240. Fax: (0273) 678133.

carries the *nifH* promoter region of *K. pneumoniae* with an NTRC binding site substituted for the NIFA site (*nifH* UAS). DNA templates were linearized when necessary with either *Eco*RI or *Pst*I.

Proteins. *A. vinelandii* NIFA and NIFL and *K. pneumoniae* core RNA polymerase and σ^{54} were purified according to previously published procedures (2, 38). Concentrations of NIFA and NIFL were calculated on the assumption that both proteins are dimers. *Escherichia coli* IHF was a kind gift of Howard Nash. The C-terminally truncated form of σ^{N} (residues 1 to 424) was provided by Wendy Cannon and Martin Buck.

In vitro transcript analysis. Single-round transcription assays were carried out in TAP buffer essentially as described previously (2). Reaction mixtures contained 5 nM template DNA (linearized with *Eco*RI when necessary), 75 nM core RNA polymerase, 200 nM σ^{54}, 200 nM NIFA, and 50 nM IHF and were incubated at 30°C for 20 min in the presence of 4 mM ATP, GTP, or ddATP to promote open complex formation. Complexes were then challenged with heparin (final concentration, 100 μg/ml), and transcripts were initiated or elongated with combinations of nucleoside triphosphates as described in the figure legends.

RNA extraction and primer extension assays on in vitro transcription reaction mixtures were carried out as described previously (4, 5). The 5′-end-labelled oligonucleotide primer for extension reactions was 5′-TTACCGTAATAGCG CATT-3′ (25).

Potassium permanganate footprinting of open complexes. $KMnO_4$ footprinting was conducted as described previously (38) with the exception that reactions were carried out in TAP buffer at 30°C under the conditions described above for in vitro transcript analysis.

To analyze inhibition of NIFA activity by NIFL, either NIFA alone or NIFA plus NIFL was incubated in TAP buffer for 10 min at 30°C prior to being added to the remaining prewarmed components of the reaction mixture. Reaction mixtures were incubated for a further 20 min to allow open complex formation.

Electrophoresis of DNA-protein complexes. Reaction mixtures (15-μl total volume) were set up and incubated for 20 min in TAP buffer as described above; 3 μl of a dye mix containing 50% glycerol, 0.1% xylene cyanol, 0.05% bromophenol blue, and 2 μg of heparin (when appropriate) was then added, and the mixture was immediately loaded onto a 4% (wt/vol) polyacrylamide gel (acrylamide/bisacrylamide ratio, 80:1) in 25 mM Tris-acetate–400 mM glycine (pH 8.6) which had been prerun at 180 V at room temperature down to a constant power of 2 W. Gels were run for 2.5 to 3 h at 100 V, dried, and analyzed by autoradiography. Complexes were quantitated with a Fujix BAS1000 phosphoimager.

Nucleoside triphosphatase assays. The release of P_i from [γ-^{32}P]ATP and [γ-^{32}P]GTP was monitored as described previously (3), except that 5-μl samples were taken after 20 min from the same reaction mixtures used to monitor open complex formation by gel retardation analysis (total reaction volume, 20 μl). The total nucleotide concentration was 4 mM, and each reaction mixture contained 0.65 μCi of [γ-^{32}P]ATP or [γ-^{32}P]GTP (specific activity, 3,000 Ci/mmol). P_i release was measured with a Fujix BAS1000 phosphoimager.

RESULTS

Influence of nucleotides on the formation and stability of open complexes. We have shown previously that ATP or GTP can catalyze the formation of open promoter complexes at the *nifH* promoter by *A. vinelandii* NIFA in a reaction which requires Eσ^{54} and is stimulated by IHF (2). A time course of the formation of heparin-stable promoter complexes in response to these nucleotides is shown in Fig. 1. In these experiments individual reaction mixtures were incubated for various times to allow complex formation on linear promoter DNA. After heparin addition, single-stranded DNA in the melted region was detected by $KMnO_4$ footprinting and the total level of complexes in each reaction was quantitated by densitometry. Control experiments showed that the addition of heparin prevented further formation of open complexes in the presence of either ATP or GTP (data not shown). Although the initial rate of complex formation was similar in the presence of either nucleotide, the maximum yield of complexes was approximately fourfold greater in the presence of GTP than in the presence of ATP. The decline in the level of complex formation from the maximum after longer incubation periods could reflect either depletion of the nucleotide substrate due to the nucleoside triphosphatase activity of NIFA or the accumulation of ADP or GDP, which are inhibitors of this activity (data not shown).

We considered the possibility that the lower yield of open promoter complexes formed in the presence of ATP compared with GTP could be indicative of a nucleotide-specific influence

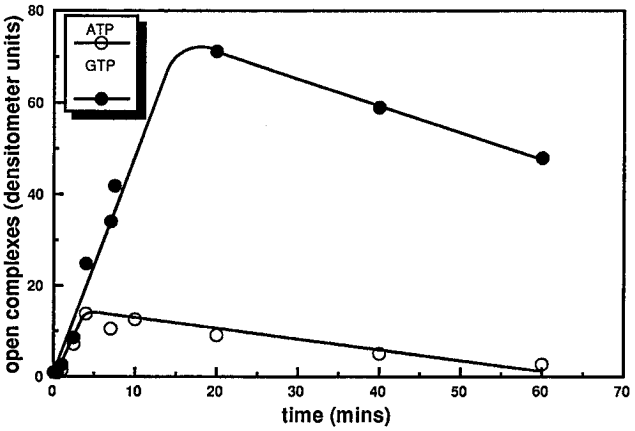


FIG. 1. Time course of open complex formation by NIFA at the *K. pneumoniae nifH* promoter on *Pst*I-linearized pNH8 DNA. Individual reaction mixtures were incubated for the times indicated on the *x* axis and then were challenged with heparin (final concentration, 100 μg/ml) and immediately probed with $KMnO_4$ as described in Materials and Methods. Reaction mixtures contained either 1 mM GTP or 4 mM ATP. After direct autoradiography, quantitative analysis of footprinting gels was carried out on a Molecular Dynamics computing densitometer by determining the peak volumes of the autoradiograms. Only exposures which fell within the linear range of the densitometer were used. Different lanes of the autoradiogram were normalized by using the intensities of three reference bands (T residues at positions −23, −59, and −107) whose reactivities were judged by visual inspection not to be influenced by open complex formation. The increase in intensity of each residue was calculated by comparison with the intensity of the residue in a reaction mixture containing $KMnO_4$-treated DNA alone. Reactive T residues between positions −9 and −1 were used for these calculations. Results shown are the means of two independent determinations.

on their stability. Preformed complexes were subjected to a heparin challenge and were then probed by treatment with $KMnO_4$ at various time intervals. When GTP was used to promote open complex formation, T residues on the top strand between positions −9 and −1 were reactive to $KMnO_4$ for up to 60 min after the heparin challenge, irrespective of the topology of the template (Fig. 2B). In contrast, complexes formed in the presence of ATP were unstable, particularly on linear DNA templates, where complexes were barely detectable after 20 min (Fig. 2A). A similar influence of DNA topology on the stability of open complexes formed at the *K. pneumoniae nifL* promoter has been observed (38), suggesting that the free energy of supercoiling may help to stabilize complexes formed on supercoiled DNA.

