Purification and in Vitro Activity of a Truncated Form of ANFA

TRANSCRIPTIONAL ACTIVATOR PROTEIN OF ALTERNATIVE NITROGENASE FROM AZOTOBACTER VINELANDII*

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The ANFA protein is the transcriptional activator of the α4-dependent anfHIDGK operon, which codes for the structural genes of the third nitrogenase system in Azotobacter vinelandii. We have purified, in soluble active form, an N-terminally truncated form of the protein, ΔANFA, which activates transcription from the anfH promoter and other α4-dependent promoters in a purified transcription system. Sequences upstream of the anfH promoter and the presence of the integration host factor protein stimulate transcription, and we have shown that ΔANFA binds to sites situated between 200 and 300 base pairs upstream of the anfH promoter. In common with other α4-dependent activators, ANFA has a highly conserved ATP binding motif in its central domain, and we have demonstrated that ATP or GTP is required for productive complex formation and that the purified truncated protein has a constitutive ATPase activity, which is presumably required to drive open complex formation.

Azotobacter vinelandii synthesizes three distinct nitrogenases, a conventional molybdenum nitrogenase, a vanadium nitrogenase, and an alternative nitrogenase that contains neither vanadium nor molybdenum. Expression of all three nitrogenase systems is repressed by high concentrations of fixed nitrogen, but the availability of metals regulates the expression of these genes: NIFA for vnfTIDGK, VNFA for vnfHIDGK, and ANFA for anfHIDGK (Joerger et al., 1991). The central domains of NIFA, VNFA, and ANFA are highly homologous and contain the ATP binding motif characteristic of other α4-dependent activators (Joerger et al., 1989a; Ronson et al., 1987). The C-terminal domains of these proteins contain a helix-turn-helix motif involved in DNA binding. The sequence of the proposed recognition helix in A. vinelandii NIFA is homologous to that conserved in other NIFA proteins and reflects a common DNA binding site (upstream activator sequence (UAS)).

ANFA is homologous to that conserved in other NIFA proteins and reflects a common DNA binding site (upstream activator sequence (UAS)). VNFA and ANFA do not contain the same recognition helix and are predicted to recognize different upstream sequences (Joerger et al., 1989a). Recently a UAS different from the conserved NIFA binding site has been proposed for VNFA (Kennedy et al., cited in Merrick (1993)), but that for ANFA remains to be identified.

In vitro studies with NIFA proteins from a number of organisms have been hindered by the very insoluble nature of these proteins when overproduced (Tuli and Merrick 1988; Santero et al., 1989; Austin et al., 1990). Although the predicted amino acid sequences of the alternative NIFAs are homologous to the family of NIFA proteins, particularly in the C-terminal region of the protein, we considered that these differences might be sufficient to change the solubility characteristics of these proteins compared with NIFA. In vivo in an enteric background, ANFA activation of the anfH promoter is dependent on the presence of the iron protein, but removal of the N-terminal domain of ANFA alleviates this requirement and produces a protein that is more transcriptionally active than the full-length form of the protein. Thus, it appears that in the absence of the iron protein the N-terminal domain has a negative role, and removal of this gives rise to a protein that has an enhanced ability to activate transcription. We have purified the N-terminally truncated ANFA in a soluble active form and characterized the requirements for transcriptional activation with α4-dependent promoters using in vitro transcription and DNA binding assays.

EXPERIMENTAL PROCEDURES

Materials

\[^{35}S\]UTP, α-[^32]P]UTP, and γ-[^32]P]ATP were purchased from Amerham Corp.

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ANFA, with respect to the predicted transcription start site promoter and the upstream regulatory region. The sequence shown positions of the -24 to -12 downstream promoter element and the had been previously shown to synthesize a transcriptionally active pro-tein. The overexpression construct (pJL2) directed the synthesis of a ing sequence under control of the tac promoter. The pEF321 construct

buffer achieved from (Joerger et al., 1992). Escherichia coli integra-
tion host factor (IHF) protein was the kind gift of Howard Nassh.

