Transcriptional regulation of molybdoenzyme synthesis in *Escherichia coli* in response to molybdenum: ModE–molybdate, a repressor of the *modABCD* (molybdate transport) operon is a secondary transcriptional activator for the *hyc* and *nar* operons

William T. Self, Amy M. Grunden,[†] Adnan Hasona and K. T. Shanmugam

Author for correspondence: K. T. Shanmugam. Tel: +1 352 392 2490. Fax: +1 352 392 5922.
e-mail: shan@micro.ifas.ufl.edu

Department of Microbiology
and Cell Science, University
of Florida, Gainesville, FL
32611, USA

Escherichia coli growing under anaerobic conditions produces several molybdoenzymes, such as formate hydrogenlyase (formate to H₂ and CO₂; *hyc* and *fdhF* genes) and nitrate reductase (*narGHJ* genes). Synthesis of these molybdoenzymes, even in the presence of the cognate transcriptional activators and effectors, requires molybdate in the medium. Besides the need for molybdopterin cofactor synthesis, molybdate is also required for transcription of the genes encoding these molybdoenzymes. In *E. coli*, ModE was previously identified as a repressor controlling transcription of the operon encoding molybdate transport components (*modABCD*). In this work, the ModE protein was also found to be a required component in the activation of *hyc-lacZ* to an optimum level, but only in the presence of molybdate. Mutant ModE proteins which are molybdate-independent for repression of *modA-lacZ* also restored *hyc-lacZ* expression to the wild-type level even in the absence of molybdate. Nitrate-dependent enhancement of transcription of *narX-lacZ* was completely abolished in a *modE* mutant. Nitrate-response by *narG-lacZ* and *narK-lacZ* was reduced by about 50% in a *modE* mutant. DNase I footprinting experiments revealed that the ModE protein binds the *hyc* promoter DNA in the presence of molybdate. ModE–molybdate also protected DNA in the intergenic region between *narXL* and *narK* from DNase I hydrolysis. DNA sequences (5' TAYAT 3' and 5' GTTA 3') found in ModE–molybdate-protected *modABCD* operator DNA were also found in the ModE–molybdate-protected region of *hyc* promoter DNA (5' GTTA-7 bp-CATAT 3') and *narX-narK* intergenic region (5' GTTA-7 bp-TACAT 3'). Based on these results, a working model is proposed in which ModE–molybdate serves as a secondary transcriptional activator of both the *hyc* and *narXL* operons which are activated primarily by the transcriptional activators, FhlA and NarL, respectively.

Keywords: molybdate, regulation, ModE, formate hydrogenlyase, *nar* operon

INTRODUCTION

Under anaerobic growth conditions, *Escherichia coli* produces several molybdoenzymes, almost all of which

serve as terminal enzymes in anaerobic respiratory pathways (Gennis & Stewart, 1996). Among the various molybdoenzymes, formate hydrogenlyase (FHL) is unique and is not a component of anaerobic respiration. The FHL complex which catalyses cleavage of formate to H₂ and CO₂, contains a formate dehydrogenase isoenzyme (FDH-H; *fdhF*), a hydrogenase isoenzyme (HYD3; *hyc*) and intermediate electron carriers also

[†]Present address: Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, USA.

Abbreviation: FHL, formate hydrogenlyase.

encoded by the *hyc* operon (Böck & Sawers, 1996). FDH-H contains selenium and molybdenum in the form of selenocysteine and molybdopterin guanine dinucleotide, respectively (Boyington *et al.*, 1997; Zinoni *et al.*, 1986).

Transcription of *fdhF* and the *hyc* operon requires FhlA as an activator and formate, the substrate for FHL, as an effector (Böck & Sawers, 1996; Maupin & Shanmugam, 1990; Schlensog & Böck, 1990; Schlensog *et al.*, 1994). Additionally, the σ^{54} -dependent *hyc* operon also requires integration host factor for maximum transcription (Hopper *et al.*, 1994). Based on DNase I-footprinting experiments, Schlensog *et al.* (1994) identified the DNA sequence to which the FhlA-formate complex binds during activation of the *hyc* operon. Besides the promoter region of the *hyc* operon, the intergenic region between *hycA* and *hycB* also contains the FhlA-binding sequence. This second FhlA-binding region is reported to be used for activation of a diverging operon, *hyp*, which encodes various proteins needed for nickel-dependent maturation of the three hydrogenase isoenzymes produced by the cell (Lutz *et al.*, 1991; Schlensog *et al.*, 1994).