To determine whether the different effects of the two nucleotides on open complex stability at the *nifH* promoter are activator specific, we performed analogous experiments using phosphorylated NTRC as the transcriptional activator. Again complexes were stable to heparin challenge when formed on linear DNA in the presence of GTP and were unstable when formed in the presence of ATP (Fig. 2C). It therefore appears that the differential effects of the nucleotides on stability are not specific to activation mediated by NIFA at this promoter.

We have noted previously that open complexes formed at the *nifL* promoter on supercoiled DNA are more extensive than those formed on linear DNA templates and show enhanced reactivity to $KMnO_4$ downstream of the transcription start site (38). Although when comparing complexes formed with GTP or ATP at the *nifH* promoter, we could not detect any significant differences in the pattern of $KMnO_4$ reactivity on the top strand (Fig. 2), three residues on the bottom strand, extending from position +2 to +4, became hyperreactive only in the presence of GTP (Fig. 3; compare lanes 2 and 3 with

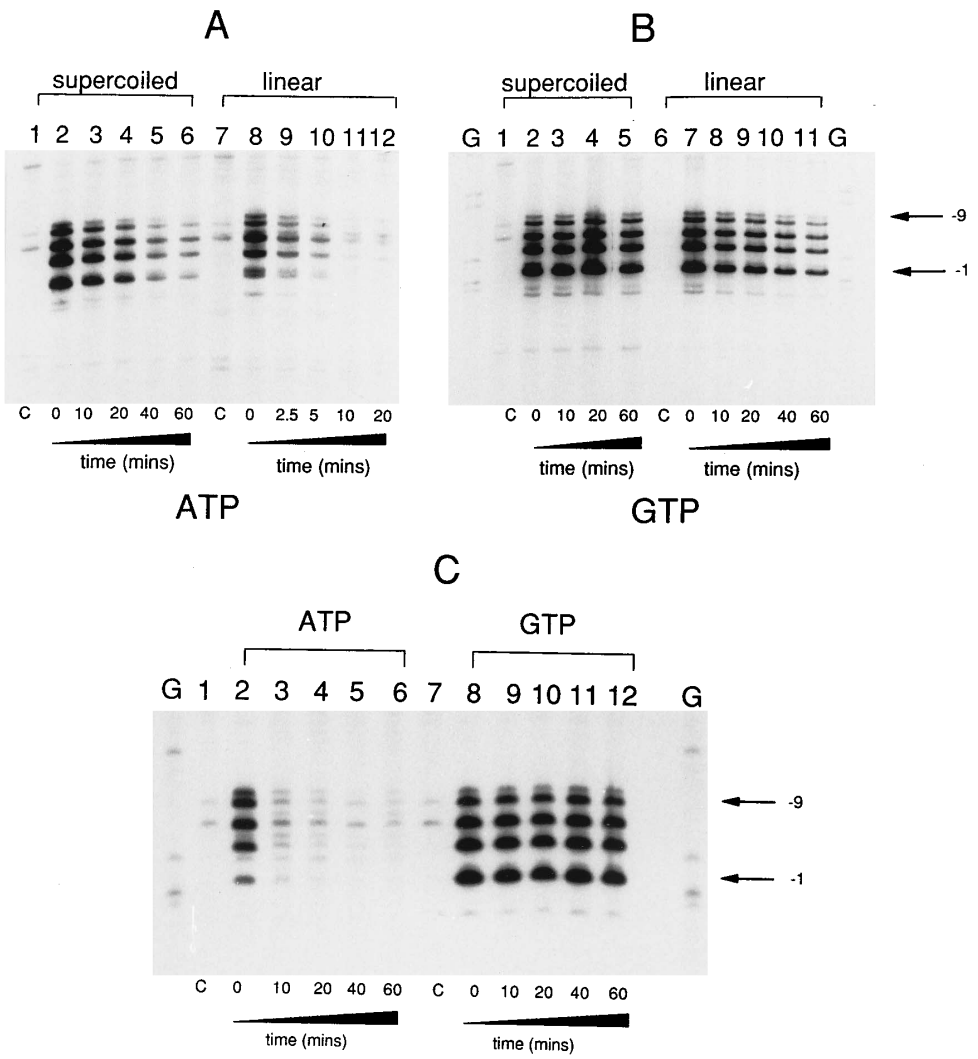


FIG. 2. Influence of nucleotides on the stability of open complexes formed at the *nifH* promoter. Open complexes were formed as described in Materials and Methods for 20 min at 30°C and then were challenged with heparin (time zero) and incubated for the times indicated beneath each lane prior to treatment with $KMnO_4$. All reaction mixtures contained $E\sigma^{54}$ and IHF, except those marked c, which are controls containing $KMnO_4$-treated DNA alone. All templates were 3′ labelled at the *Bam*HI site to analyze the top strand. Lanes G, guanine-specific chemical sequencing ladders. (A) Reaction mixtures contained 200 nM NIFA and 4 mM ATP. The template DNA was either supercoiled pNH8 (lanes 1 to 6) or *Pst*I-linearized pNH8 DNA (lanes 7 to 12). Lanes 1 and 7, $KMnO_4$-treated DNA alone. (B) Reactions were as in panel A except that the nucleotide was 1 mM GTP. Supercoiled DNA was present in lanes 1 to 5, and linear pNH8 DNA was present in lanes 6 to 11. $KMnO_4$-treated DNA alone was present in lanes 1 and 6. (C) Reaction mixtures contained 600 nM NTRC and 7.5 mM carbamoyl phosphate to promote phosphorylation of this activator. The template DNA was *Pst*I-digested pJES409. The nucleoside triphosphate was either 4 mM ATP (lanes 2 to 6) or 4 mM GTP (lanes 8 to 12). Lanes 1 and 7, $KMnO_4$-treated DNA alone.

lanes 5, 6, and 7). Moreover, this enhanced reactivity was also observed when GTP was added to heparin-treated complexes that had been preformed in the presence of ATP (Fig. 3, lanes 8 to 13). This result indicates that the presence of GTP can influence the structure of a preformed open complex, and since this extended open complex can be formed in the presence of heparin, it seems likely that GTP hydrolysis by the activator is not required for its formation.