Transcription Assays—Single round transcription assays with puri-ified proteins were carried out in either acetate buffer (50 mM Tris acetate, pH 7.8, 100 mM potassium acetate, 5 mM magnesium acetate, 27 mM ammonium acetate, 1 mM dithiothreitol) or chloride buffer (50 mM Tris-HCl, pH 7.8, 10 mM magnesium chloride, 100 mM potassium chloride, 0.1 mM EDTA, 0.1 mM dithiothreitol, 50 µM bovine serum albumin). Template DNA (10 ng) was preincubated at 37 °C for 20 min with core RNA polymerase (75 µM; ε4; 230 µM; ATP, GTP, and CTP (0.4 µM); and ANFA at the concentrations indicated. Transcription synthesis was initiated by adding heparin (100 µg/ml) and either [α32P]UTP or [α35S]UTP and unlabeled UTP (final UTP concentration, 12 µM) in a reaction volume of 50 µl. After 10 min, the reaction was terminated by adding an equal volume of stop mix (5 mM ammonium acetate, 100 mM EDTA). RNA transcripts were phenol extracted, ethanol precipitated, and then run on 6% polyacrylamide sequencing gels. The 5′- or 3′-end radiolabeled bands were identified by autoradiography of the dried gel. Transcripts were quantitated either by densitometric scanning the bands corresponding to full-length transcripts or by excision of radioactive bands containing full-length transcripts and counting in Cocktail T (BDH) in a LKB Wallac scintillation counter.

DNA Templates—For the transcription assays, the plasmid containing the full-length anfH promoter (pSAS9) was constructed by cloning a 431-bp HindIII fragment carrying the downstream promoter element and the region upstream of this to -405 (see Fig. 1c) into the HindIII site of the transcription vector pTE103 (Elliot and Geiduschek, 1984). pSAS9 was derived from this by deleting the upstream region from the NruI site at -53 to the BamHI site in the polylinker in pTE103. Where indicated, supercoiled template DNA was linearized by digestion with ScaI. Other transcription templates (pRD968, pRD981, and pTEST4) were as described previously (Austin et al., 1987; Whitehall et al., 1992).

DNA templates for the footprinting reactions were constructed by cloning the 450-bp BamHI-HindIII fragment from pSAS9 into pTZ18 to give pSAS10 and pTZ19 to give pSAS11. Single-stranded template DNA from these constructs was extended with [32P]-5′-end labeled primers to produce duplex DNA templates for dimethyl sulfate and DNAse I footprinting. Oligonucleotide primers were 5′-CACAATGATGCGCCGTT-3′ from -404 to -389 for pSAS10, and 5′-CCAGGTAAACCGAGGACC-3′ from -116 to -132 for pSAS11.

Footprinting—Footprinting reactions were carried out in acetate buffer as used for the transcription assays in a final volume of 50 µl. Template DNA and ANFA were incubated together at the concentration in the figure legends for 20 min at 30 °C before adding the footprinting reagent. Dimethyl sulfate and DNAse I footprints were carried out as described by Buck and Cannon (1992).

ATPase Assays—Measurement of ATP hydrolysis was carried out as described by Austin and Dixon (1992).

RESULTS

Purification of N-terminally Truncated ANFA—Attempts to purify the full-length form of the ANFA protein from A. vin-elandii were unsuccessful due to the tendency of the overpro-duced protein to precipitate when exposed to conditions neces-sary for chromatography. As the in vitro data indicated that the removal of the N-terminal domain of the protein enhanced its ability to activate transcription and eliminated its dependence on the presence of the iron protein,7 we considered that an N-terminally deleted form of the protein might be more amen-i-able to purification and characterization in vitro. We con-structed a plasmid in which the N-terminal domain of approxi-mately 20 kDa was deleted by fusion of the first 17 codons of

ANFA. Protein concentrations were determined using Coomassie Blue G-250 reagent (Pierce Chemical Co.) and also estimated from densitometric scanning of stained bands on SDS gels. The most highly purified protein preparations were judged to be at least 99% pure with no other contaminating bands by visual inspection of overloaded Coomassie Blue-stained SDS gels. Native gel electrophoresis and Western blotting of native gels was carried out on a PhastSystem (Pharmacia). Molecular masses were estimated by comparison with protein markers for nondenaturing gel electrophoresis (Sigma). The molar concentration of ANFA was calculated assuming the protein to be a dimer in solution.

