Journal of Bacteriology, July 1993, p. 4267–4273
0021-9193/93/144267-07$02.00/0
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Vol. 175, No. 14

MINIREVIEW

Prokaryotic Enhancer-Binding Proteins Reflect Eukaryote-Like Modularity: the Puzzle of Nitrogen Regulatory Protein C

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INTRODUCTION

Eukaryotic enhancer-binding proteins are often modular in design in that they are composed of physically separable domains that can function independently of one another (18, 46, 55). Detailed analyses have identified regions involved in specific DNA recognition and others involved in activation of transcription. In fact, in many instances, combining a domain from one protein with a second domain from another has produced a chimeric protein that demonstrates the expected functional properties of each parent (3, 19, 21, 22). As outlined below, members of a family of prokaryotic enhancer-binding proteins are also modular in structure. However, one member of the family, the NTRC protein (nitrogen regulatory protein C; also called NRI) of enteric bacteria, is apparently an exception. Comparison of the sequence of NTRC with that of other activators and with the sequence and structure of the factor for inversion stimulation (FIS) reveals a likely explanation for the puzzling properties of NTRC.

ACTIVATORS OF σ^{54}-HOLOENZYME

We are studying prokaryotic enhancer-binding proteins that activate transcription by σ^{54}-holoenzyme, an alternative holoenzyme form of RNA polymerase (Fig. 1). σ^{54}-holoenzyme and its activators have been identified in two major divisions of the bacteria (previously called Eubacteria), the purple bacteria and their relatives (also called *Proteobacteria*) (38, 39, 63, 67, 74) and the gram-positive bacteria (11). Activators of σ^{54}-holoenzyme bind to enhancer sites or to upstream or downstream activation sequences and contact the polymerase at a promoter by DNA looping (5, 6, 12, 44, 57, 58, 64, 69). They activate transcription by catalyzing isomerization of closed complexes between σ^{54}-holoenzyme and a promoter, in which the DNA remains double-stranded, to transcriptionally productive open complexes, in which the DNA is locally denatured around the transcriptional start site (49, 54, 60). To catalyze open complex formation, these activators must hydrolyze ATP or another nucleoside triphosphate (41, 54, 70). At some promoters, activators may also stimulate the initial binding of polymerase in closed complexes (1).

Among the 11 activators of σ^{54}-holoenzyme for which sequence information is available, three have been particularly well studied: the NTRC protein, the NIFA protein (nitrogen-fixation protein A), and the DCTD protein (dicarboxylate transport protein D), all of which are found in members of the purple bacteria. Of these, only the NTRC protein has been purified and studied extensively in vitro. Like eukaryotic enhancer-binding proteins, activators of σ^{54}-holoenzyme have several domains (7, 13, 53, 71). Most of those from the purple bacteria have three domains (regions). The amino-terminal domain is regulatory (32, 52, 53, 73) and is a true domain in that it is joined to the remainder of the protein by a protease-sensitive flexible linker (13, 32, 75). The central domain appears to be directly responsible for nucleotide hydrolysis and transcriptional activation (4, 8, 23, 24, 40, 70), and the carboxy (C)-terminal domain contains a helix-turn-helix DNA-binding motif (9, 10, 13, 48, 50). Homology among activators of σ^{54}-holoenzyme is found in the central and C-terminal domains but is highest in the central domain (Fig. 2) (7, 13, 59).

