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Chimeric Transcriptional Activators Generated In Vivo from VnfA and AnfA of *Azotobacter vinelandii*: N-Terminal Domain of AnfA Is Responsible for Dependence on Nitrogenase Fe protein

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In vivo recombinants generating chimeras between the transcriptional activators VnfA and AnfA of *Azotobacter vinelandii* were constructed by cloning their structural genes in tandem and selecting against a conditionally lethal gene inserted between them. The parent molecules differ in their promoter specificities and in that AnfA, but not VnfA, requires the Fe protein of nitrogenase for its activity. Chimeras with fusion junctions in the N-terminal half of the central domain were found to be inactive, probably as a result of misfolding. All chimeras carrying the C-terminal domain of AnfA showed the corresponding promoter specificity, supporting the model which ascribes promoter specificity to the DNA-binding properties of the C-terminal domain. None of the chimeras showed the dependence on Fe protein typical of AnfA, including one which composed 82% of AnfA with only a short segment of VnfA at the N terminus. Deleting the N-terminal domain of AnfA gave a fully active protein which was also independent of Fe protein. This indicates that the N-terminal domain has an inhibitory effect on activity which is relieved by Fe protein.

Azotobacter vinelandii possesses three genetically distinct nitrogenases (for a review, see reference 4). Each comprises an electron transport component, the Fe protein, and a tetrameric or hexameric protein that contains a complex metal cluster, the site of N_2 reduction. The preferred enzyme, encoded by *nifHDK*, contains a FeMo cofactor and corresponds to the enzyme found in most other nitrogen-fixing bacteria. When molybdenum is absent, a vanadium nitrogenase encoded by *vnfH* and *vnfDGK* can be synthesized, and in the absence of both molybdenum and vanadium, a third enzyme encoded by *anfHDGK* which contains only iron is found. The *nifH*, *vnfH*, and *anfH* genes encode the Fe proteins of the three systems.

The promoters of the nitrogenase structural genes (and ancillary genes such as *nifEN* and *vnfEN*, which are required for cofactor synthesis) are recognized by RNA polymerase (E) containing σ^{54} (or σ^{N}), which needs an additional activator protein for catalysis of open complex formation. Each nitrogenase system in *A. vinelandii* has its own activator: NifA, VnfA, and AnfA for the Mo, V, and Fe nitrogenases, respectively (2, 14). Activators that drive transcription initiation by $E\sigma^{54}$ typically comprise three domains (8). The conserved C-terminal domain binds to enhancer sequences, bringing the activator into the vicinity of the promoter. The strongly conserved central domain has a catalytic function, driving DNA strand separation by $E\sigma^{54}$ in an ATP- and GTP-dependent reaction (for a review, see reference 18). The N-terminal domains, which are thought to have a regulatory role, can be very different, or even missing. Some, such as that of NtrC, compose the phosphoacceptor domain of two-component systems and as such are relatively well characterized (for a review, see reference 19). The precise function of VnfA and AnfA has not been established, but both carry a possible metal-binding site close to their N termini, which could mediate regulation by Mo or V or provide a redox center (14, 21).

VnfA and AnfA are similar for their entire length (61% amino acid identity) but differ both in the promoters they recognize and in that AnfA appears to require the presence of functional Fe protein of either the Mo or V system for its activity (15, 21). We have used a novel strategy for generating genetic chimeras in vivo to construct hybrid activators from VnfA and AnfA. Comparing the activation profiles of these chimeras with those of the parent molecules indicates that promoter specificity is determined by the C-terminal region of the molecule and suggests that the requirement for Fe protein shown by AnfA is a function of its N-terminal domain, which is confirmed by deletion of the entire domain.