Core RNA polymerase and ε4 from K. pneumoniae were purified as described previously (Whitehall et al., 1992). Escherichia coli integra-
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ANFA-A 1.2-kilobase pair EcoRI-HindIII fragment from pEF3213 carrying the anfA gene with 625 bp of the coding sequence

FIG. 1. a diagrammatic representation of the domain structure of ANFA; b, position of fusion junction between lacZ and anfA. The first seventeen codons of lacZ are fused to the SpH1 site in the Q-linker region in anfA. The coding sequence of anfA starts with the alnine residue at position 209 in the protein. c, DNA sequence of the anfH promoter and the upstream regulatory region. The sequence shown (Joerger et al., 1989b) is that spanning the two HincII sites from +27 to -405 with respect to the predicted transcription start site (+1). The positions of the -54 to -12 downstream promoter element and the potential IHF binding site are underlined.

Methods

Purification of Proteins: Overexpression and Purification of Truncated ANFA—A 1.2-kilobase pair EcoRI-HindIII fragment from pEF321 carrying the anfA gene with 625 bp of the coding sequence was fused into HindIII site of the SpH1 vector in the Q-linker region in anfA. The coding sequence of anfA starts with the alnine residue at position 209 in the protein. a, DNA sequence of the anfH promoter and the upstream regulatory region. The sequence shown (Joerger et al., 1989b) is that spanning the two HincII sites from +27 to -405 with respect to the predicted transcription start site (+1). The positions of the -54 to -12 downstream promoter element and the potential IHF binding site are underlined.

were cloned into a 1.2-kilobase pair EcoRI-HindIII fragment from pEF321 carrying the anfA gene with 625 bp of the coding sequence that was subsequently cloned into the SpH1 vector in the Q-linker region in anfA. The coding sequence of anfA starts with the alanine residue at position 209 in the protein. a, DNA sequence of the anfH promoter and the upstream regulatory region. The sequence shown (Joerger et al., 1989b) is that spanning the two HincII sites from +27 to -405 with respect to the predicted transcription start site (+1). The positions of the -54 to -12 downstream promoter element and the potential IHF binding site are underlined.

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In Vitro Activity of Bacterial Transcription Factor

Transcriptional Activation at anfH Promoter in Vitro—The purified truncated ANFA (ΔANFA), was able to activate the A. vinelandii anfH promoter in a single round transcription assay. The anfH promoter region is shown in Fig. 1c. The downstream promoter element contains the −12 to −24 recognition sequence for σ45 holoenzyme, and there is a potential IHF binding site (5'-TTTCAATTGATTG-3') between −89 and −100 with respect to the transcription start site. The IHF DNA binding consensus is 5'-(A/T)ATCAAN4TTNG-3', so this potential site contains only one mismatch. We used a supercoiled DNA template (pSA8) in which the anfH promoter and 400 bp of the region upstream of the promoter containing the putative ANFA UAS and IHF sites have been cloned upstream of the strong T7 transcriptional terminator in the transcription vector pTE103.

Transcripts initiating at the predicted anfH transcription start site and terminating at the T7 terminator would be 330 nucleotides in length. We compared the ability of ΔANFA to activate transcription in either a chloride-based or acetate-based buffer system. The use of acetate instead of chloride enhances DNA-protein interactions and facilitates open complex formation. In the presence of core RNA polymerase and σ45, a full-length transcript of the expected length was observed in both buffers (Fig. 3c). Transcription was detectable in acetic buffer by at least 50% at limiting protein concentrations, compared with that in chloride buffer, and occurred at a lower protein concentration. Transcription was detectable in acetic buffer between 10–25 nM ΔANFA compared with 50 nM in chloride buffer. Therefore, acetic buffer was used in further experiments with the anfH promoter.

Transcriptional activation by ΔANFA at the anfH promoter was not sensitive to the incubation temperature of the assay. Performing the assays at 37 or 30 °C did not influence the level of transcript synthesized (data not shown). Formation of productive complexes at the anfH promoter required a nucleoside triphosphate. Productive complexes were formed when ATP, GTP, and CTP were present together in the assay before the heparin challenge. When these were added after the addition of heparin, no transcripts were made (Fig. 3d). Addition of either ATP or GTP individually supported formation of productive complexes, but CTP did not.