SEPARATION OF DNA BINDING AND TRANSCRIPTIONAL ACTIVATION

As with many eukaryotic enhancer-binding proteins, it has been possible to demonstrate with two activators of σ^{54}-holoenzyme (NIFA and DCTD) that the central domain is an independent functional unit in that transcriptional activation and DNA binding are truly separable. In each case, the C-terminal (DNA-binding) domain of the protein was re-

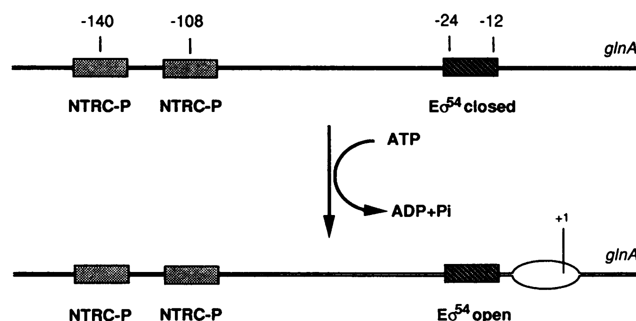

FIG. 1. Formation of open complexes at the *glnA* promoter of *Salmonella typhimurium*. Like other activators of σ^{54}-holoenzyme ($E\sigma^{54}$), NTRC catalyzes the isomerization of closed complexes between this polymerase and a promoter to open complexes in a reaction that requires hydrolysis of ATP (see text for details). Although NTRC must be phosphorylated (NTRC-P) to hydrolyze ATP (52, 53), this is not true of all activators (38). The *glnA* promoter and enhancer-like binding sites for NTRC are indicated with boxes. Numbers above the NTRC-binding sites indicate positions of their centers with respect to the transcriptional start site at +1. Promoter recognition sequences are located at approximately −12 and −24. The *glnA* gene encodes glutamine synthetase.

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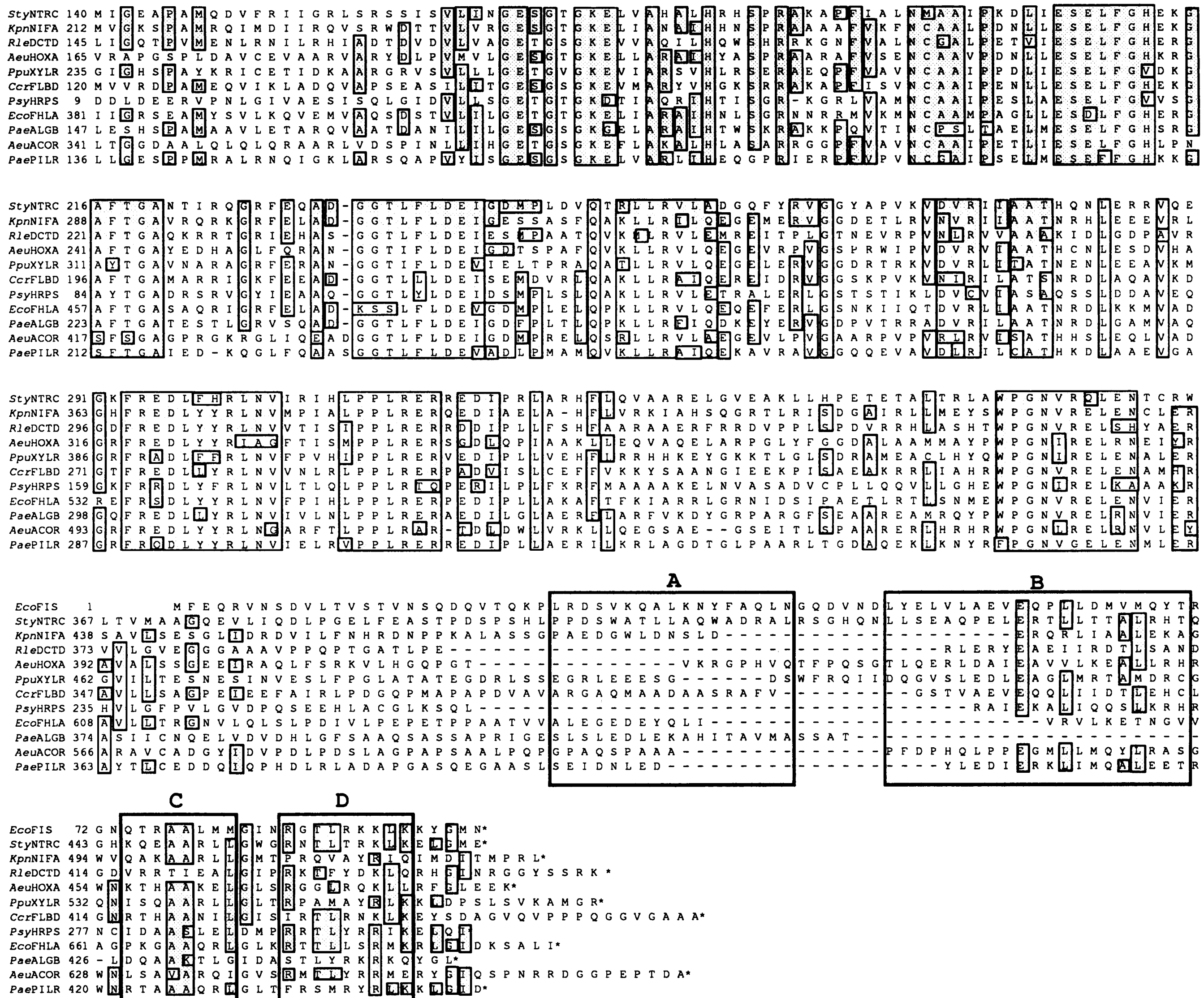