GTP is the initiating nucleotide for *nifH* transcription. One explanation for the increased stability of open complexes formed with GTP is that this nucleotide is the initiator for transcription at the *nifH* promoter, its presence giving rise to a more extensive melted region. However, there is some disagreement in the literature concerning the precise transcription start site of the *nifH* promoter. Previous S1 nuclease and reverse transcriptase mapping studies of *nifH* mRNA made both in vivo and in vitro (5, 8, 31, 35) suggested that transcription is initiated at positions corresponding to two G residues on the top strand (marked with asterisks in Fig. 4A). However, more recent experiments labelling RNA in vitro with nucleoside [γ-^{32}P]triphosphates (28) have indicated that transcription can be initiated only with ATP at this promoter (at the site marked with an arrow in Fig. 4A). In order to resolve this discrepancy, we have determined the transcription start site by using both reverse transcriptase mapping and in vitro RNA labelling procedures. Primer extension experiments with in vitro-synthesized RNA indicated that the start site corresponded to two G residues on the top strand, as shown previously, irrespective of whether open complex formation was catalyzed by ATP or GTP prior to a heparin challenge (Fig. 4B, lanes 1 and 2). The same start sites were utilized on supercoiled as well as linear DNA templates (data not shown). No

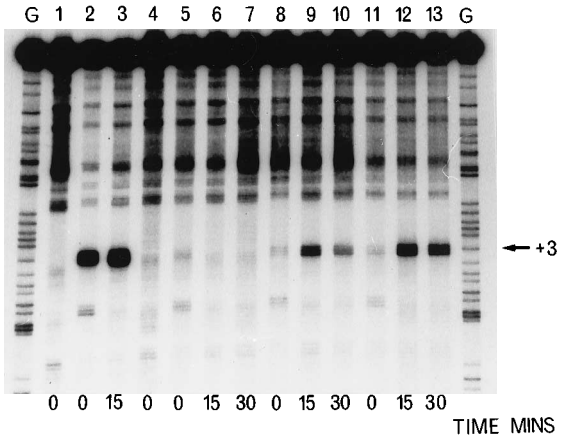


FIG. 3. Influence of nucleotides on the reactivity of open complexes to $KMnO_4$ on the bottom strand. The template DNA was the 240-bp *Eco*RI-*Bam*HI fragment from pNH8, which was 3′ labelled at the *Eco*RI site. Reaction mixtures were incubated for 20 min at 30°C in the presence of either 4 mM GTP (lanes 1 to 3) or 4 mM ATP (lanes 4 to 13). Complexes were then challenged with heparin (time zero) and footprinted with $KMnO_4$ after a further incubation period of 0, 15, or 30 min as indicated beneath each lane. Reaction mixtures in lanes 4 to 7 contained no additional nucleotide, whereas in lanes 8 to 13, GTP was added to the mixture at the same time as the heparin challenge (100 μM GTP in lanes 8 to 10 and 1 mM GTP in lanes 11 to 13). Lanes 1 and 4, control reactions with template DNA and nucleotide but without proteins. Lanes G, guanine-specific chemical sequencing reactions.

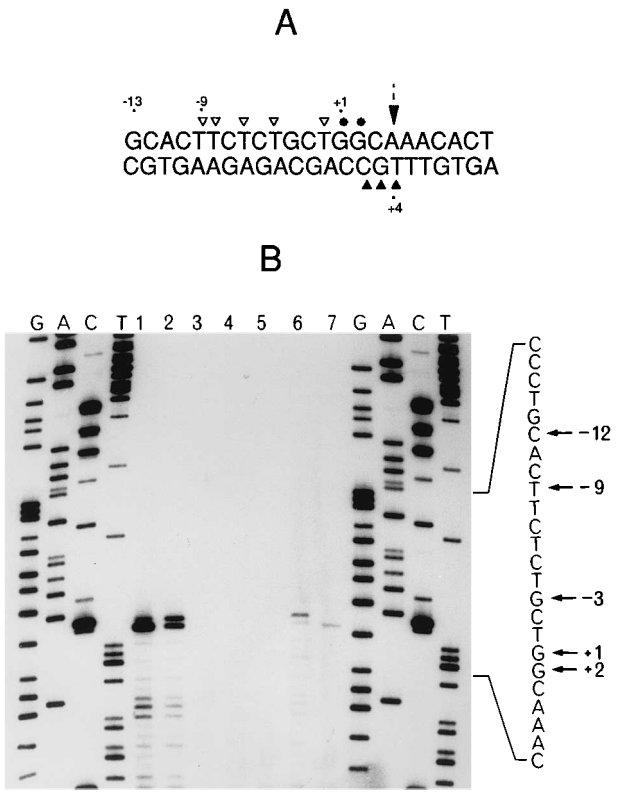


FIG. 4. Sequence of the *K. pneumoniae nifH* promoter around the transcription start site. Open triangles show residues reactive to $KMnO_4$ in open complexes formed with either ATP or GTP, whereas closed triangles indicate residues which are reactive only in the presence of GTP. The unexpected hyperreactivity of the G residue at position −3 to $KMnO_4$ treatment and piperidine cleavage presumably reflects considerable structural distortion. Asterisks indicate transcription start sites identified in this study and by others. The dotted arrow indicates the start site reported in reference 28. (B) Primer extension analysis of in vitro *nifH* transcripts. Products in lanes 1 to 6 were derived from in vitro transcription reactions with *Eco*RI-linearized pNH8 DNA incubated in the presence of either 4 mM ATP (lanes 1 and 4) or 4 mM GTP (lanes 2, 3, 5, and 6) to promote open complex formation. All reaction mixtures contained $E\sigma^{54}$, with the exception of those in lanes 3 and 4, in which σ^{54} was replaced by a C-terminally truncated derivative (residues 1 to 424). Reaction mixtures also contained NIFA and IHF, with the exception of those in lane 5 (no NIFA) and lane 6 (no IHF). After 20 min of incubation, transcripts were extended by addition of a mixture of all four nucleoside triphosphates (400 μM). RNA was extracted from the reaction mixtures and analyzed by primer extension mapping as described in Materials and Methods. The product in lane 7 is derived from RNA isolated from *K. pneumoniae* grown under nitrogen-fixing conditions. Lanes marked G, A, T, and C, dideoxy sequencing reactions (bottom strand) with the same end-labelled primer. The corresponding sequence of the top strand is shown to the right.

extension products were detected when $E\sigma^{54}$ was replaced by holoenzyme containing a truncated form of the sigma factor or when NIFA was omitted from the reactions (Fig. 4B, lanes 3 to 5). The signal decreased as expected when IHF was omitted (Fig. 4B, lane 6), and the in vivo start site mapped to the second of the two G residues (Fig. 4B, lane 7). In agreement with these results, the in vitro-synthesized RNA became labelled in the presence of [γ-^{32}P]GTP but not [γ-^{32}P]ATP irrespective of whether the template DNA was linear or supercoiled (Fig. 5A). When the transcription assay mixture contained ATP, CTP, [α-^{32}P]UTP, and the dinucleotide GpG, short (18-nucleotide) transcripts were detected in the absence of GTP (data not shown). Since the first available G residue downstream of the start sites is located at position +19, this result suggests that GpG can act as the initiator. Our findings are therefore fully consistent with previous data indicating that transcription is initiated by GTP at this promoter, and we have no explanation for the apparent involvement of ATP as reported previously (28).

