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Intramolecular signal transduction within the FixJ transcriptional activator: *in vitro* evidence for the inhibitory effect of the phosphorylatable regulatory domain

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ABSTRACT

FixJ is a phosphorylatable 'response regulator' controlling the transcription of the key nitrogen fixation genes *nifA* and *fixK* in *Rhizobium meliloti*. Sequence and genetic analyses indicated that FixJ comprises an N-terminal phosphorylatable regulatory domain, FixJN, and a C-terminal transcriptional activator domain, FixJC. We have now overexpressed and purified the FixJC protein and show that it is fully active in an *in vitro* transcription system with purified RNA polymerase. FixJC appeared to act synergistically with RNA polymerase at the *nifA* promoter. Furthermore FixJC was more active *in vitro* than the full-length dephosphorylated FixJ protein. Therefore activity of FixJC is inhibited by FixJN within the FixJ protein. This inhibition is relieved by phosphorylation of FixJN. Such a negative mode of intramolecular signal transduction may be generalizable to other response regulators.

INTRODUCTION

Two-component regulatory systems are signal transduction devices widely spread among eubacteria (see ref. 1 for a recent review). They have been found both in Gram-negative and in Gram-positive bacteria (and also, recently, in eukaryotes, refs. 2–5), and are involved in such diverse physiological responses as chemotaxis, sporulation, photosynthesis, pathogenesis, antibiotic resistance, symbiosis, regulation of carbon, nitrogen and phosphate assimilation, osmoregulation, etc. Moreover, each organism contains a number of two-component regulatory systems operating concurrently: for instance not fewer than 19 such systems have already been identified in *Escherichia coli*.

This formidable versatility results from the modular structure of two-component regulatory systems (Figure 1). The first component, a 'sensor', interacts with a signal molecule *via* a discrete, signal-specific sensor domain; this sensor domain modulates the kinase and/or phosphatase activities of a conserved histidine kinase domain. The second component, a 'response regulator', contains a conserved domain which is phosphorylated on an aspartate residue by the kinase domain of the sensor component; this phosphorylatable domain regulates in turn the response catalyzed by a discrete, output-specific activator domain.

This modular organization is exemplified by the oxygen-sensitive FixLJ system involved in the regulation of symbiotic nitrogen fixation in *Rhizobium meliloti* (6). The oxygen signal is directly sensed by the haem-containing domain of FixL and transduced to the FixL kinase domain (7–9). Oxygen-regulated phosphorylation (10,11) of the N-terminal regulatory domain of FixJ (FixJN) then modulates the activity of the C-terminal transcriptional activator domain (FixJC). In previous papers we proposed that FixJC is the transcriptional activator domain of FixJ (6,12), and that its activity is modulated negatively by FixJN (12), on the basis of genetical results and sequence analysis. The latter conclusion was based on the phenotype of *Rhizobium* strains overexpressing FixJC, which expressed higher levels of the target *nifA* gene than isogenic strains overexpressing FixJ (12). Here we establish this model using an *in vitro* transcription system with the purified FixJC activator domain. We demonstrate that the FixJN regulator domain negatively regulates FixJC within the FixJ protein. Such a negative mode for intramolecular signal transduction suggests a hypothesis which accounts for the evolutionary adaptability of two-component response regulators.

MATERIALS AND METHODS

Purification of FixJC

The FixJC-expressing plasmid pDK330 (12) was introduced into *E.coli* strain DH5. The resulting strain was grown to stationary phase at 37°C in 15 l LB medium containing 50 μg/ml ampicillin. Cells (60 g) were harvested by tangential filtration and resuspended in 60 ml cold TEB buffer (20 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 1 mM β-mercaptoethanol). The cell suspension was sonicated, debris were centrifuged, and the crude extract in the supernatant was fractionated between 40% and 60% ammonium sulphate saturation. After centrifugation, the protein

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pellet was dialyzed overnight in Spectra/Por 3 tubing (3,500 M_r cut-off, Spectrum) against 2 l TEB containing 100 mM KCl. The resulting extract was applied on a Sephacryl S-100 column (5×45 cm) equilibrated in the same buffer at a flow-rate of 5 ml/min. Fractions containing FixJC were monitored on High Density Phastgels (Pharmacia), pooled, diluted with an equal volume of TEB, and applied on an S-Sepharose column (1.6×15 cm) equilibrated in TEB at a flow-rate of 2 ml/min. FixJC was eluted with a 300 ml linear gradient from 0 to 1 M KCl. Fractions containing FixJC were combined, diluted with an equal volume of TED buffer (20 mM Tris−HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM dithiothreitol) and applied on a Mono S HR 5/5 column (Pharmacia) equilibrated in TED buffer at a flow-rate of 1 ml/min. 10 ml TED buffer containing 0.1 M KCl were applied, followed by a 15 ml linear gradient from 0.1 to 0.25 M KCl in TED. At this stage fractions containing FixJC were contaminated with one protein species of higher molecular weight. This contaminant was removed by gel permeation chromatography on an S-200 Sephacryl column (2.2×82 cm) equilibrated in TED containing 0.1 M KCl at a flow-rate of 1 ml/min. Homogeneous FixJC was concentrated by ultrafiltration through Amicon membranes (3,000 M_r cut-off), dialysed against TED containing 100 mM KCl and 50% glycerol and stored at −20°C. Protein concentrations were estimated by the method of Bradford (13).