FIG. 2. Alignment of the central and C-terminal domains of 11 activators of σ^{54}-holoenzyme and alignment of their C-terminal domains with the entire sequence of FIS from *E. coli*. The 11 activators shown are all of those for which a complete sequence has been published. Alignments are taken from the references cited below. Numbers at the beginning of each row indicate the number of residues from the first amino acid in the protein; the C-terminal residue is followed by a star. Boxes labeled A, B, C, and D correspond to the α-helices in FIS as determined by X-ray crystallography (Fig. 3) (34, 35, 77). Shaded amino acids are those that are identical in at least five activators, and shaded amino acids in FIS are identical to those in at least five activators. Dashes indicate gaps, which are those introduced in the references cited below to optimize the alignment between activators, particularly in their C-terminal domains. We made manual attempts to increase the number of amino acid identities between individual activators and FIS and found that this could be accomplished only by increasing the number of gaps and not by changing the location of the gap. The last four letters of the designations at the left indicate the acronym for the activator, and the first three letters indicate the organism: *Sty*NTRC, *S. typhimurium* NTRC; *Kpn*NIFA, *K. pneumoniae* NIFA (7); *Rle*DCTD, *Rhizobium leguminosarum* DCTD (59); *Aeu*HOXA, *Alcaligenes eutrophus* HOXA (15); *Ppu*XYLR, *Pseudomonas putida* XYLR (26); *Ccr*FLBD, *Caulobacter crescentus* FLBD (56); *Psy*HRPS, *Pseudomonas syringae* HRPS (20); *Eco*FHLA, *E. coli* FHLA (43, 61); *Pae*ALGB, *Pseudomonas aeruginosa* ALGB (76); *Aeu*ACOR, *A. eutrophus* ACOR (36); *Pae*PILR, *P. aeruginosa* PILR (27). Activators are from members of the α (*Rle* and *Ccr*), β (*Aeu*), and γ (*Sty*, *Kpn*, *Ppu*, *Psy*, *Eco*, and *Pae*) subgroups of the purple bacteria (63, 74). In cases where the sequence of a given activator has been published for organisms in addition to the one shown (DCTD from *R. meliloti* [28], NIFA from *Azotobacter vinelandii* [2] and the "alternative NIFA proteins" VNFA and ANFA [29] from *A. vinelandii*), our conclusions with respect to gaps hold for all of them. Where the NIFA proteins from *K. pneumoniae* and *A. vinelandii* have gaps, the NIFA proteins from *Rhizobium meliloti* (7), *R. leguminosarum* biovar *viciae* (17), *R. leguminosarum* biovar *trifolii* (25), and *Bradyrhizobium japonicum* (66) have cysteine-containing interdomain linkers that have been implicated in mediating their sensitivity to molecular oxygen (17). Alignments of NTRC from different organisms with FIS are shown in Fig. 4; all NTRCs have the central domain homology indicated in this figure for NTRC from *S. typhimurium* (13, 31, 47, 53, 68). Mutant NIFA proteins from *K. pneumoniae* and *R. meliloti* that were truncated after residues 458 and 489, respectively, retained activity (14, 23), as did a mutant DCTD protein from *R. leguminosarum* that was truncated after residue 401 (24; see text for details). A chimeric protein that joined residue 386 of DCTD to residue 496 of NIFA from *R. meliloti* (the latter not shown) (52b) was also active (28, 40, 52b).