The increased stability and extended nature of the open complex formed in the presence of GTP could indicate the formation of a stable ternary complex, although previous experiments with the σ^{54}-dependent *glnAp*$_2$ promoter have suggested that the synthesis of a transcript of at least 7 nucleotides is required in order to form the ternary complex (28). We investigated whether rifampin, which inhibits the transition from binary to stable ternary complexes, would inhibit the further elongation of transcripts formed in the presence of different combinations of nucleotides. The presence of GTP alone did not give rise to a rifampin-resistant complex, whereas the addition of GTP and CTP resulted in some complexes being rifampin resistant (Fig. 5B; compare lanes 1 and 2 with lanes 3 and 4). The presence of GTP and CTP would be expected to allow synthesis of the trinucleotide GGC (Fig. 4A), but in abortive initiation experiments (data not shown), some transcripts of 9 nucleotides or greater were present, indicating either that this nucleotide combination may be contaminated or that misincorporation occurs (23). In the presence of GTP, CTP, and ATP, most complexes were rifampin resistant, as might be expected (28) (Fig. 5B, lanes 5 and 6) since this combination should allow synthesis of a 9-nucleotide transcript (Fig. 4A). We therefore conclude that the presence of GTP alone does not allow the formation of a stable ternary complex at this promoter.

Gel retardation analysis of open promoter complexes. Gel mobility shift assays were used to detect DNA-protein interactions at the *nifH* promoter and to investigate the potential role of nucleotides in the formation of nucleoprotein complexes. We expected that complexes which had undergone the transition to the open promoter form would be resistant to heparin challenge in a band shift assay. When complexes were formed in the presence of ATP and challenged with heparin

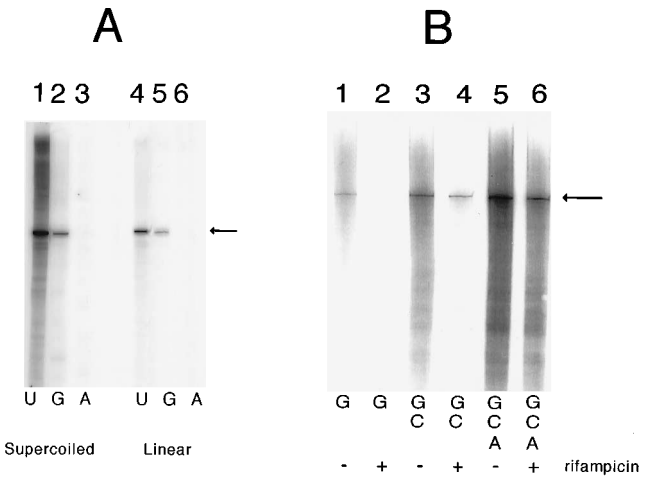


FIG. 5. Determination of the initiating nucleotide and rifampin sensitivity of initiated complexes. (A) Open complexes were formed in the presence of 4 mM ddATP on supercoiled pNH8 (lanes 1 to 3) or *Eco*RI-linearized pNH8 DNA (lanes 4 to 6). After 20 min of incubation, heparin was added together with a mixture all four ribonucleoside triphosphates (60 μM each) to initiate transcription. These mixtures also contained either 5 μCi of [α-^{32}P]UTP (lanes 1 and 4), 10 μCi of [γ-^{32}P]GTP (lanes 2 and 5), or 10 μCi of [γ-^{32}P]ATP (lanes 3 and 6). After 5 min, a chase mix of each nucleotide (400 μM) was added, and the reaction mixtures were incubated for a further 10 min to allow complete elongation. Transcripts were precipitated with ethanol and analyzed by electrophoresis on a 6% sequencing gel. (B) Open complexes were formed on supercoiled pNH8 DNA as described for panel A and challenged with heparin after 20 min of incubation. [α-^{32}P]GTP (10 μM) was then added to initiate transcription (lanes 1 and 2), with the addition of CTP (100 μM) (lanes 3 and 4) or CTP plus ATP (100 μM each) (lanes 5 and 6) to promote the formation of short RNA chains of 2, 3, and 9 bases, respectively. After 5 min of incubation, the reactions in lanes 2, 4, and 6 were challenged with rifampin (200 μM). All reaction mixtures were then incubated for a further 5 min prior to the addition of a chase mix of all four ribonucleoside triphosphates (400 μM each). Reaction mixtures were then incubated for a further 10 min prior to ethanol precipitation and analysis as described above.

prior to loading of the gel, a retarded species, designated c4, whose presence was dependent on Eσ^{54} and NIFA was formed (Fig. 6A). Formation of the c4 complex was also stimulated by IHF (Fig. 6B; compare lanes 7 and 9 with lanes 11 and 13). These requirements, in addition to the observation that the c4 complex was not detected in the absence of nucleoside triphosphates (Fig. 6B, lane 5) suggest that it represents the heparin-resistant open complex. No significant difference in the mobilities of complexes formed in the presence of ATP and those formed in the presence of GTP were noticeable, although as expected from the footprinting studies, the yield of the c4 complex was approximately fourfold greater in the presence of GTP. When c4 bands were excised from the gel and subjected to $KMnO_4$ treatment in the gel slice, T residues in the melted region of the promoter showed the characteristic pattern of reactivity found in the open complex (data not shown).

In the absence of heparin we detected two further complexes, c1 and c2, whose presence was not dependent on nucleotides (Fig. 6B, lanes 4, 8, and 12). Complex c1 is formed in the presence of IHF alone (data not shown), whereas the retarded species designated c2 may represent a complex mixture of closed complexes, open complexes, and multiply bound molecules which are not resolved in this gel system. $KMnO_4$ treatment of c2 complexes excised from the gel revealed that open complexes were present only in those bands originating from reactions which contained nucleotides (data not shown). As expected under the buffer conditions used (9), we did not detect binding of Eσ^{54} alone to the promoter, and a stable bound species was not observed when 200 nM NIFA was present in addition to Eσ^{54} (Fig. 6B, lanes 2, 6, and 10), even though this concentration of NIFA is sufficient to saturate open complex formation. IHF is therefore required for the formation of the c2 and c4 complexes.

Adenosine nucleotides influence specific inhibition of NIFA activity by NIFL. We have previously shown that *A. vinelandii* NIFL, when overproduced under aerobic nitrogen-rich growth conditions and purified from *E. coli*, inhibits open complex formation by NIFA in the presence of ATP (2). Similar results were obtained when heparin-resistant complexes were analyzed by gel retardation, since formation of complex c4 was inhibited when NIFL was present in reaction mixtures containing ATP (Fig. 7A, lanes 1 to 4). The presence of NIFL had no effect on the mobility of complex c1 or c2, indicating that NIFL does not interfere with the binding of IHF or NIFA to DNA (data not shown). However, when GTP was used to promote the formation of open complexes, NIFL did not inhibit the presence of complex c4 (Fig. 7A, lanes 5 to 8). These results were confirmed by $KMnO_4$ footprinting of open complexes. NIFL inhibited the formation of open promoter complexes by NIFA in the presence of ATP on both linear and supercoiled DNA templates but had no effect on the formation of complexes in the presence of GTP (Fig. 7B and C). As observed previously (2), the inhibitory effect of NIFL was specific, since NIFL did not influence the formation of open promoter complexes by NTRC in the presence of ATP.