In vitro transcription assays

Single round transcription assays were performed as described previously (14) in a 20 µl incubation mixture containing 600 nM (2 units) purified RNA polymerase (Boehringer), various concentrations of purified FixJC or FixJ, and 25 ng of supercoiled template DNA. We used as templates plasmids pJMR500, pJMR400 and pJMR300, which contain the *lacUV5*, *nifA* and *fixK* promoters, respectively (14). When appropriate, the FixJ protein was phosphorylated in the presence of 20 mM acetyl-phosphate (Sigma) as described (14). Run-on transcription assays were performed in identical conditions, except that heparin was omitted and elongation time was reduced from 12 min to 6 min.

RESULTS

Purification of FixJC

Preliminary experiments with *E.coli* strain DH5 containing the FixJC-encoding plasmid pDK330 (12) showed that this strain synthesized amounts of FixJC (predicted M_r = 8.27 kDa) sufficient to be readily detected on a Coomassie-stained SDS−polyacrylamide gel of crude extracts. We purified the FixJC protein from this strain, taking advantage of both the small size of this molecule and its basicity (predicted pI = 10.5). Thus cation exchange chromatography (on S-Sepharose and Mono S) proved to be particularly effective for purification of FixJC. The resulting protein, which was more than 95% pure (Figure 2), behaved like a monomeric protein in gel permeation chromatography.

FixJC activates transcription of *nifA* and *fixK in vitro*

The purified FixJC protein was tested for activation of various promoters in a single-round transcription assay developed by Reyrat *et al.* (14). In this system characteristic transcripts of 340 nt, 495 nt and 370 nt, are synthesized from the *lacUV5*, *nifA* and *fixK* promoters on templates pJMR500, pJMR400 and pJMR300, respectively (Figure 3). The FixJC protein exerted

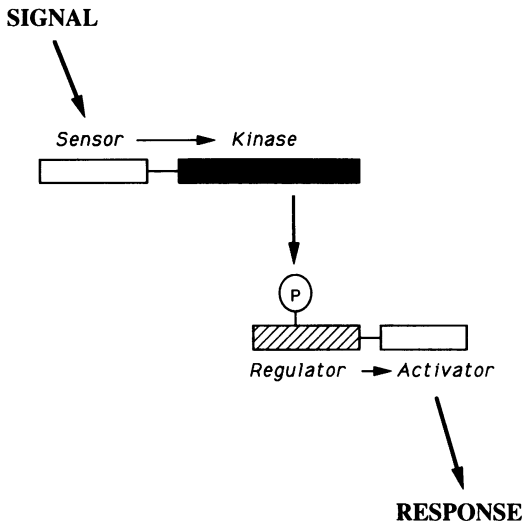

Figure 1. Modular structure of two-component regulatory systems. The signal is sensed by a sensor module and transduced to the kinase module. The phosphorylation state of the regulator module determines in turn the intensity of the response catalysed by the activator module.

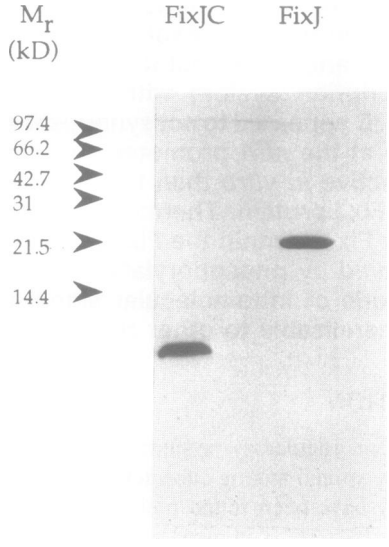

Figure 2. Purified FixJC. Coomassie-stained 16.5% SDS−polyacrylamide gel (42) of purified FixJC and FixJ.