moved without eliminating transcriptional activation (2a, 4, 23, 24, 40) (see legend to Fig. 2) and, conversely, in the case of NIFA the isolated C-terminal domain retained the ability to bind specifically to DNA (9, 37, 41, 48, 50). (In both of these cases the N-terminal regulatory domain has also been removed, and the isolated central domain retains the ability to activate transcription [2a, 23, 24, 40].) Moreover, a functional chimera has been constructed between the N-terminal and central domains of DCTD and the C-terminal domain of NIFA (28, 40, 52a) (see legend to Fig. 2). This chimera appears to have the DNA-binding specificity of NIFA and retains the ability to activate transcription. Although transcriptional activation by the chimeric protein has been demonstrated only in vivo, activation by the central domains of NIFA (2a) and DCTD (21a) have recently been demonstrated in vitro as well.

Several groups have attempted to demonstrate that transcriptional activation and DNA-binding functions could also be separated for the NTRC proteins from enteric bacteria (10, 14, 62), since these proteins are well characterized in vitro. A particular effort was made to demonstrate transcriptional activation by mutant forms of NTRC lacking the C-terminal DNA-binding domain, since it has been an outstanding question in both prokaryotic and eukaryotic systems whether an enhancer-binding protein can activate transcription from solution. Although the isolated C-terminal domain of NTRC retains the ability to bind to DNA (14, 33, 54a, 62) the remainder of the protein has lost the ability to activate transcription both in vivo and in vitro (10, 14, 53a, 62). Why should NTRC differ from other activators of σ^{54}-holoenzyme? We postulate that activators of σ^{54}-holoenzyme must be able to dimerize in order to activate transcription and that the salient difference between NTRC from enteric bacteria and other activators is a difference in the location of major dimerization determinants.