MATERIALS AND METHODS

Plasmid construction. The plasmids used in this work are listed in Table 1. Standard techniques were used to construct pEF29, which carries *vnfA* and *anfA* in tandem separated by a conditionally lethal marker. The 1,727-bp *Xho*I fragment carrying *anfA* was excised from pJJ289 (13) and cloned first into pBGS18 (23) cut with *Sal*I, such that the translation start was adjacent to the *Bam*HI site of the polylinker. The *Bam*HI-*Hin*dIII fragment was then cloned into pJJ288 (13), which carries *vnfA* transcribed from the *lacZ* promoter, and a 3.8-kb *Bam*HI fragment carrying the Km^r gene and the *sacB* gene of *Bacillus subtilis* (22) inserted at the *Bam*HI site separating the two genes, yielding pEF29.

The *lacZ'-'anfA* fusion plasmid, pEF321, which encodes a truncated AnfA lacking the N-terminal regulatory domain (Δ^RAnfA), was constructed by inserting a 917-bp *Sph*I fragment cut from pJJ289 into the *lacZ*α-complementing vector pMD238, fusing the first 20 codons of *lacZ* to *anfA* in the correct reading frame. The *Sph*I fragment does not carry the final 19 codons of *anfA*, which were added by substituting the *Afl*III-*Hin*dIII fragment from pJJ289 for the shorter one in the initial construct, to give pEF321.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic or genotype	Reference or source
Strains		
E. coli 71-18	Δ(*lac-pro*) *supE thi* F'[*proAB lacI*^{q}]	12
E. coli YMC12	Δ*lacU169 hutC*$_{K. pneumoniae}$ *ntrC*::Tn*5 thi hsr endA*	1
K. pneumoniae KP5022	*hisD2 hsdR1 nif*$^+$	24
K. pneumoniae CK260	*hisD2 hsdR1 rpsL4 nifH2260*	7
Plasmids		
pTZ18R	*lacZ*α-complementing phagemid	17
pMD238	pACYC184-based *lacZ*α-complementing vector	11
pJJ288	pMD238 carrying *vnfA* expressed from *lacZp*	13
pJJ289	pMD238 carrying *anfA* expressed from *lacZp*	13
pUM24	pUC4 carrying a *sacB*-Km^r cassette	22
pEF29	pMD238 carrying *vnfA* and *anfA* in tandem separated by a *sacB*-Km^r cassette	This work
pEF30-36	pMD238 carrying *vnfA'-'anfA* chimeras (see Fig. 2 for details)	This work
pEF321	pMD328 carrying truncated *anfA*	This work
pPW165	*vnfE'-lacZ* fusion in IncP vector	P. Woodley
pPW173	*vnfH'-lacZ* fusion in IncP vector	P. Woodley
pMD324	*vnfD'-lacZ* fusion in IncP vector	13
pAG322	*anfH'-lacZ* fusion in IncP vector	13
pRD577	*glnAp2-lacZ* fusion	6

Assay of activator function. The β-galactosidase activity of whole cells carrying activator plasmids and transcriptional *lacZ* fusions to the *vnfH*, *vnfD*, *vnfE*, or *anfH* promoters was measured in *Klebsiella pneumoniae* strains grown anaerobically at 29°C under nitrogen starvation to activate nitrogenase expression, as described by Jacob and Drummond (13). Activation of *glnAp2* was measured in *Escherichia coli* YMC12 grown in Luria-Bertani (LB) medium to early stationary phase, also at 29°C, since pRD577 undergoes runaway replication at 37°C (6).

Western blots (immunoblots). *E. coli* YMC12 strains carrying activator plasmids were grown as described for the *glnAp2* activation assays, harvested, and lysed in Laemmli buffer, and the extracts were run on sodium dodecyl sulfate-polyacrylamide gels. The denatured proteins were electrophoretically transferred to an Immobilon-N membrane (Millipore) and exposed to a rabbit polyclonal antiserum raised against AnfA. Cross-reacting material was detected by use of an immunogold silver staining kit, as described by the manufacturers (Amersham).