no detectable effect on the transcription of the *lacUV5* promoter which was used as a control. On the other hand the *nifA* and *fixK* promoters were completely inactive in transcription assays containing RNA polymerase holoenzyme but lacking a transcriptional activator, as determined previously (14−16). These promoters were transcribed only in the presence of an activator protein, which could be either FixJ (14,16) or purified FixJC (Figure 3). The fact that FixJC activated the *nifA* and *fixK* promoters is fully consistent with previous genetical results (12). In addition we note that the isolated FixJC domain activates the *nifA* promoter at the very same position as the full-length FixJ protein, since the same discrete molecular species were obtained

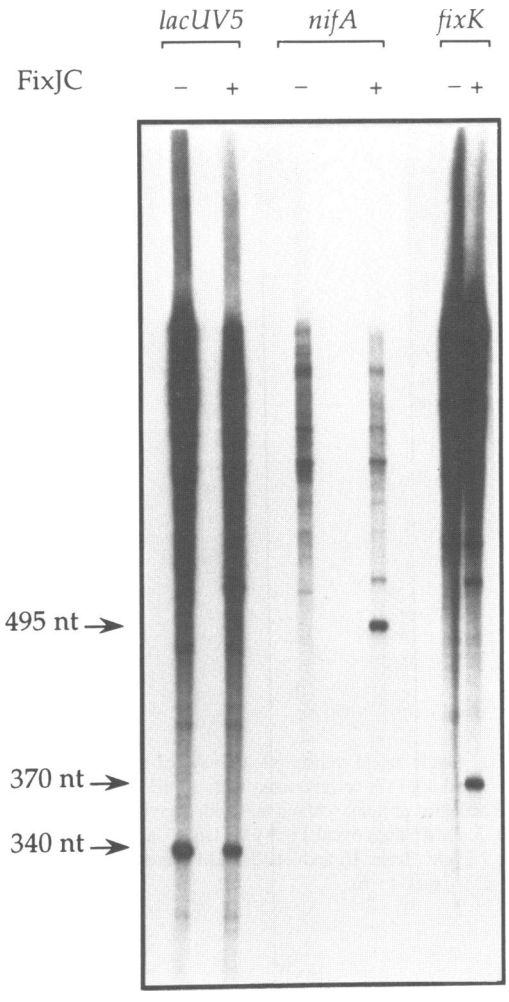

Figure 3. FixJC activates transcription of *nifA* and *fixK in vitro*. Single round transcription assays were performed with templates pJMR500 (*lacUV5*), pJMR400 (*nifA*) and pJMR300 (*fixK*), without or with FixJC (80 μM). The autoradiogram was exposed for different times for the three promoters.

with either protein (Figure 4a). Thus FixJC-mediated transcription initiation of *nifA* is as precise as FixJ-mediated initiation. This result conclusively demonstrates that FixJC carries the entire transcription activation function of FixJ, including any determinant of the specificity for the *nifA* promoter.

FixJN inhibits FixJC activity within the FixJ protein

To investigate the mode of regulation of FixJC by FixJN, we compared the activity of FixJC with that of FixJ. Titration of the *nifA* promoter with increasing amounts of FixJC revealed a sharp response of promoter activity between 1.8 μM and 3.5 μM FixJC (Figure 4a). Maximal activity of the *nifA* promoter was obtained at 3.5 μM of FixJC monomer, versus 95 μM of FixJ monomer (Figure 4b). Thus FixJC is more active than FixJ *in vitro* in single-round transcription assays. It is however conceivable that FixJ might act more effectively than FixJC at another stage, for instance at re-initiation of transcription. To test this possibility, we carried out similar titration experiments measuring run-on transcription of the *nifA* promoter (Figure 5). These titration experiments confirmed that FixJC is approximately 40-fold more active than native FixJ, since maximal promoter activity was obtained at 2.3 μM FixJC versus 100 μM FixJ.

We then proceeded to compare the transcriptional activity of FixJC with that of phosphorylated FixJ. Indeed it has been shown previously (14,16) that phosphorylation stimulates the transcriptional activity of FixJ. Here phospho-FixJ was obtained chemically using acetyl-phosphate as a phosphodonor (14,16). In agreement with previous results (14), phosphorylation of FixJ stimulated its activity considerably since maximal activity was obtained at 5.5 μM instead of 95 μM FixJ monomer (Figure 4). Mock-treatment of FixJC with acetyl-phosphate, on the other hand, had little or no effect. Thus isolated FixJC is more active than the native full-length protein and approximately as active as phospho-FixJ. These results show that the FixJN regulatory domain acts negatively on the FixJC activator domain within the FixJ protein, which inhibition is relieved by phosphorylation.