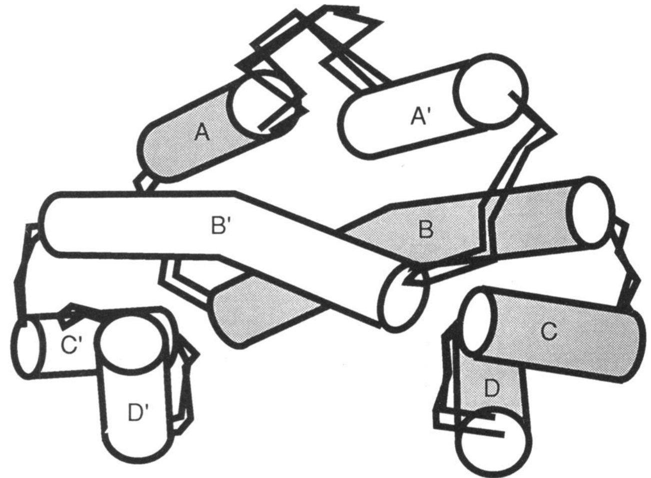

FIG. 3. Proposed three-dimensional structure of the FIS dimer from *E. coli* (34, 35, 77). Alpha-helices in the two monomers are designated A, B, C, and D and A', B', C', and D', respectively. (The first 19 residues of FIS, which were apparently disordered in the crystals, are not indicated.) The C and D (C' and D') helices are the DNA-binding helices. The two monomers associate by interdigitating their A and B helices. Each of the two long B helices that cross in the center of the dimer makes extensive van der Waals contacts and hydrogen bonds with the A' helix of the opposite monomer. In addition, four main chain hydrogen bonds connecting the N terminus of helix B with the C terminus of helix C' stabilize the dimer, with each helix C' being, in effect, an extension of helix B of the opposite subunit. Presumably, it is the loss of or decrease in the latter contacts that accounts for monomerization of forms of NTRC that lack the helices corresponding to the C and D helices or just the D helix, respectively (see text and legend to Fig. 4). Reprinted with permission from reference 77.

HOMOLOGY BETWEEN THE C-TERMINAL DOMAINS OF NTRC AND FIS

At present, little is known about the molecular architecture of activators of σ^{54}-holoenzyme, there being no X-ray or nuclear magnetic resonance-derived structures. However, there are recently-published crystal structures for the FIS protein of *Escherichia coli* (34, 35, 77) (Fig. 3), which show homology uniquely to the C-terminus of NTRC (16, 30) and not to that of other activators of σ^{54}-holoenzyme (Fig. 2 and 4). (FIS was named for its ability to stimulate site-specific inversion when bound to recombinational enhancers, one of its many activities [16].) The C terminus of NTRC from enteric bacteria is 36% identical to FIS across 50 amino acid residues that are deemed essential for dimerization and DNA recognition by FIS (helices B, C, and D) (34, 35, 77) (Fig. 3 and 4). The C terminus of NTRC from several members of the α-purple bacteria is 44% identical to FIS over this region (Fig. 4). The dimeric structure of FIS appears to require the intercalation of helices A and B of the two monomers (Fig. 3). Helices C and D constitute the helix-turn-helix DNA-binding motif. We have noted that all activators of σ^{54}-holoenzyme other than NTRC lack amino acid residues required to form the intercalating helices of FIS, i.e., they lack residues in the A and/or B helices (Fig. 2).

There are several lines of evidence that the major dimerization determinants for NTRC proteins of enteric bacteria are structurally homologous to those for FIS and lie in the C-terminal region of the protein, whereas those for other activators do not. (i) The C-terminal domains of the *E. coli* and *Klebsiella pneumoniae* NTRC proteins (residues 398 to 468 and 380 to 469, respectively) bind to sites with dyad symmetry and retain the ability to repress transcription from appropriate promoters in vivo (14, 62). (ii) The purified C-terminal domain of NTRC from *Salmonella typhimurium* (residues 380 to 469) recognizes a single DNA-binding site (17 bp with dyad symmetry) as a dimer (assessed in a gel mobility shift assay by formation of mixed heterodimers with full-length NTRC) and has approximately normal binding affinity for such a site (32a, 33, 54a). Moreover, the isolated C-terminal fragment is a dimer in solution, as assessed by gel filtration chromatography and sedimentation equilibrium ultracentrifugation. In fact, the major dimerization determinants of NTRC from *S. typhimurium* have been shown to reside in its C-terminal domain, as evidenced by the fact that the rate of homodimer dissociation to monomers is the same for the C-terminal fragment as for full-length NTRC (32a, 33). By contrast, a C-terminal fragment of NIFA from *K. pneumoniae* (residues 459 to 525), which can bind to DNA only at very high concentrations (41), is largely monomeric in solution (37). (iii) A mutant NTRC protein with an amino acid substitution (A410E) in the putative helix corresponding to the A helix of FIS (Fig. 2 and 4) and the isolated C-terminal fragment derived from it are largely monomeric in solution (63a). (iv) A mutant form of the *Salmonella* NTRC protein in which the C-terminus is truncated at the middle of the putative helix corresponding to helix A of FIS (Δ412–469) is a monomer in solution (53a). (In each of the