Nucleotide-specific inhibition of NIFA activity by NIFL cannot be explained on the basis of the differential stability of open promoter complexes, since we have shown previously that open complex formation in the presence of GTP, CTP, and ATP (which normally results in the presence of rifampin-resistant complexes in the absence of NIFL [Fig. 5B]), is also susceptible to NIFL inhibition (2). When ATP was added to the reaction mixtures in addition to GTP, some inhibition by NIFL was observed even at a high ratio of GTP to ATP (Fig. 7D and E). Inhibition in reaction mixtures containing GTP was also observed when the nonhydrolyzable analog of ATP, ATPγS, was added, but the inhibition was greater in the presence of ADP (Fig. 7D and E). Control experiments with NIFA alone indicated that the adenosine nucleotides inhibited open complex formation to some extent (the maximum inhibition observed was 24% with ADP), but inhibition increased significantly when both NIFL and NIFA were present (at least 92% inhibition with ADP). NIFL-mediated inhibition was not observed when CTP, UTP, GDP, GTPγS, or AMP was present in addition to GTP (Fig. 7D), implying that the requirement for inhibition is specific to ADP and ATP. We considered the possibility that ADP is a more potent effector of NIFL-mediated inhibition than ATP and that the inhibition of NIFA activity seen in the presence of ATP is due to formation of ADP by the catalytic activity of NIFA. Accordingly, when we ensured that ADP formed in the reaction was rapidly converted to ATP, by adding an ATP-generating system consisting of a mixture of creatine kinase and creatine phosphate, NIFL-mediated inhibition was not observed (Fig. 7D).

Inhibition of the nucleoside triphosphatase activity of NIFA by NIFL. One way in which NIFL could inhibit NIFA activity is by modulating the catalytic activity of this activator. We have found that nucleoside triphosphatase activity copurifies with *A. vinelandii* NIFA and that the rate of ATP hydrolysis by the protein is similar to that of GTP. In contrast, purified NIFL had no detectable nucleoside triphosphatase activity (data not shown). However, when NIFL was added to reaction mixtures containing native NIFA, the ATPase activity was inhibited, coincident with a similar decrease in the presence of heparin-

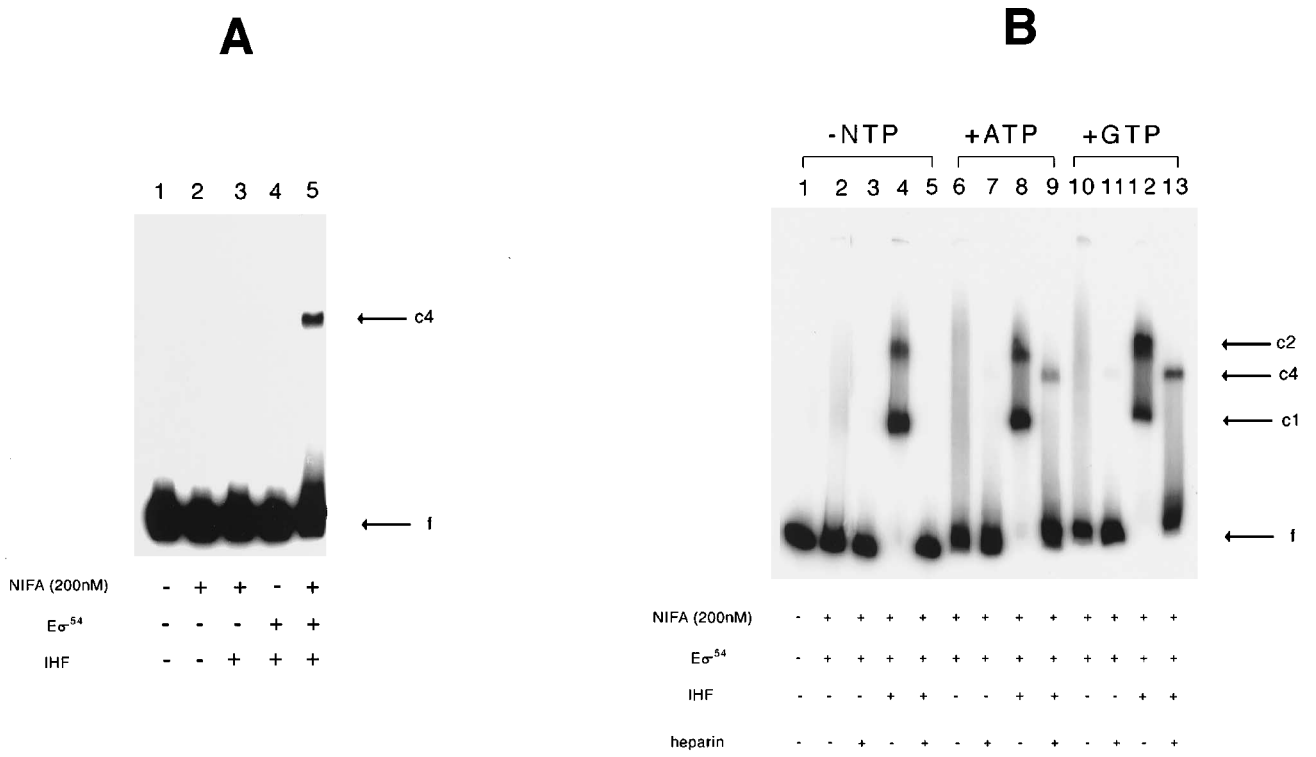
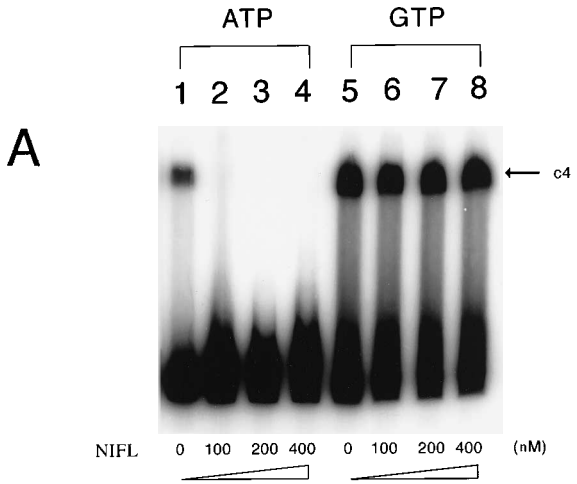