In order to determine whether sequences upstream of the anfH promoter were required for transcriptional activation, we constructed a template in which the sequence from −53 to −405 was deleted. The ability of ΔANFA to activate transcription from the full-length and deleted promoter was compared in acetic buffer and is shown in Fig. 3, a and b. Activation from the deleted promoter required a higher protein concentration, 50 nM compared with 10–25 nM for the full-length promoter (Fig. 3a). Transcription from the full-length promoter was maximal at 200 nM protein, and no further increase in transcription was observed at higher protein concentrations. Transcription from the deleted promoter increased more slowly with increasing
**In Vitro Activity of Bacterial Transcription Factor**

**Fig. 3.** *In vitro* transcription from the *anfH* promoter. a, single round transcription assays were carried out in acetate buffer as described under "Methods." Template DNA (10 nM) was preincubated at 37 °C for 20 min with core RNA polymerase (75 nM α4 (230 nM), ATP, GTP, and CTP (0.4 mM) and ΔANFA at the concentrations indicated to allow open complexes to form. A single round of transcription was initiated by adding heparin (100 μg/ml), [35S]UTP, and unlabeled UTP (12 μl) in a reaction volume of 50 μl. RNA transcripts were processed and visualized by autoradiography as described under "Methods." Transcript levels are arbitrary units of transcription obtained by densitometrically scanning the bands corresponding to the full-length transcript. ○, template DNA (pSA8) was the full-length *anfH* promoter; ■, template DNA (pSA9) was the deleted promoter lacking the upstream sequences -53 to -405 with respect to the transcription start site. ▲, reaction conditions were the same as in Fig. 2a except that transcription was initiated using [32P]UTP (12 μl) and quantitated by determination of the radioactivity in excised bands corresponding to the full-length transcripts as described under "Methods." ○, template DNA was pSA8; ■, template DNA was pSA9. c, comparison of transcription from the full-length *anfH* promoter in either acetate (○) or chloride (●) buffer (see "Methods"). Reaction conditions and transcript processing and quantitation were as for Fig. 2a. d, productive complexes at the full-length *anfH* promoter were allowed to form in acetate buffer in the presence of 0.4 mM ATP, GTP, and CTP (lane 2), in the absence of nucleoside triphosphates (lane 3), or in the presence of 4 mM ATP (lane 4), GTP (lane 5), or CTP (lane 6). ΔANFA was present at 100 nM except in lane 1. Incubation conditions, transcript processing, and autoradiography were as described in Fig. 2a. e, *in vitro* transcription was carried out using the full-length *anfH* promoter (pSA8) in acetate buffer. Template DNA (10 nM) was linearized as described under "Methods." IHF was used at 50 nM. Incubation conditions transcript processing and densitometric analysis were as described in Fig. 2a. ○, -IHF; ■, +IHF. f, reaction conditions were the same as in Fig. 3e except that transcription was initiated using [32P]UTP (12 μl) and quantitated by determination of the radioactivity in excised bands corresponding to full-length transcripts as described under "Methods." Lane 1, no ΔANFA; lanes 2, 4, 6, and 8, 100 nM ΔANFA; lanes 3, 5, 7, and 9, 500 nM ΔANFA. Counts/min in each transcript after deducting the background value obtained from lane 1 were as follows. Lane 2, 2215; lane 3, 6985; lane 4, 1560; lane 5, 4114; lane 6, 8018; lane 7, 10779; lane 8, 1126; lane 9, 2704.
protein concentration. By 500 nM protein, the level of transcription was the same from both DNA templates (Fig. 3b). Thus the region upstream of the anfH promoter contains sequences stimulatory for transcription, which were shown to include the ANFA UAS and potential IHF binding site. We examined the effect of purified IHF protein on transcriptional activation from the anfH promoter. Using 50 nM IHF, there was only a very small stimulation of transcription with the supercoiled template between 10 and 25 nM protein (data not shown). However, the same concentration of IHF strongly stimulated transcription from a linear template containing the full-length promoter. The stimulation was greatest at low protein concentrations (Fig. 3c). On linear DNA in the presence of 50 nM IHF, transcription was observed at 50 nM ΔANFA, and a 3-4-fold stimulation was observed at 100 nM protein. In the absence of IHF, transcription from the linear template was only detected above 100 nM ΔANFA. Transcription was not stimulated when the deleted promoter, which lacks the potential IHF binding site as well as the ANFA UAS, was used as the DNA template. An example of the primary data is shown in Fig. 3f.