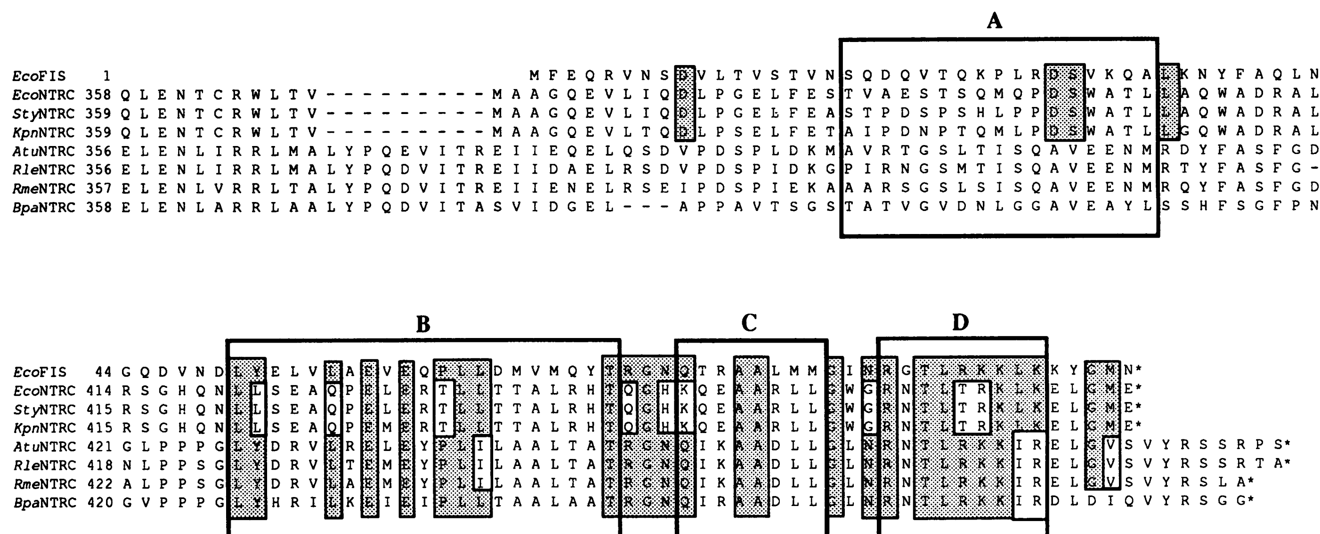

FIG. 4. Alignment of the C-terminal domains of NTRC proteins from different organisms with FIS from *E. coli*. With the exception of NTRC from *Rhodobacter capsulatus* (31), which does not appear to function with σ^{54}-holoenzyme (35a), all of the NTRC proteins for which a complete sequence has been published are included. Numbers and boxes labeled A, B, C, and D are as described for Fig. 2. Shaded amino acids are those that are identical with residues in FIS. NTRC proteins from enteric bacteria, members of the γ subgroup of the purple bacteria, are as follows: *Eco, E. coli* (45); *Sty, S. typhimurium*; and *Kpn, K. pneumoniae* (13). They were aligned with one another by using GENALIGN, a multiple sequence alignment program (IG Suite 5.4 by IntelliGenetics, Inc. [1991]). NTRC proteins from members of the α subgroup of the purple bacteria are as follows: *Atu, Agrobacterium tumefaciens* (68); *Rle, R. leguminosarum* biovar *phaseoli* (47); *Rme, R. meliloti* (65); *Bpa, Bradyrhizobium parasponium* (53). They were also aligned with one another by using GENALIGN. To align NTRC proteins from enteric bacteria with those from α-purple bacteria, it was necessary to introduce a gap in the NTRCs from the enteric bacteria. We have arbitrarily placed this gap after amino acid identities characteristic of the central activation domain (Fig. 2). FIS was aligned with the NTRC proteins manually. The C-terminal residue of FIS is aligned precisely with the C-terminal residue of each NTRC protein from the enteric bacteria, which allows good alignment of the DNA-binding helices C and D; the C-terminal residue of FIS is aligned differently with the C terminus of each NTRC protein from the α-purple bacteria to maximize amino acid identities in the C and D helices. Mutant NTRC proteins from *S. typhimurium* that were truncated after position 453 or 444, respectively, retained residual activity in vitro, whereas the protein truncated after residue 411 did not (see text). Mutant NTRC proteins from *K. pneumoniae* that were truncated after residue 381 or 411, respectively, were inactive in vivo (see text). A mutant NTRC protein from *S. typhimurium* with the substitution A410E retained residual activity in vitro. Although there is no published sequence for the *B. japonicum* NTRC protein, the sequence of the *ntrC* gene was reported to be 86% identical to that from *B. parasponium* (42, 53), and restriction sites were conserved between the two. We infer from the restriction map that the truncated NTRC protein from *B. japonicum* that retained activity in vivo ended with residue 400 (42). However, the mobility of the truncated protein on a sodium dodecyl sulfate-polyacrylamide gel does not appear to be congruent with this, and a second NTRC protein that was truncated at the same position could not be detected. As discussed by Martin et al. (42), the latter observations may be accounted for by differences in the NPTII cassettes that were inserted adjacent to *ntrC* sequences.