RESULTS

Generation of chimeras. Recombinants between *vnfA* and *anfA* were isolated by cloning the two genes in tandem separated by a cartridge carrying Km^r and the *sacB* gene of *B. subtilis* (22), which confers sensitivity to sucrose, and plating *E. coli* 71-18 carrying this construct on LB agar containing 5% sucrose. Resistant colonies arose at a frequency of about 10^{-5}, the majority being Km^s and containing plasmids which appeared on the basis of restriction analysis to carry *vnfA'-'anfA* recombinants. However, about 40% were Km^r, and restriction analysis revealed rearrangements of pEF29 which disrupted *sacB*. To avoid studying siblings resulting from the same recombination event, a number of individual Km^r pEF29 transformants, each of which presumably originated from a single intact molecule, were grown in LB lacking kanamycin and plated separately on LB agar containing sucrose. One SacB$^-$ Km^s colony with the plasmid restriction pattern characteristic of the desired recombinants was then chosen from each plate, and the *vnfA-anfA* junction in 11 of these was sequenced, either by using a set of primers homologous to regions strongly conserved in the *anfA* and *vnfA* coding sequences or by cloning restriction fragments carrying fusion junctions into M13mp18 (27). This yielded seven different recombination junctions, six of them in the region encoding the well-conserved central domain (Fig. 1). All were in the correct reading frame in regions of strong homology. Three of the junctions were obtained twice or more in independent events, but this did not appear to reflect maxima in the local base sequence homology.

Properties of the VnfA-AnfA chimeras. The requirement of AnfA for nitrogenase Fe protein can be satisfied in *K. pneumoniae* grown on dinitrogen (13). To determine the promoter specificity of our constructs, a set of β-galactosidase assays was therefore carried out with *lacZ* fusions to the *vnfH*, *vnfE*, *vnfD*, and *anfH* promoters in *K. pneumoniae* KP5022 cultured anaerobically under severe nitrogen limitation (Table 2). As expected, AnfA does not activate the *vnfH*, *vnfD*, or *vnfE* promoters. VnfA, on the other hand, does activate the *anfH* promoter to some extent, consistent with the presence of VnfA-binding sites upstream from the *anfH* promoter (25a). The active chimeras drove the *anfH'-'lacZ* fusion but failed to activate the *vnf* promoters, presumably because they carry the DNA-binding domain of AnfA.

The chimeras with fusion junctions in the N-terminal half of the central domain (pEF30, -31, and -32) proved to be inactive in these conditions. In an attempt to demonstrate activator function in these constructs, we examined *glnAp2-lacZ* expression from pRD577 (6) in *E. coli* YMC12, which carries a mutation in *ntrC*, the physiological activator of *glnAp2*. This promoter is particularly easy to activate and can be driven by a wide variety of activators, even when their binding sites are absent from the upstream region, probably because the run of Ts between positions −15 and −17 facilitates binding of Eσ^{54} (5, 6). However, *glnAp2* was not activated by the inert chimeras. AnfA was also inactive under these assay conditions, while VnfA and Δ^RAnfA activated *glnAp2* strongly (Table 3).

To examine the possible dependence of the chimeric activators on nitrogenase Fe protein, their function was examined in *K. pneumoniae* CK260, which carries a point mutation in *nifH*, the structural gene for the Fe protein. The *nifH* mutation, which eliminates Fe protein activity in crude extracts, reduced activation by AnfA about fourfold (Table 4). None of the chimeras showed a comparable dependence on *nifH*, even pEF30, which carries 82% of the coding region of *anfA*. The fusion junction in pEF30 occurred in the regulatory domain (Fig. 1), and it was not clear whether the short N-terminal VnfA sequence present in the construct actively relieved *nifH* dependence or whether the entire chimeric N-terminal domain was misfolded and hence devoid of function. We therefore constructed a *lacZ'-'anfH* fusion which removed the N-terminal domain as far as the Q linker (26) between the N-terminal domain of *anfA* and the catalytic central domain (see Materials and Methods and Fig. 1). The activity of the truncated protein, Δ^RAnfA, was higher than that of full-length