**DNA Binding by ΔANFA—DNase I and dimethyl sulfate footprinting of the anfH promoter was carried out using labeled DNA templates containing 400 bp of the region upstream of the predicted transcription start site and including the downstream promoter element.** Fig. 4a shows the dimethyl sulfate protection data on both the top and bottom strand with increasing concentrations of ΔANFA. On the top strand (Fig. 4a, lanes 1–5), the major site of protection is the GG doublet at -216 and -217 with the two G residues at -225 and -226 being strongly hypermethylated. The G at position -268 is also protected, while that at -270 is hypermethylated. Further weaker sites of protection and hypermethylation are observed upstream of -300. On the top DNA strand, the G residues at -302 and -313...
are weakly protected, while on the bottom strand the two Gs at -305 and -306 are weakly hypermethylated. In both types of footprint, protection is observed at 250 nM protein, and the sites are fully protected above 750 nM ANFA. The methylation protection data are summarized in Fig. 4b and indicate that there are at least two and probably three binding sites for ANFA upstream of -200, which are located approximately 40 base pairs apart. Examination of the sequences protected from dimethyl sulfate by ANFA do not show any obvious dyad symmetry characteristic of DNA-binding proteins which bind as dimers. However, alignment of the protected regions shows some conservation of the sequences involved, and this is shown in Fig. 4c. There is a totally conserved 5'-GGTA motif in each potential site within the following consensus: 5'-CnGG-(cg)(cg)nGGTA. There does not appear to be a corresponding symmetrical sequence in any of the three putative sites, although there is an inverted repeat of the GGTA motif in the most downstream site at position -225. Therefore, these may represent half-sites for ANFA binding.

Transcriptional Activation at glnP2 and nifLp—The effectiveness of ANFA as a transcriptional activator was also examined at other σ54-dependent promoters. We compared the ability of the protein to activate the K. pneumoniae glnP2 and nifL promoters and a mutant nifL promoter, (nifL74), with a G to T transition at -26, increasing the homology to glnP2 and to the consensus Eσ54 binding site. This mutation increases the formation of closed promoter complexes with Eσ54 in the absence of an activator protein (Whitehall et al., 1992). In acetate buffer, all three promoters were activated by ANFA and gave the expected 420 (nifL) or 440 (glnP2) nucleotide transcript when assayed in a single round transcription assay as described for the anfl promoter (Fig. 6). Transcripts were observed at 100 nM protein, were σ54-dependent, and required the presence of a nucleoside triphosphate for open complex formation. Transcription also occurred at the same protein concentrations on a linear glnP2 promoter template in acetate buffer (Fig. 6, a and b). In chloride buffer transcript levels from the glnP2 promoter were similar to those in acetate buffer, but no transcripts were made from the nifL promoter even at 400 nM protein. At the nifL74 promoter, a low level of transcription was observed above 200 nM protein when assayed in chloride buffer (Fig. 6, c and d). The glnP2 promoter forms a stable closed complex with Eσ54 in the absence of the activator, while the nifL promoter interacts only weakly with Eσ54 alone and requires presence of the activator to stabilize closed complex formation (Minchin et al., 1989). In acetate buffer, which enhances DNA-
protein interactions, ΔANFA can activate efficiently all three promoters. In chloride buffer, the requirements for open complex formation are more stringent, and the inability of the protein to activate the nifL compared with the glnAp2 promoter may reflect the difference in the promoter's ability to form a stable closed complex. Although the nifL74 mutation increases the promoter's homology to glnAp2 and the Ed4 consensus binding sequence, it does not overcome the requirement for acetate buffer to stimulate transcription by ΔANFA at the concentrations tested.