latter cases, oligomerization state was assessed by gel filtration.)

There are also several indications that activators of σ^{54}-holoenzyme must be able to dimerize and, at least in some cases, to form higher-order oligomers (54a, 72) to activate transcription. The C-terminal deletion form of NTRC mentioned above fails to activate transcription in vitro and also fails to hydrolyze ATP, although it is normally phosphorylated within its amino-terminal regulatory domain (53a). Other similar C-terminal deletion forms of NTRC from *K. pneumoniae* fail to activate transcription in vivo (10). However, shorter C-terminal deletions of the *Salmonella* protein, i.e., of helices corresponding to the D helix or the C and D helices of FIS (see legend to Fig. 4), which also yield forms of NTRC that are largely monomeric in solution (this can be rationalized based on the crystal structure of FIS [see legend to Fig. 3]), retain some ability to hydrolyze ATP and activate transcription in vitro (53a). The same is true for the A410E substitution form of NTRC (63a). We presume that the high concentrations of these proteins necessary to demonstrate transcriptional activation (>200 nM) are sufficient to drive the formation of some dimer. Finally, although it was possible to produce an active chimera between the C-terminal domain of NIFA and an upstream region of DCTD (see legend to Fig. 2) (20a, 28, 40), it was not possible to do so with upstream regions of NTRC (14). Failure to obtain active chimeras between NIFA and NTRC might be explained by the fact that both portions of the chimera were monomeric.

LOCATION OF DIMERIZATION DETERMINANTS IN NTRC AND OTHER ACTIVATORS: MODULARITY

We predict that most activators of σ^{54}-holoenzyme which lack amino acid residues that constitute the dimerization region of FIS will have major dimerization determinants in their central domains and hence that their isolated central domains, like those of NIFA and DCTD, will activate transcription. These activators will probably be of a modular nature, like eukaryotic enhancer-binding proteins. The NTRC protein of enteric bacteria, the paradigmatic activator in this family, appears not to be completely modular due to a nonconserved location of its major dimerization determinants in the C-terminal domain. However, like other members of the family, the NTRC protein of enteric bacteria can apparently activate transcription without binding to DNA, as evidenced by the fact that forms of NTRC lacking the