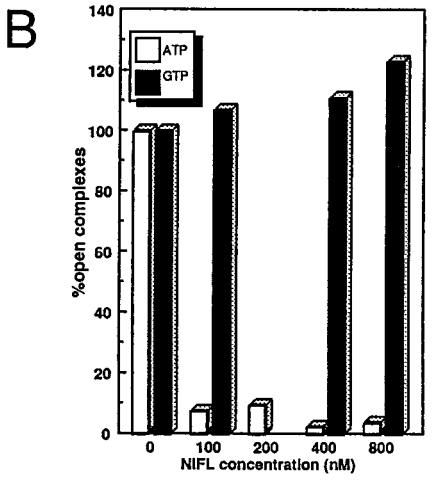
FIG. 6. Nucleoprotein complexes analyzed by gel retardation analysis. Reaction mixtures were incubated for 20 min and then loaded on a native gel as described in Materials and Methods. The DNA template was the *Eco*RI-*Bam*HI fragment from pNH8, labelled at the *Bam*HI site. (A) Requirements for the formation of heparin-resistant complexes. All reaction mixtures contained ATP (4 mM), and heparin was added immediately prior to loading of the gel as described in Materials and Methods. NIFA was present in lanes 2, 3, and 5, $E\sigma^{54}$ was present in lanes 4 and 5, and IHF was present in lanes 3, 4, and 5. (B) Influence of heparin and nucleotides on complex formation. Nucleotides added to reaction mixtures: lanes 1 to 5, none; lanes 6 to 9, ATP (4 mM); lanes 10 to 13, GTP (4 mM). NIFA and $E\sigma^{54}$ were present in all reaction mixtures except that in lane 1. IHF was present in lanes 4, 5, 8, 9, 12, and 13, and heparin was added prior to loading of samples in lanes 3, 5, 7, 9, 11, and 13.

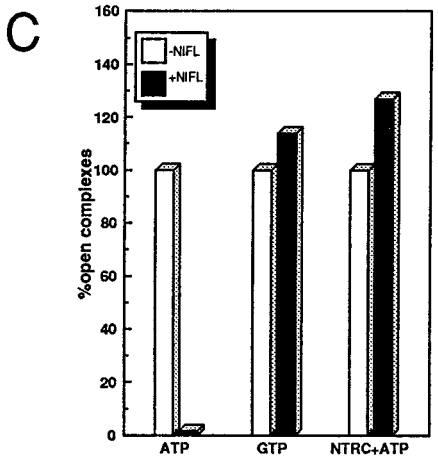
resistant open complexes (Fig. 8A). Inhibition was apparently specific to NIFA, since no inhibition of the ATPase activity or open complex formation by NTRC was detected (Fig. 8B). Specific inhibition of the nucleoside triphosphatase activity of NIFA by NIFL provides evidence that this activity is a property of the NIFA protein itself and is not due to the presence of contaminants. When only GTP was present, NIFL reduced GTPase activity by 50% at relatively high NIFL concentrations, and no inhibition of open complex formation was observed (Fig. 8C). However, when both ATP and GTP were present, the GTPase activity of NIFA was inhibited more strongly at high protein concentrations; open complex formation was also inhibited, in agreement with our finding that adenosine nucleotides are required for inhibition by NIFL (Fig. 8D). The steady-state data in Fig. 8 suggest that the GTPase activity of NIFA is not limiting for open complex formation, but confirmation of this will require a more detailed kinetic approach.

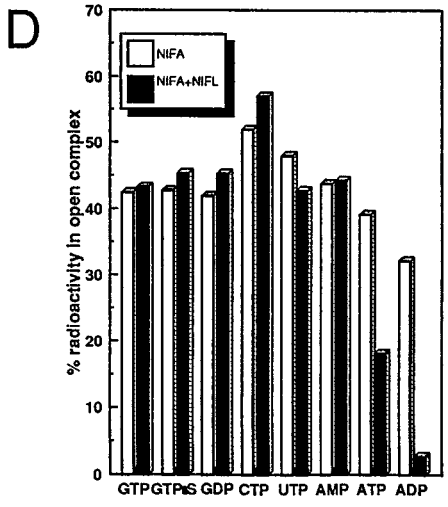
DISCUSSION

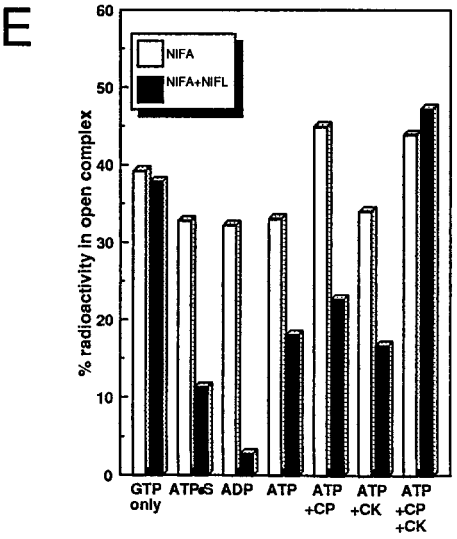
Analysis of the role of nucleotides in transcriptional activation at σ^{54}-dependent promoters is complicated by the requirement for nucleoside triphosphates in catalysis of the isomerization step and the subsequent involvement of nucleotides in transcription initiation. We find significant differences in the stabilities of open complexes preformed by NIFA at the *K. pneumoniae nifH* promoter in the presence of GTP and those formed in the presence of ATP. This difference is particularly marked on linear DNA templates when further open complex formation is prevented by addition of heparin, which presumably competes for binding of IHF and other proteins not specifically bound in the open complex. Complexes formed on negatively supercoiled DNA were more stable, possibly because they are stabilized by the free energy of DNA supercoiling, as suggested from studies of the *K. pneumoniae nifL* promoter (38, 39). Our results suggest that two different types of open complex are formed. Complexes formed in the presence of GTP are more extensive than those formed with ATP, and in the former case the melted region appears to extend downstream of the transcription start site, in agreement with our confirmation that GTP is the initiating nucleotide at the *nifH* promoter. The transition of RNA polymerase into the initiated conformation in the presence of GTP may strengthen polymerase-promoter contacts, perhaps as a consequence of a conformational change which realigns the catalytic subunits and stabilizes the complex. Alternatively, base pairing with a melted single strand may stabilize the open complex. Although the presence of GTP is predicted to allow synthesis of the dinucleotide GpG and hence formation of the first phosphodiester bond, the rifampin chase experiments suggest that GTP does not promote the transition from the promoter-polymerase binary complex to the stable ternary complex. There may be some differences between the nature of the initiated complex formed with $E\sigma^{54}$ and that of the complex formed with $E\sigma^{70}$, since individual nucleotides or the initiator GpA does not influence the rate of dissociation of open complexes formed at the *lac*UV5 promoter (34), although dissociation is significantly reduced if the nucleotide combination allows a short (6-mer) RNA to form, suggesting that open complexes


A
ATP
GTP
1 2 3 4 5 6 7 8
c4
NIFL 0 100 200 400 0 100 200 400 (nM)


B
ATP
GTP
%open complexes
0 100 200 400 800
NIFL concentration (nM)