ATP Hydrolysis by ΔANFA—ANFA, in common with all other α4-dependent activator proteins, has a highly-conserved central domain that is predicted to interact with Ed4 during transcriptional activation and contains a putative nucleotide binding site (Ronson et al., 1987). The NTRC activator protein has been shown to have a phosphorylation-dependent ATPase activity that is stimulated by site-specific DNA binding (Weiss et al., 1991; Austin and Dixon, 1992). To characterize the potential ATPase activity of ΔANFA, the amount of inorganic phosphate released from incubations containing [γ32P]ATP and highly-purified ΔANFA was measured under different conditions. We compared ATP hydrolysis in chloride and acetate buffer at 25 °C. In both buffers there were similar levels of ATP hydrolysis by the protein alone (Fig. 7, a and b). Addition of supercoiled plasmid DNA stimulated the ATPase activity of the protein in both buffers, but this presumably occurs by nonspecific DNA binding as the stimulation was observed irrespective of whether or not the DNA contained the anfH promoter upstream region with the ANFA binding sites present (Fig. 7, a and b). The ATPase activity of the protein was linear with increasing protein concentration up to 1 μM protein at 3 mM ATP (Fig. 7c). Fig. 7d shows a time course of ATP hydrolysis that is linear for at least 1 h at 300 nm protein.

discussion

We have purified in soluble active form an N-terminally truncated form of the ANFA protein from A. vinelandii. The protein, although soluble, still demonstrated the tendency, common to a number of α4-dependent activators and in particular NFA proteins, to aggregate and precipitate during purification. However, the truncated protein was significantly more amenable to purification than the full-length protein, which precipitated after chromatography on heparin agarose. The full-length protein may also require the presence of the iron protein or anaerobic conditions to maintain activity during purification. The purified truncated ANFA exhibited some heterogeneity in native molecular weight, being at least a dimer in solution but with some higher molecular weight species present. This may represent an equilibrium between the dimeric and tetrameric forms of the protein. The purified protein activated the anfH promoter in in vitro transcription assays, transcription being stimulated by the presence of acetate ions in the buffer and by sequences upstream of the -12 to -24 promoter element. The DNA binding experiments indicate that there are at least two and possibly three binding sites for ANFA upstream of position -200. The space between these is approximately 40 base pairs, which should allow four helical turns between each to orient them on the same face of the DNA helix. Although there does not appear to be any α4-dimd symmetry at these sites, conserved sequences are identifiable in the regions protected from dimethyl sulfate. As there is only one known anf promoter, anfH, it is not possible to make sequence comparisons to aid identification of a consensus binding site for ANFA. However ANFA is known to activate the promoter of A. vinelandii nifH promoter. This gene product is required for all

1 M. Drummond, personal communication.

FIG. 7. ATP hydrolysis by ΔANFA. a and b show a comparison of ATP hydrolysis in acetate and chloride buffer. Reactions containing 300 nm ΔANFA were incubated in either acetate (a) or chloride (b) buffer at 25 °C in the presence (filled bars) or absence (open bars) of supercoiled plasmid DNA (10 nm). Hydrolysis was initiated by the addition of 3 μM [γ32P]ATP (95 cpdpmol ATP), and incubation was continued for 1 h. 1-μl aliquots were removed for analysis of phosphate release by thin layer chromatography as described by Austin and Dixon (1992). DNA was the full-length anfH promoter construct, pSA9 (gray bars), or the deleted promoter, pS9 (black bars). c, effect of ΔANFA concentration of ATP hydrolysis. Reactions containing the indicated concentrations of ΔANFA were incubated in acetate buffer in the absence of DNA. Reaction conditions and analysis of phosphate release were as for a and b. d, time course of ATP hydrolysis by ΔANFA. 300 nm ΔANFA was incubated in a reaction volume of 10 μl at 25 °C. Hydrolysis was initiated by the addition of 3 μM [γ32P]ATP (95 cpdpmol ATP). 1-μl samples were removed at the times indicated and diluted with 9 μl of 0.5% SDS, 2 mm EDTA. 1-μl aliquots were analyzed for phosphate release as described under "Methods."
three nitrogenase systems, and two binding sites for NIF A promoter have been identified upstream of the -12 to -24 region of this promoter (Joeger and Bishop, 1988). However, examination of the sequence upstream of the promoter does not reveal any homology with the conserved sequences found in the **nifH** promoter. Therefore, this region of the putative ANFA binding site is not conserved in the **nifB** promoter.