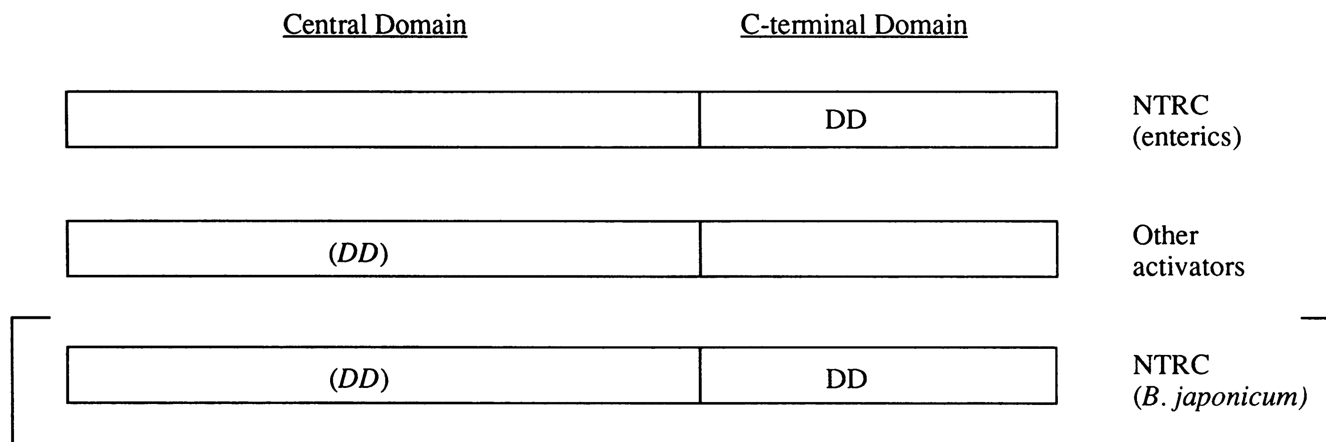

FIG. 5. Location of dimerization determinants in activators of σ^{54}-holoenzyme. DD indicates the presence of dimerization determinants in the C-terminal domain of NTRC that correspond to dimerization determinants in FIS, and (*DD*) indicates the presence of putative dimerization determinants in the central domain of other activators. Although the central domains of NIFA (2a) and DCTD (21a) are active in vitro, both are aggregated, and hence it has not been possible to determine the oligomerization state of the active species. Work from the laboratory of the late Barry Chelm indicates that the central domain of NTRC from *B. japonicum* is active in vivo (42; however, see legend to Fig. 4). If this is the case, we would postulate that the NTRC protein of *B. japonicum* (and possibly other α-purple bacteria) has two sets of dimerization determinants: one in its C-terminal domain that corresponds to dimerization determinants in FIS, and one in its central domain that corresponds to dimerization determinants in other activators.

DNA-binding helices retain a low residual ability to activate transcription in vitro (53a).

EVOLUTIONARY CONSIDERATIONS

E. Morett has constructed evolutionary trees based on sequences of all activators of σ^{54}-holoenzyme and on sequences of their separate domains (47a, 51). He finds that the C-terminal domain of NTRC from members of the α-purple bacteria shows greater amino acid identity to FIS from *E. coli* than it does to the C-terminus of NTRC from enteric bacteria, which are γ-purple bacteria. Inference of the ancestral structure of the C-terminal and central domains of activators of σ^{54}-holoenzyme in the purple bacteria will require additional biochemical studies and determination of the sequences of NTRC, other activators, and FIS from additional members of this group, e.g., members of the δ-subgroup, which is on a different branch of the purple bacterial tree than the other subgroups (39, 74).

We thank Enrique Morett for sharing unpublished data, Steve Finkel and Reid Johnson for helpful discussion on the structure of FIS, and Greg Martin for information on the truncated NTRC protein from *B. japonicum* that was studied in the laboratory of the late Barry Chelm. We thank Norm Pace for valuable criticism of the manuscript.

This work was funded by NIH grant GM38361 to S.K.

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