FixJC and RNA polymerase act synergistically at the *nifA* promoter

The FixJ/FixJC titration experiments above were conducted at a relatively high RNA polymerase molarity (600 nM, ref. 14). This high molarity was required to allow the detection of a *nifA* transcript activated by native non-phosphorylated FixJ. It is however possible that FixJC or phosphorylated FixJ may require lower amounts of RNA polymerase. We therefore titrated the *nifA* promoter with increasing amounts of RNA polymerase, at a molarity of activator protein (20 μM) sufficient for native FixJ-dependent transcription (Figure 6). The results showed that 100 nM RNA polymerase sufficed for FixJC-dependent activation, but did not allow activation of the *nifA* promoter at the same molarity of native FixJ: 600 nM RNA polymerase was required. When, however, FixJ was phosphorylated, transcription proceeded effectively at 150 nM RNA polymerase. Thus phosphorylation of FixJ, or the use of FixJC, makes the *nifA* promoter active at lower RNA polymerase concentrations than required with native FixJ. This indicates that FixJC and RNA polymerase act synergistically at the *nifA* promoter.

DISCUSSION

Modular structure of the FixJ transcriptional activator

The *in vitro* evidence presented here conclusively establishes the modular structure of the FixJ protein, as was initially proposed on the basis of homology analysis (6, 17), and later confirmed by genetic analysis (12). The C-terminal domain of FixJ (FixJC) is the transcriptional activator domain: it is sufficient for the specific activation of the *nifA* and *fixK* promoters. Activation of the *nifA* promoter by FixJC involves a synergistic effect with RNA polymerase. Indeed inhibition of transcription activation by FixJN could be overcome in the presence of high amounts of RNA polymerase (Figure 6). Conversely, FixJC or phosphorylated FixJ allowed *nifA* transcription to proceed at lower RNA polymerase concentrations. Thus FixJC potentiates the action of RNA polymerase at the *nifA* promoter.

The N-terminal domain of FixJ (FixJN) is dispensable for promoter activation and thus plays a regulatory, not a mechanistic function. It is of interest to understand how FixJC activity is regulated by FixJN within the FixJ protein. Our *in vitro* system allowed us to establish that FixJN acts negatively on FixJC activity, which provides a mechanistic basis for signal transduction within the FixJ molecule. This is consistent with previous genetical results, because *Rhizobium* strains