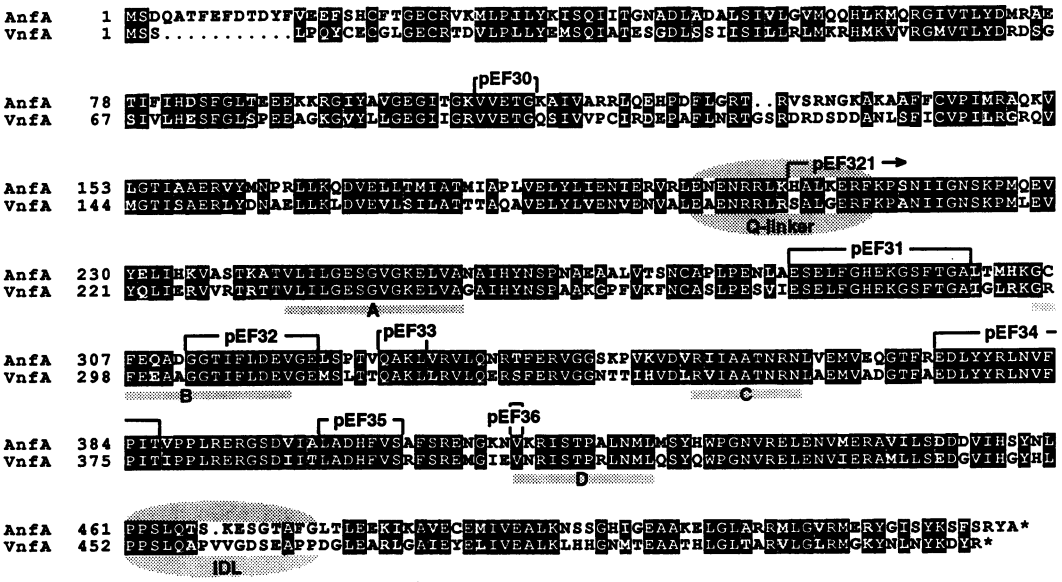

FIG. 1. Alignment of the VnfA and AnfA proteins. Blocks of homology within which recombination occurred carry the names of the corresponding plasmids above, and the site at which AnfA is truncated in pEF321 is indicated by a superscript arrow. Lettered bars beneath the alignment delineate the motifs characteristic of the very disparate nucleic acid-dependent ATPases described by Koonin (16). The approximate position and extent of the Q linker and the interdomain linker separating the catalytic and DNA-binding domains are shaded and marked Q-linker and IDL, respectively. The ATP- and GTP-binding site corresponds to motif A.

AnfA (Tables 2 and 4) and was not affected by the *nifH* mutation in CK260 (Table 4), suggesting that the N-terminal domain has an inhibitory effect on activity which is relieved by Fe protein.

The abundance in the *E. coli* YMC12 strains of material cross-reacting with a polyclonal antiserum raised against AnfA was estimated by Western blotting (Fig. 2). VnfA could not be detected with this serum (Fig. 2, lane 1). Full-length AnfA was readily detectable (Fig. 2, lane 2), and Δ^RAnfA was at least as abundant (lane 3), but none of the chimeric proteins could be detected, even that encoded by pEF30, which carries all the AnfA epitopes present in the truncated form.

DISCUSSION

The construction of chimeric genes using in vivo recombination provides a useful tool for the functional analysis of protein families. Weber and Weissmann (25) used recircularization of linear DNA following transformation to generate such recombinants, but we have found the method we describe here much more efficient. Since recombination necessarily occurs in regions of high homology, local secondary structure of the fusion protein is likely to remain unperturbed, but the fold of a chimeric domain can nevertheless be disrupted by the failure of residues well separated in the sequence to pack precisely in the protein interior. The alternative approach of engineering chimeras in vitro by exchanging intact domains can founder on the difficulty of designing the fusion junction correctly, since the region between domains is often poorly conserved (9).