Availability of an electron acceptor, like nitrate, increases the growth rate and cell yield of anaerobically growing *E. coli*. For this shift to nitrate respiration, the cell requires the presence of nitrate, the substrate for the molybdoenzyme nitrate reductase, absence of dioxygen, as well as intracellular molybdate (Gennis & Stewart, 1996; Gunsalus, 1992). Nitrate reductase is encoded by the genes in the *narGHJl* operon. The mechanism of regulation of the *narGHJl* operon has been thoroughly investigated and recently reviewed (Darwin & Stewart, 1996). Transcription of this operon requires the FNR, NarX and NarL proteins. The NarX protein is the histidine protein kinase and the NarL protein is the response regulator in the transcriptional activation of the *narGHJl* operon (Schroder *et al.*, 1994; Walker & DeMoss, 1993; Williams & Stewart, 1997). The NarX protein, besides transferring the phosphate after auto-phosphorylation to the NarL protein, also serves as a phosphatase of NarL-P to modulate the level of *narGHJl* operon expression (Schroder *et al.*, 1994; Walker & DeMoss, 1993). The dual activity of NarX to phosphorylate/dephosphorylate NarL permits the concerted synthesis of nitrate reductase in the presence of nitrate as a terminal electron acceptor.

Transcription of the genes encoding molybdoenzymes also requires the presence of molybdate in the cytoplasm (Cotter & Gunsalus, 1989; Iuchi & Lin, 1987; Pascal *et al.*, 1982; Rosentel *et al.*, 1995; Schlensog *et al.*, 1989; Stewart, 1988). The mechanism by which molybdate availability is transduced to transcription of genes encoding the molybdoenzymes is not known. Investigation of the molybdate-dependent regulation of transcription of operons encoding molybdoenzymes is complicated by the requirement of molybdopterin guanine dinucleotide for the enzymic activity of the gene products. The fact that the *hyc* operon does not code for

a molybdoprotein (Böhm *et al.*, 1990; Gladyshev *et al.*, 1994) allows the molybdate-dependent regulation studies to be conducted independently of the need for molybdate for activity of the gene product.

The *modABCD* operon, encoding molybdate-transport components, is also regulated by the availability of molybdate (Miller *et al.*, 1987; Rech *et al.*, 1995; Rosentel *et al.*, 1995). The molybdate-dependent repression of the *modABCD* operon is mediated through ModE (Grunden, 1996; Grunden *et al.*, 1996; McNicholas *et al.*, 1996; Walkenhorst *et al.*, 1995). It has been shown that the ModE-molybdate complex is the true repressor of the *modABCD* operon and apparently the ModE protein serves as a molybdate sensor in *E. coli* (Grunden & Shanmugam, 1997).

The presence of ModE as a molybdate sensor raises interesting questions about the role of this protein in regulating transcription of molybdoenzyme operons like *narGHJl*. ModE was shown to bind to the upstream region of the *moa* (molybdopterin synthesis) and *dms* (dimethylsulfoxide reductase) operons of *E. coli* (McNicholas *et al.*, 1997; McNicholas *et al.*, 1998). Although ModE apparently plays a role in the regulation of these two operons, the mechanism of this regulation is not known and these studies are also complicated by the presence of two promoters in the *dms* operon (McNicholas *et al.*, 1998).

In this paper, we present evidence that the ModE protein is a secondary transcriptional activator for the *hyc* and *narXL* operons which are primarily activated by the transcriptional activators, FhlA and NarL-P, respectively.

METHODS

Bacterial strains. The bacterial strains, phages and plasmids used in this study are listed in Table 1.