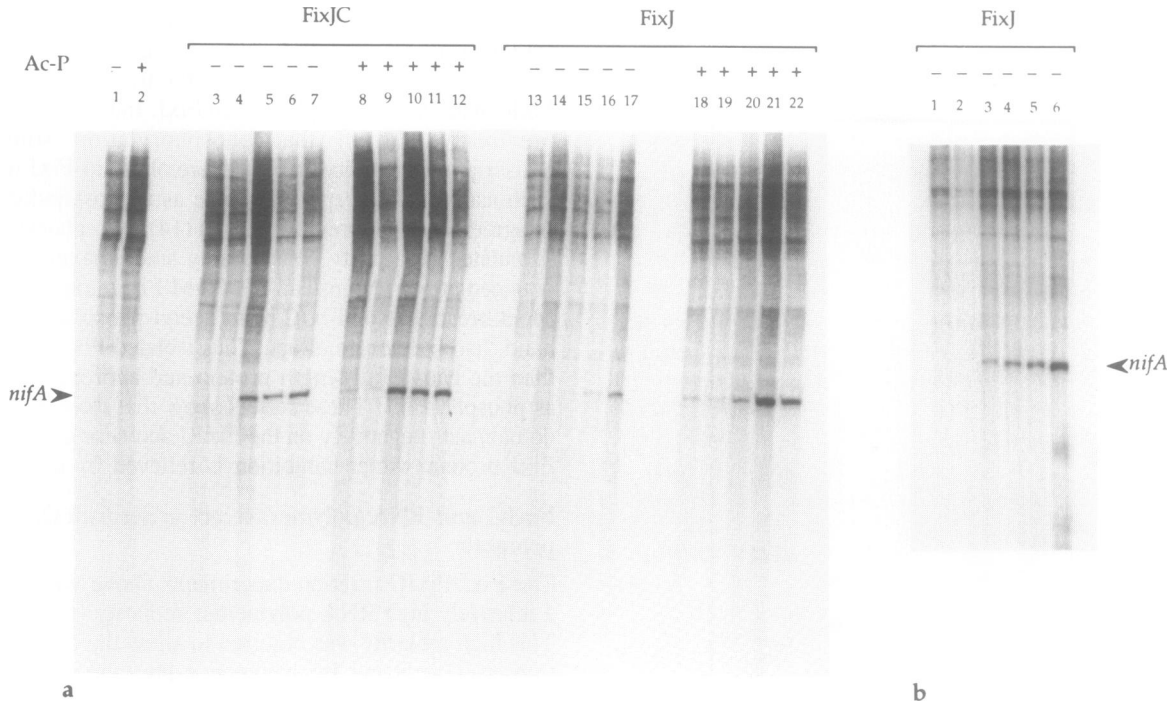

Figure 4. FixJC and phospho-FixJ activate *nifA* transcription at lower molarities than native FixJ. Single round transcription assays of the *nifA* promoter on the pJMR400 template, using either FixJC or FixJ. (**a**) The proteins were either untreated (lanes 3–7 and 13–17) or pretreated with 20 mM acetyl-phosphate (lanes 8–12 and 18–22). Lanes 1 and 2 are negative control transcription experiments in the absence of activator protein, without and with acetyl-phosphate, respectively. Molarities of FixJC monomer were: lanes 3 and 8, 0.9 μM; lanes 4 and 9, 1.8 μM; lanes 5 and 10, 3.5 μM; lanes 6 and 11, 9 μM; lanes 7 and 12, 18 μM. Molarities of FixJ monomer were: lanes 13 and 18, 0.55 μM; lanes 14 and 19, 1.1 μM; lanes 15 and 20, 2.2 μM; lanes 16 and 21, 5.5 μM; lanes 17 and 22, 11 μM. (**b**) Extended titration with FixJ, at molarities of 2.2 μM, 5.5 μM, 11μM, 22 μM, 55 μM and 95 μM (lanes 1–6).

overexpressing FixJC expressed higher levels of *nifA* than isogenic strains overexpressing FixJ (12). However, when expressed at physiological levels in *Rhizobium*, FixJC was only two-fold more active than FixJ on the *nifA* promoter in the absence of FixL (12). In the present work we found that FixJC was fully active on the *nifA* promoter at a molarity *ca.* 40-fold lower than FixJ. Therefore the relatively low *in vivo* activity of FixJC, when expressed at physiological levels in *Rhizobium*, does not result from a lower affinity of FixJC for the *nifA* promoter target. Instead it presumably results from a faster turn-over of the FixJC protein, as compared with wild-type FixJ.

Intramolecular signal transduction in response regulators

Two classes of models have been invoked to explain signal transduction in two-component response regulators (1). One class of models postulates changes in aggregation state of the regulators upon phosphorylation, which would be active as oligomers. The second class of models involves direct contacts between the phosphorylated regulator domain and the output domain, which could in principle be either stimulatory or inhibitory. Note that these two classes of models are not necessarily mutually exclusive.

In this paper we have provided evidence for the latter model, because the FixJN regulator domain exerts an inhibitory effect upon activity of the FixJC output domain. In this model phosphorylation brings about a conformational change of the regulator domain, sufficient to relieve inhibition of the output domain. Previously such a model was based on *in vivo* evidence, in the cases of transcriptional activation by FixJ (12), DctD (18)

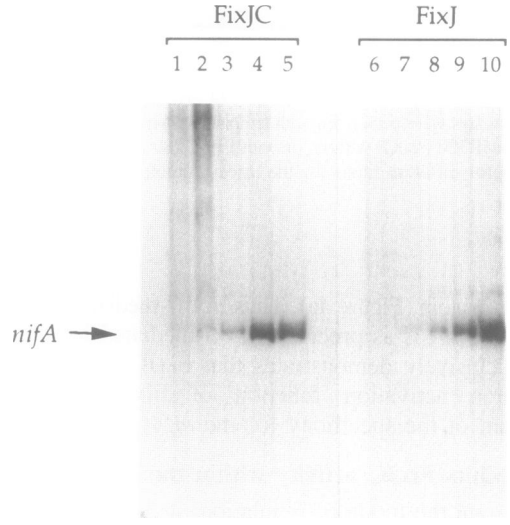

Figure 5. Run-on transcription assay of the *nifA* promoter with FixJC or FixJ. Molarities of FixJC were 0.28 μM, 0.57 μM, 1.15 μM, 2.3 μM and 4.6 μM (lanes 1 through 5). Molarities of FixJ were 5 μM, 10 μM, 20 μM, 50 μM and 100 μM (lanes 6–10).

and Spo0A (19). *In vitro* evidence was also available for the CheB methylesterase, because proteolytic cleavage of the phosphorylatable domain results in a 15-fold enhancement of esterase activity (20). However, to our knowledge, no *in vitro* evidence to support