AnfA could be detected on Western blots with a polyclonal antibody raised against the full-length protein. VnfA was undetectable with the same antibody, possibly because it is not synthesized in sufficient amounts, but more probably because it does not cross-react with the serum. The truncated form of the activator, Δ^RAnfA, which has the *lacZ* translation start, appears to accumulate to slightly higher levels than the full-

TABLE 2. Promoter specificity of VnfA, AnfA, and their truncated and chimeric derivatives

Plasmid	Activator	β-Galactosidase activity of promoter fusions in *K. pneumoniae* KP5022[a]			
		pAG322 *anfH'-lacZ*	pPW173 *vnfH'-lacZ*	pMD324 *vnfD'-lacZ*	pPW165 *vnfE'-lacZ*
pMD238	Vector	45	16	8	4
pJJ288	VnfA	138	149	194	106
pJJ289	AnfA	634	15	23	4
pEF30	VnfA-AnfA	254	13	20	5
pEF31	VnfA-AnfA	46	10	10	4
pEF32	VnfA-AnfA	44	18	12	3
pEF33	VnfA-AnfA	41	17	13	3
pEF34	VnfA-AnfA	390	15	15	4
pEF35	VnfA-AnfA	351	13	5	5
pEF36	VnfA-AnfA	504	16	13	5
pEF321	Δ^RAnfA	839	14	9	4

[a] β-Galactosidase activities of cells grown anaerobically under nitrogen limitation, expressed in Miller units.

TABLE 3. Activation of *glnAp2* by VnfA, AnfA, and their truncated and chimeric derivatives in *E. coli* YMC12

Plasmid	Activator	β-Galactosidase activity of *glnAp2-lacZ* (pRD577)[a]
pMD238	Vector	91
pJJ288	VnfA	636
pJJ289	AnfA	95
pEF30	VnfA-AnfA	318
pEF31	VnfA-AnfA	120
pEF32	VnfA-AnfA	100
pEF33	VnfA-AnfA	95
pEF34	VnfA-AnfA	165
pEF35	VnfA-AnfA	378
pEF36	VnfA-AnfA	1,714
pEF321	Δ^RAnfA	4,057

[a] β-Galactosidase activities of cells grown in LB medium at 29°C, expressed in Miller units. Standard derivations were ±15% on average.

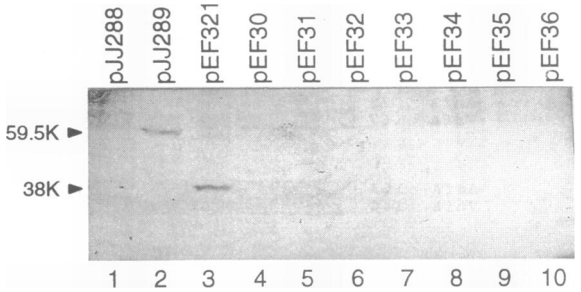

FIG. 2. Western blot of activator proteins in *E. coli* YMC12 using antiserum raised against AnfA. Lane 1, VnfA; lane 2, AnfA; lane 3, truncated form of AnfA; lanes 4 to 10, VnfA-AnfA chimeras. The arrows to the left indicate the expected molecular masses (in kilodaltons) of the full-length and truncated forms of AnfA.

length protein, since although it lacks all the epitopes of the N-terminal domain, it gives at least as strong a signal on Western blots.

The chimera encoded by pEF30, on the other hand, was undetectable and must be present at much lower levels than Δ^RAnfA, since it carries all the same epitopes. This could be either because the *vnfA* translation start is much less effective than that of *lacZ* or because the entire structure is destabilized by the chimeric N-terminal domain. The chimeras encoded by pEF31-36 may be present at comparably low levels, but since they lack some epitopes present in Δ^RAnfA, our data do not show this conclusively.

Three of the chimeras between VnfA and AnfA do not activate transcription under any of the conditions tested, presumably either because they are misfolded and degraded or because functional residues of VnfA and AnfA fail to complement each other. The varying effectiveness of the active chimeras can be explained in similar terms.

All the active chimeras drive transcription from the *anfH* promoter but fail to activate the *vnf* promoters. We attribute this specificity to the presence of the intact DNA-binding domain of AnfA in the chimeric constructs, since unlike *glnAp2* the *anfH* promoter requires upstream sequences and hence presumably activator binding for activation (7a). We have shown previously that attaching the DNA-binding domain of VnfA to AnfA is sufficient to transfer the corresponding promoter specificity and DNA-binding properties (13), and the reciprocal constructs we describe here indicate that the reverse is also true.