The region upstream of the **nifH** promoter also contains an IHH consensus binding site located between the -12 to -24 downstream promoter element and the upstream binding sites for ANFA. Purified IHH protein stimulates transcription from the linear **anfH** promoter by ANFA, but the supercoiled promoter shows no IHH dependence except at very low concentrations of ANFA. This is in contrast to NIF activation of the *K. pneumoniae nifH* promoter where IHH stimulates transcription of the supercoiled promoter (Hoover et al., 1990). The IHH site and potential ANFA binding sites are located considerably further upstream of the -12 to -24 region than their counterparts in the **K. pneumoniae nifH** promoter (Buck et al., 1988; Hoover et al., 1990). One possibility for the lack of IHH dependence of the supercoiled promoter is that there is an increased flexibility due to the length of the upstream region and so a greater likelihood of the bound activator contacting Er²⁴ at the promoter. Linearization of the DNA may reduce this flexibility, and activation then becomes more dependent on IHH for loop formation. Alternatively, the ability of Er²⁴ to bind the promoter may influence the dependence on IHH for activation.

At ANFA concentrations above 200 nm, transcription from the **anfH** promoter is not stimulated by specific DNA binding, and activation occurs equally well from either the full-length or UAS-deleted promoter. At high activator concentrations, interactions between ANFA and Er²⁴ can occur either in solution or by non-specific DNA binding, so there is much less dependence on the presence of the UAS. Transcriptional activation from the **glnAp2** and **nifL** promoters occurs in the absence of a UAS and requires a higher concentration of ANFA to activate transcription than the full-length **anfH** promoter. The **glnAp2** promoter can form a stable closed complex with Er²⁴ in the absence of the activator (and at the **anfH** promoter protection of the -12 to -24 region by Er²⁴ from DNaseI is observed in the absence of ANFA). In contrast, stable closed complexes at **nifL** are only detected in the presence of the activator (Minchin et al., 1989). In acetate buffer, which facilitates open complex formation, all three promoters are activated efficiently by ANFA. However, in chloride buffer, where requirements for open complex formation are more stringent, the **nifL** promoter is not activated by ANFA, but the **anfH** and **glnAp2** promoters are, although activation from the both the full-length and UAS-deleted **anfH** promoter is reduced. These differences may reflect, at least in part, the ability of the promoter to form a closed complex with Er²⁴ and the requirement for the activator to stabilize this complex.

In common with other Er²⁴-dependent activator proteins ANFA requires a nucleoside triphosphate to form productive complexes with promoter DNA. ATP or GTP will support complex formation, this requirement being similar to that for the NTRC protein (Lee et al., 1993). NTRC possesses a phosphorylation and DNA-dependent ATPase activity that is required to catalyze open complex formation (Weiss et al., 1991; Austin and Dixon, 1992). The ANFA protein has a constitutive ATPase activity that, by analogy to NTRC, is presumably required to drive formation of open complexes, and it seems likely that there is a common mechanism by which ATP hydrolysis is employed to catalyze open complex formation by Er²⁴-dependent activator proteins (Kustu et al., 1989). The levels of ATPase activity of ANFA are roughly similar to those achieved by phosphorylated NTRC and higher than those of S160F NTRC, a constitutively active mutant NTRC protein (Popham et al., 1989; Dixon et al., 1991; Austin and Dixon, 1992). The ATPase activity of ANFA differs from that of NTRC in that it occurs equally well in both acetate and chloride buffer systems, whereas that of NTRC is greatly stimulated by acetate buffer. ATP hydrolysis by ANFA also increases linearly with increasing protein concentrations, in contrast to phosphorylated NTRC, which shows a nonlinear increase in both the presence and absence of DNA. We have interpreted this response as a mechanism whereby ATP hydrolysis is stimulated by cooperative interactions between phosphorylated NTRC dimers bound to multiple binding sites on DNA (Austin and Dixon, 1992). The ATPase activity of the ANFA protein is not greatly stimulated by the presence of DNA and may not be so tightly coupled to DNA binding as in the case of NTRC. However, the ATPase activity of the full-length ANFA protein may be modulated by the presence of the N-terminal domain just as phosphorylation of the N-terminal domain regulates the ATPase activity of NTRC. In the case of ANFA, it may be the reduction-oxidation state of the potential metal binding site in the N-terminal domain that influences the ATPase activity of the catalytic domain of the protein. Purification of the full-length protein will facilitate understanding of the role of the N-terminal domain in the transcriptional activity of the protein.

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