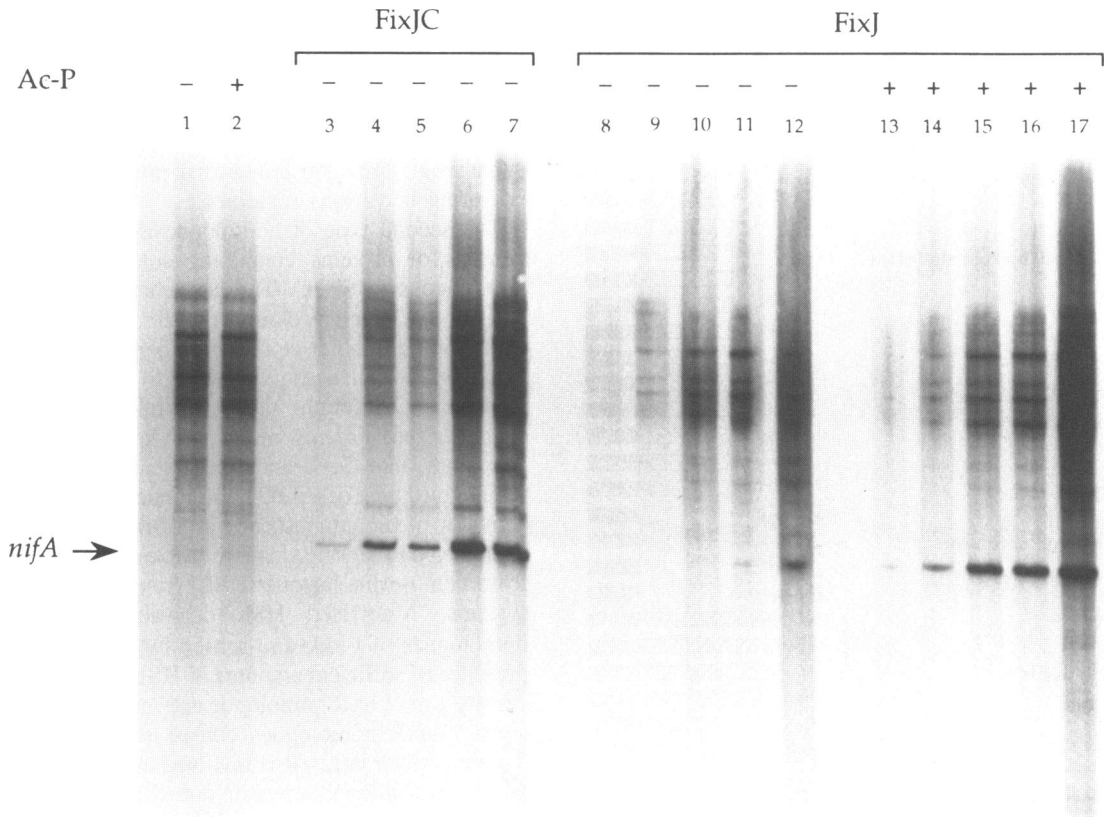

Figure 6. FixJC and phospho-FixJ act synergistically with RNA polymerase at the *nifA* promoter. Single round transcription assays of the *nifA* promoter at 20 μM of FixJC or FixJ monomer. The proteins were either untreated (lanes 3–12) or pretreated with 20 mM acetyl-phosphate (lanes 13–17). Lanes 1 and 2 are negative control transcription experiments in the absence of activator protein, without and with acetyl-phosphate, respectively. Molarities of RNA polymerase holoenzyme were: lanes 3, 8 and 13, 50 nM; lanes 4, 9 and 14, 100 nM; lanes 5, 10 and 15, 150 nM; lanes 6, 11 and 16, 200 nM; lanes 1, 2, 7, 12 and 17, 600 nM.

this model was available for a response regulator that functions as a transcriptional activator. This *in vitro* evidence we have now obtained for FixJ.