TABLE 4. Effects of a *nifH* mutation on the activities of VnfA, AnfA, and their truncated and chimeric derivatives in *K. pneumoniae*

Plasmid	Activator	β-Galactosidase activity of *anfH'-lacZ* (pAG322) in indicated strain[a]	
		KP5022 (*nif*$^+$)	CK260 (*nifH*)
pMD238	Vector	67	68
pJJ288	VnfA	301	356
pJJ289	AnfA	828	197
pEF30	VnfA-AnfA	380	442
pEF31	VnfA-AnfA	73	85
pEF32	VnfA-AnfA	83	73
pEF33	VnfA-AnfA	81	63
pEF34	VnfA-AnfA	437	351
pEF35	VnfA-AnfA	688	497
pEF36	VnfA-AnfA	1,314	1,135
pEF321	Δ^RAnfA	1,786	1,511

[a] β-Galactosidase activities of cells grown anaerobically under nitrogen limitation, expressed in Miller units.

By comparing activator function in *K. pneumoniae* wild-type and *nifH* mutant backgrounds, we have further confirmed that the activity of AnfA, but not VnfA, is dependent on the presence of nitrogenase Fe protein in the cell, as first proposed by Joerger et al. (15). This dependence may not be absolute, because we observed a residual AnfA activity in the *nifH* point mutant CK260 as well as in other *nifH* strains including two Tn*5* insertion mutants (data not shown). Joerger et al. (15) described a more dramatic reduction of AnfA activity in a *nifH vnfH* mutant of *A. vinelandii* with *anfA* in its chromosomal location, and in our system enhanced expression of *anfA* from the *lac* promoter on a multicopy plasmid may mask the effect of the *nifH* mutants to some extent.

Removing the N-terminal domain of AnfA relieved the dependence on Fe protein and gave an activator that appeared more effective than full-length AnfA even in derepressing conditions. This is unlikely to reflect the slightly enhanced intracellular concentration of Δ^RAnfA, since the same raised levels of activity were observed when the copy number of the truncated gene was reduced by a factor of about 10 (data not shown). However, it might mean that the *K. pneumoniae* Fe protein interacts less effectively with AnfA than do the Fe proteins of the *A. vinelandii* nitrogenases. In any case, the N-terminal domain of AnfA clearly has a negative regulatory function. This is reminiscent of *Rhizobium meliloti* NifA, removal of whose N-terminal domain enhances activity (3). Removing the N-terminal domain of *K. pneumoniae* NifA, on the other hand, reduces activity three- or fourfold, and in the case of NtrC the N-terminal domain in its phosphorylated form is absolutely required for activation (9). These differences may be understood in terms of a model in which the regulatory domain provides a switchable conformational constraint on the catalytic domain, the activity of the truncated form being that of a relaxed conformation.

In the case of AnfA, the properties of the truncated protein indicate that this switch is thrown, directly or indirectly, by the Fe protein. The possibility raised by Joerger et al. (15) that Fe protein is required for *anfH* transcript stability can probably be discounted. One possible reason for the dependence of AnfA activity on Fe protein is that it provides a means of inducing synthesis of the third nitrogenase in response to vanadium deprivation (20, 21). The V nitrogenase Fe protein supports AnfA function in *A. vinelandii* and continues to be synthesized in the absence of vanadium (15), while the VFe apoprotein is

degraded in the absence of its cofactor. In these conditions, it is suggested, AnfA provides an alternative electron acceptor, and its reduced form drives transcription of the third system (20, 21). If this model is correct, the N-terminal domain of AnfA may have a redox function, and Premakumar et al. (21) have found that mutating Cys21 and Cys26 of AnfA eliminates its transcriptional activation, which they interpret in such terms. Our data indicate that these mutations lock the N-terminal domain into its inhibitory conformation rather than nullifying its function.

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