C
-NIFL
+NIFL
%open complexes
ATP
GTP
NTRC+ATP


D
NIFA
NIFA+NIFL
% radioactivity in open complex
GTP GTPγS GDP CTP UTP AMP ATP ADP


E
NIFA
NIFA+NIFL
% radioactivity in open complex
GTP only
ATPγS
ADP
ATP
ATP +CP
ATP +CK
ATP +CP +CK

may be stabilized by the process of abortive initiation (10, 34). In contrast, whereas open complexes formed at the T7 A1 promoter are heparin resistant, the abortive transcription complex is heparin sensitive (23). Although our results strongly suggest that GTP stabilizes open complexes as a consequence of its role as the initiating nucleotide, we cannot rule out the possibility that NIFA in the presence of GTP reduces the dissociation rate of $E\sigma^{54}$ in open complexes via a direct protein-protein interaction, as has been observed for the catabolite gene activator protein-RNA polymerase association at the *lac* promoter (33). In the latter case the influence of GTP would not be specific to NIFA, because we also found that GTP stabilizes open complexes formed by NTRC. The gel retardation studies confirmed our previous observations (2) that there is a stringent requirement for IHF in the formation of open promoter complexes on linear DNA fragments. Although open complexes formed in the presence of ATP are relatively unstable to heparin challenge in solution, we were able to detect ATP-dependent complexes in the gel retardation assay following heparin challenge. However, in this case the heparin was added immediately prior to loading of the gel, and there is a possibility that complexes are stabilized within the gel matrix.

Our data strongly suggest that adenosine nucleotides are specifically required for the inhibitory activity of NIFL. Since ADP is a more potent effector than ATP and promotes inhibition in the absence of ATP, it seems extremely unlikely that inhibition involves phosphotransfer between NIFL and NIFA as observed in conventional two-component regulatory systems. Furthermore, NIFL does not apparently exhibit autophosphorylation or protein kinase activity, and current evidence suggests that sensory transduction between NIFL and NIFA occurs via stoichiometric protein-protein interactions (2, 15, 21, 40). Adenosine nucleotides, particularly ADP, may therefore influence complex formation between NIFL and NIFA, either by binding specifically to NIFL or, for example, by altering the conformation of NIFA and thus rendering it more susceptible to the inhibitory activity of NIFL.

It is possible that the sequence homology between NIFL and other members of the histidine protein kinase family is indicative of a conserved nucleotide binding site located in the C termini of these proteins (27a). Interestingly, the sporulation protein SpoIIAB from *Bacillus subtilis*, which is a serine protein kinase, also shows homology to this putative nucleotide-binding pocket and, like NIFL, interacts stoichiometrically with its partner SpoIIAA in response to adenosine nucleotide levels (1, 24). In this case the ATP/ADP ratio influences the partner with which SpoIIAB can interact. In the presence of ADP, SpoIIAB associates with SpoIIAA, whereas ATP favors complex formation between SpoIIAB and the sigma factor σ^F, thereby inhibiting σ^F activity (1). A similar model could be proposed for NIFL, in which ADP favors complex formation between NIFL and NIFA and ATP stimulates interaction between NIFA and $E\sigma^{54}$. However, in this system, partner switching would be a property associated with the activator NIFA rather than with the negative regulator NIFL.

The nucleotide-binding pocket located within the central domain of NIFA might provide another site for sensing the level of adenosine nucleotides. According to this model, the ADP-bound form of NIFA might favor a conformation which facilitates interaction with NIFL, in contrast to the ATP-bound form. However, in this case the conformational switch would be specific to the ATPase cycle, since no inhibition by NIFL occurs in the presence of GDP or GTP. Our evidence suggests that when the inhibitory complex between NIFL and NIFA is formed, the nucleoside triphosphatase activity of the activator is inhibited. Thus, NIFL apparently can modulate the activity of native NIFA by inhibiting its catalytic activity. This presumably involves a specific interaction between NIFL and NIFA, since NIFL does not inhibit the ATPase activity of NTRC.

Whatever mechanism is involved in the formation of the inhibitory NIFL-NIFA complex, it is clear that our data with the *A. vinelandii* native NIFL and NIFA proteins are markedly different from those obtained with the refolded form of *K. pneumoniae* NIFL and derivatives of *K. pneumoniae* NIFA. First, *K. pneumoniae* NIFL does not show a nucleotide-specific requirement for inhibition of either the maltose-binding protein–NIFA fusion or the central domain of NIFA (6, 21). Second, in contrast to our results, the nucleoside triphosphatase activity of the central domain of *K. pneumoniae* NIFA is not inhibited by NIFL, even though refolded *K. pneumoniae* NIFL does inhibit transcriptional activation by this isolated domain (6). Although these apparent contradictions can be rationalized on the basis of differences between the *Azotobacter* and *Klebsiella* proteins, we favor the view that the isolated central domain and the maltose-binding protein fusion forms of NIFA may interact with NIFL differently from the native NIFA protein. This hypothesis is strongly supported by the observation that removal of the amino-terminal domain of *K. pneumoniae* NIFA increases its susceptibility to inhibition by NIFL in vivo in the absence of oxygen and fixed nitrogen (13). The amino-terminal domain may thus play a regulatory role in sensing conformational changes in NIFL, perhaps allowing interaction only with a conformer of NIFL which is

FIG. 7. Nucleotide-specific requirement for the inhibition of NIFA activity by NIFL. (A) Influence of NIFL on formation of heparin-resistant complex c4 on a native gel. Template DNA was the *Eco*RI-*Bam*HI fragment from pNH8, labelled at the *Bam*HI site. All reaction mixtures contained $E\sigma^{54}$ and IHF. NIFA (200 nM final concentration) and NIFL (final concentration indicated beneath each lane) were preincubated in TAP buffer for 10 min prior to addition to the remaining prewarmed components of the reaction mixture. ATP (4 mM) was present in reaction mixtures loaded in lanes 1 to 4, and GTP (4 mM) was present in those loaded in lanes 5 to 8. Reaction mixtures were incubated for 20 min and challenged with heparin prior to gel loading. (B) Influence of NIFL on open complex formation by NIFA as determined by $KMnO_4$ footprinting. Reactions were set up as described for panel A, with the NIFL concentrations indicated on the *x* axis. ATP or GTP was present at 4 mM. Reactive residues in the open complex were analyzed as described for Fig. 1 with the exception that the bands were quantitated on a Fujix BAS 1000 phosphoimager. Results were normalized to 100% with respect to open complexes formed in the presence of NIFA alone. (C) Reactions were set up and analyzed by $KMnO_4$ footprinting as described for panel B with the exception that the DNA template was supercoiled pNH8 DNA. When included, NIFL was present at 200 nM. Reactions marked ATP and GTP contained 200 nM NIFA and the indicated nucleoside triphosphate (400 mM). Control reaction mixtures contained NTRC (600 nM) phosphorylated with carbamoyl phosphate (10 mM); open complexes were generated with ATP (4 mM) (indicated as NTRC+ATP). Results were normalized to 100% with respect to open complexes formed in the absence of NIFL. (D) Specificity of the nucleotide requirement for NIFL inhibition. Open complex formation was analyzed by quantitation of complex c4 on native gels. Reactions were set up as described for panel A with the exception that all reaction mixtures contained either GTP (4 mM) or GTP (3.8 mM) together with an additional nucleotide (0.2 mM) as indicated on the *x* axis. Preincubation mixtures contained either NIFA or NIFA plus NIFL. The final concentration of each of these proteins in the reaction mixture was 200 nM. Complexes were quantitated on the phosphoimager and expressed as the percentage of the total radioactivity in each lane as complex c4. (E) Influence of an ATP-regenerating system on NIFL inhibition of NIFA activity. Reactions were set up and analyzed as described for panel D. All reaction mixtures contained GTP, either alone (4 mM) or with an additional nucleotide (0.2 mM) as indicated on the *x* axis. In each case the total nucleotide concentration was 4 mM. Addition of creatine phosphate (CP) (12 mM) and/or creatine kinase (CK) (20 U/ml) is also indicated on the *x* axis.