This negative mode for intramolecular signal transduction is particularly interesting in the light of recent results demonstrating a similar effect within the σ^{70} RNA polymerase subunit which determines promoter-binding specificity (21). Although σ^{70} specifically recognizes promoter sequences, the isolated protein does not bind DNA, and normally specific binding is observed only when σ^{70} is incorporated into RNA polymerase holoenzyme. However it was recently shown that specific binding of isolated σ^{70} with promoter DNA could be obtained, provided a large amino-terminal domain called 'region 1' is deleted (21,22). Thus region 1 inhibits the DNA-binding function of the C-terminal part of σ^{70}. This inhibition is relieved within the RNA polymerase holoenzyme molecule, presumably due to a conformational change of region 1. The parallel that we draw with FixJ is all the more relevant as the FixJC transcriptional activator domain is homologous to σ^{70} 'region 4' (12) which recognizes promoter −35 sequences (21,23,24). Thus an analogous mechanism operates both in FixJ and in σ^{70} to regulate transcription activation or DNA-binding in a negative fashion. It should be noted, however, that σ region 1 does not appear to share sequence similarity with the FixJN regulatory domain. Thus signal transduction mechanisms within both molecules can be considered analogous, but not necessarily homologous. Analogous negative regulatory mechanisms appear to operate also in a large spectrum of prokaryotic and eukaryotic regulatory proteins, including the AraC transcriptional activator (25), the glucocorticoid receptor (26), the c-Myb transcriptional activator (27), cAMP- and cGMP-dependent protein kinases (28) and protein kinase C (29). It is our working hypothesis that this very general regulatory mechanism, already demonstrated for FixJ, CheB, DctD and Spo0A, is at the basis of signal transduction in most bacterial response regulators.

Shuffling of the transcriptional activator module during evolution of eubacteria

The FixJC domain is known to be homologous to several bacterial transcriptional activators (12,17). We now update this protein family and find that domains homologous to FixJC are arranged in a wide variety of combinations (Figure 7).

The first type of FixJC homologue consists of 'response regulators' homologous to FixJ over its entire length. These proteins contain an additional domain which is phosphorylated on a conserved aspartate residue, which results in activation of transcription of target genes by the FixJC-homologous domain (reviewed in ref. 1). For instance it was recently demonstrated

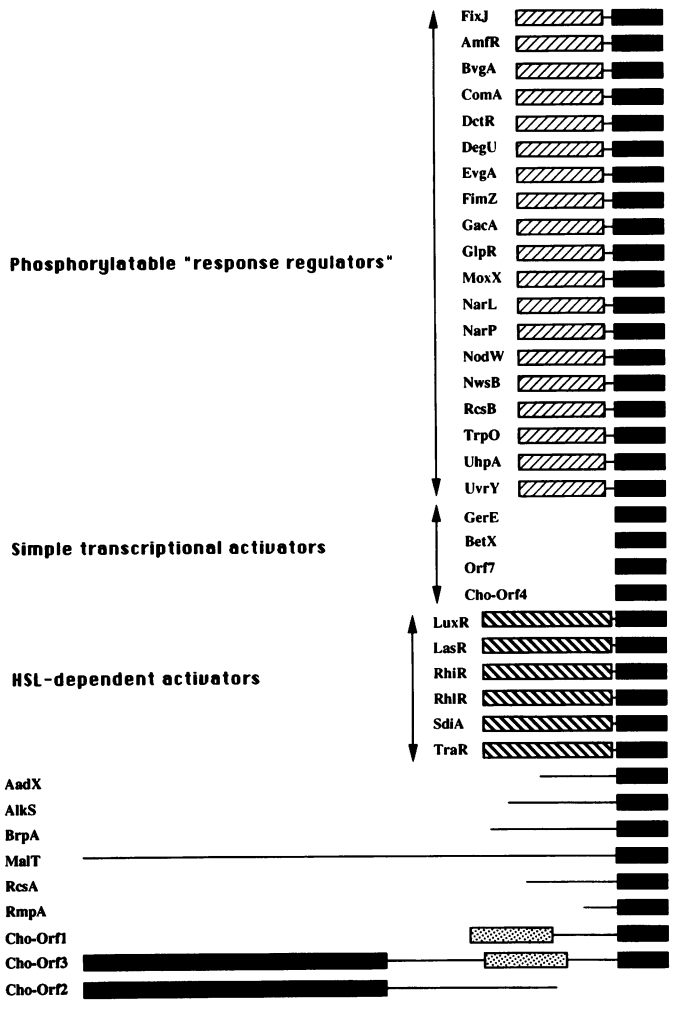

Figure 7. The FixJC family of transcriptional activators. In addition to previously identified members (12), this rapidly growing protein family now includes: (**a**) Phosphorylatable 'response regulators': *Streptomyces griseus* AmfR (a regulator of aerial mycelium formation, ref. 43); *Rhodobacter capsulatus* DctR (a regulator of dicarboxylate transport genes, ref. 44); *E.coli* EvgA (45), FimZ (a regulator of *fimbriae* biosynthesis, also found in *Salmonella typhimurium*, refs. 46, 47) and NarP (a nitrite-dependent regulator, ref. 48); *Pseudomonas fluorescens* GacA (which plays a role in secondary metabolite production, ref. 49); *Pseudomonas aeruginosa* GlpR (an activator of glycerol metabolism, ref. 50); *Paracoccus denitrificans* MoxX (a putative regulator of methanol dehydrogenase, ref. 51); *Bradyrhizobium japonicum* NwsB (a supressor of the homologous *nodW* gene, ref. 52). (**b**) Simple transcriptional activators: an ORF linked to the *betT* gene of *E.coli* (named BetX on this figure; SWISS-PROT entry YAHA_ECOLI, ref. 53); an open reading-frame (ORF) in a *fimbria* biosynthetic region of a *S.typhimurium* virulence plasmid (Orf7, ref. 54); an ORF upstream of a *Streptomyces* cholesterol oxidase operon (Cho-Orf4, ref. 38). (**c**) HSL-dependent activators: LasR, an activator of virulence functions in *P.aeruginosa* (55,56); *Rhizobium leguminosarum* RhiR (57); RhlR, a regulator of rhamnolipid synthesis in *P.aeruginosa* (58); SdiA, an inhibitor of cell division in *E.coli* (59); TraR, an activator of Ti plasmid transfer in *Agrobacterium tumefaciens* (60,61). (**d**) Other activators: an ORF linked to *Salmonella choleraesuis aadA* gene (named AadX on this figure, ref. 62); the AlkS activator of *Pseudomonas oleovorans* alkane catabolic genes (63); the BrpA activator of the *Streptomyces hygroscopicus* bialaphos biosynthetic pathway (64); the RmpA virulence factor of *Klebsiella pneumoniae* (65); and two ORFs upstream of a *Streptomyces* cholesterol oxidase operon (Cho-Orf1 and Cho-Orf3, ref. 38). Domains showing homology within each family are schematized by boxes with similar shadings. Transcriptional activator domains homologous to FixJC are symbolized by the darkest shading; phosphorylatable domains homologous to FixJN are lightly hatched; HSL-sensing domains are strongly hatched. This analysis was performed using the BLAST network service of the National Center for Biotechnology Information (Bethesda).

that phosphorylation of the FixJ homologue ComA results in a considerable increase of its affinity for target sequences (30). It is evident that phosphorylatable response regulators of the FixJ family are found in a tremendous diversity of regulons, and in a wide variety of bacteria. It seems also likely that each eubacterial organism contains a number of FixJ homologues operating in various regulons.

The second type of homologue is the most simple, since it consists of proteins containing solely the activator domain, homologous to FixJC. The best characterized member of this class is the GerE protein from *Bacillus subtilis*, involved in expression of mother-cell-specific genes during sporulation (31). Three other sequences were found to have a similar one-domain structure. Although these proteins have not yet been characterized, it seems safe to predict that they are transcriptional activators, like GerE and FixJC.

The third type of FixJC homologue, exemplified by the LuxR activator of bioluminescence in *Vibrio fischeri* (32), appears to be involved in sensing small diffusible intercellular signals derived from homoserine lactone (HSL) (reviewed in ref. 33). In these systems, *N*-acylated HSL derivatives are synthesized by homologues of LuxI, and sensed by LuxR homologues. In the presence of sufficient amounts of HSL (which occurs at high cell density), the LuxR homologue activates the expression of target genes. LuxR homologues consist of a conserved N-terminal domain, which in LuxR is involved in HSL sensing (34,35), and of a transcriptional activator domain homologous with FixJC. Consistent with our results, the latter domain in LuxR is active *per se*, as was shown by Choi and Greenberg (36,37) who used an *in vivo* assay for LuxR activity. Interestingly, the LuxR transcriptional activator domain is regulated negatively by the HSL-sensing, N-terminal domain. Thus a similar, albeit heterologous, intramolecular signal transduction mechanism operates in both the FixJ and the LuxR families of transcriptional activators.

Several other bacterial proteins exhibit homology to the FixJC transcriptional activator domain (Figure 7). Each of them possesses an N-terminal domain which is unrelated to FixJN or LuxRN. Finally an intriguing set of genes has been sequenced in the vicinity of a *Streptomyces* cholesterol oxidase operon (38). It contains four open reading frames, three of which show homology to FixJC. The domain arrangement of the corresponding proteins exhibits extensive shuffling (Figure 7).

It is thus evident that, during the evolution of eubacteria, activator domains homologous to FixJC have been recruited for a wide variety of physiological functions, and in numerous different protein contexts. This domain thus behaves like an evolutionarily autonomous modular unit in eubacteria, specialized in transcriptional activation. Evolution of proteins from combinations of autonomous modules is a recurrent theme in the analysis of protein structures (see for instance refs. 1,17,39–41). It is remarkable that signal transduction mechanisms in eubacteria should also proceed from such a combinatorial logic of sensor, regulator and activator modules.

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