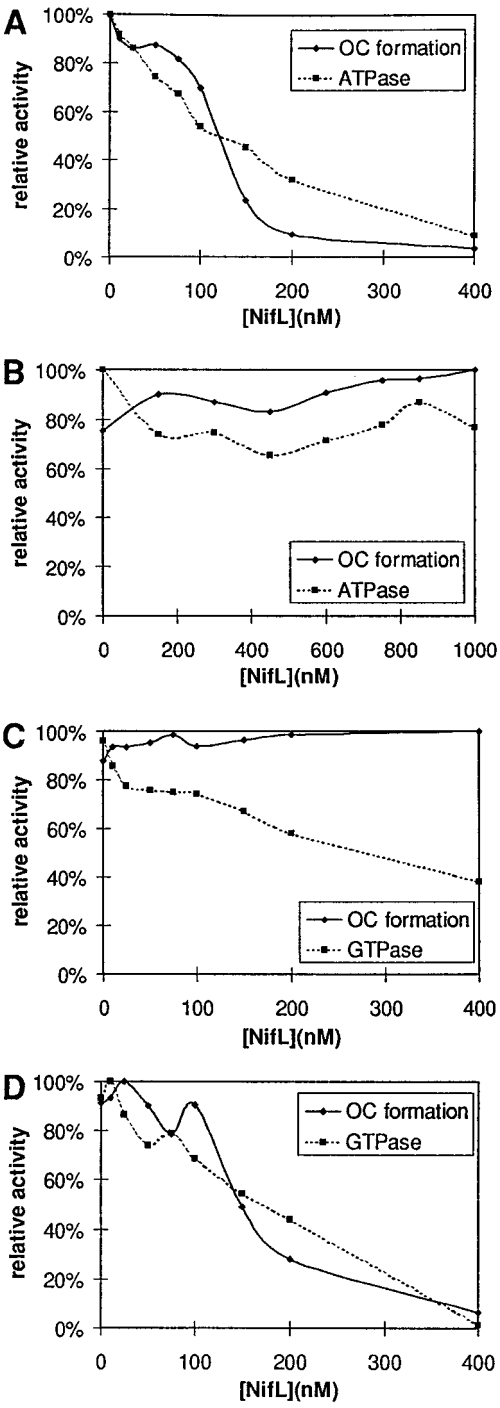


FIG. 8. Inhibition of the nucleoside triphosphatase activity of NIFA by NIFL. Both open complex (OC) formation and nucleoside triphosphatase activity (open lines) were analyzed with the same reaction mixture, as described in Materials and Methods. (A) Reaction mixtures contained 4 mM ATP and NIFA (200 nM). ATPase activity in the absence of NIFL was 130 pmol of P_i per min per pmol of NIFA (100%). Open complex formation was quantitated by using the *Eco*RI-*Bam*HI fragment from pNH8; 15% of total radioactivity was in complex c4 in the absence of NIFL (100%). (B) Reaction mixtures contained 4 mM ATP and 600 μM NTRC phosphorylated with carbamoyl phosphate (10 mM). ATPase activity in the absence of NIFL was 4.3 pmol of P_i per min per pmol of NTRC (100%). Open complex formation was measured by using the *Eco*RI-*Bam*HI fragment from pJES409; 22% of total radioactivity was in complex c4 in the absence of NIFL (100%) (C) Reaction mixtures contained 4 mM GTP and NIFA (200 nM); 100% GTPase activity was 116 pmol of P_i per min per pmol of NIFA. Open complex formation was assayed on the *Eco*RI-*Bam*HI fragment from pNH8; 50% of total radioactivity was in complex c4 in the absence of NIFL (100%) (D) Reaction mixtures contained 3.8 mM GTP, 0.2 mM ATP, and NIFA (200 nM); 100% GTPase activity was 88 pmol of P_i per min per pmol of NIFA. Open complex formation was measured as described for panel C; 44% of total radioactivity was in complex c4 in the absence of NIFL (100%).

stabilized by the presence of the appropriate adenosine nucleotides. This domain could therefore act as a conformational switch, preventing interaction with NIFL under derepressing conditions. Although the experiments with truncated NIFA proteins indicate that the N-terminal domain is not the sole target for NIFL inhibition, this domain may modulate the catalytic activity of the central domain in response to NIFL. It would thus appear that the inhibitory effect of NIFL on NIFA may involve both inhibition of its catalytic activity and the potential to disrupt interactions between NIFA and $E\sigma^{54}$.

Although we have evidence for an adenosine nucleotide switch controlling the activity of *A. vinelandii* NIFL and NIFA in vitro, the physiological significance of this response is unclear at present. Perturbations in external oxygen concentration and changes in nitrogen status are likely to influence the energy status, and consequent changes in the ATP/ADP ratio could therefore modulate the activity of NIFL and NIFA in vivo in response to either oxygen or fixed nitrogen. However, there are conflicting data concerning the effect of these perturbations on the ATP/ADP ratio in diazotrophs (16). Indeed, one report suggests that the ATP/ADP ratio decreases markedly in both *A. vinelandii* and *K. pneumoniae* under N-limiting conditions (36), which according to our in vitro studies would lead to inactivation of NIFA by NIFL. Clearly there is a need for further experimentation to characterize the influence of energy status on the regulation of nitrogen fixation. Since NIFL appears to respond independently to oxygen and fixed nitrogen (11, 17, 32), it is most probable that its activity is controlled by other signals in addition to adenosine nucleotides.

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ADDENDUM IN PROOF

The previous observation (28) that transcripts from the *K. pneumoniae nifH* promoter are initiated at the A residue marked in Fig. 4A has now been corrected by Berger et al. (D. K. Berger, F. Narberhaus, H.-S. Lee, and S. Kustu, J. Bacteriol. **177:**191–199, 1995).

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