Media and growth conditions. Media used for bacterial growth have been described previously (Lee *et al.*, 1985, 1990; Rosentel *et al.*, 1995). L-broth (LB), which served as rich medium, was supplemented with glucose (0.3%; LBG), sodium formate (15 mM; LBF), sodium molybdate (1 mM; LB + Mo) or sodium nitrate (30 mM), as needed. Whenever required, glucose minimal medium was supplemented with sodium molybdate at a final concentration of 0.1 mM. Sodium nitrate (30 mM) was present in all media used to culture *nar-lacZ* fusion strains. Antibiotics, as needed, were included in media at the following concentrations: ampicillin, 100 $\mu\text{g ml}^{-1}$; tetracycline, 30 $\mu\text{g ml}^{-1}$; chloramphenicol, 50 $\mu\text{g ml}^{-1}$ (plates), 10 $\mu\text{g ml}^{-1}$ (liquid); kanamycin, 50 $\mu\text{g ml}^{-1}$.

Genetic and molecular biological experiments. These were performed essentially as previously described (Grunden *et al.*, 1996; Maupin & Shanmugam, 1990; Rosentel *et al.*, 1995). Lambda phages, isolated from various strains, were transduced into strain BW545 and single lysogens were selected as described by Rosentel *et al.* (1995) and further verified by comparing the levels of β -galactosidase activity produced by the cultures in both the presence and absence of nitrate to that of the original lysogens. All other mutations were transduced

Table 1. Bacterial strains, phages and plasmids used in this studyAll bacterial strains are derivatives of *E. coli* K-12

Strain, phage or plasmid	Relevant genotype	Source/reference
Strains		
BL21(DE3)	λ -lacUV5-T7 gene 1	F. W. Studier*
BW545	$\Delta(lacU)169$ <i>rpsL</i>	Laboratory collection
MC4100(λ PC50)	<i>araD139</i> $\Delta(argF-lac)U169$ <i>relA1</i> <i>rpsL150</i> <i>flb-5301</i> <i>deoC1</i> <i>ptsF25</i> $\lambda\Phi(narG'-lacZ)$	R. Gunsalus†
MRi93	<i>pcnB80</i> <i>zad2084::Tn10</i>	CGSC 7066
RK4353	MC4100 <i>gyrA219 non-9</i>	V. Stewart‡
RK5278	RK4353 <i>narL215::Tn10</i>	V. Stewart‡
VJS720	<i>modB247::Tn10</i>	V. Stewart‡
VJS2009	$\Delta(trpEA)2$ $\lambda\Phi(narX'-lacZ)$	V. Stewart‡
VJS4809	$\lambda\Phi(narK'-lacZ)$	V. Stewart‡
SE1910	BW545 $\Delta(modE-Km)2$	Grunden <i>et al.</i> (1996)
SE1978	BW545 $\Delta modF-Km$	This study
SE1980	BW545 $\Delta modEF-Km$	This study
SE2163	SE2176 $\Delta(modE-Km)2$	SE2176 $\times$ P1(SE1910)
SE2176	BW545 $\lambda\Phi(narG'-lacZ)$	This study
SE2200	SE2176 $\Delta(modE-Km)2$ <i>modB::Tn10</i>	SE2176 $\times$ P1(WS178)
SE2202	SE2176 <i>modB::Tn10</i>	SE2176 $\times$ P1(VJS720)
SE2213	SE2176 <i>narL::Tn10</i>	SE2176 $\times$ P1(RK5278)
AH78	AH122 $\Delta(modE-Km)2$	AH122 $\times$ P1(SE1910)
AH79	AH121 $\Delta(modE-Km)2$	AH121 $\times$ P1(SE1910)
AH106	AH122 <i>narL::Tn10</i>	AH122 $\times$ P1(RK5278)
AH107	AH121 <i>narL::Tn10</i>	AH121 $\times$ P1(RK5278)
AH121	BW545 $\lambda\Phi(narK'-lacZ)$	This study
AH122	BW545 $\lambda\Phi(narX'-lacZ)$	This study
AH179	SE2202 <i>cys</i>	Spontaneous chromate ^R
WS160	BW545 λ WS1	This study
WS161	WS160 $\Delta(modE-Km)2$	WS160 $\times$ (P1)SE1910
WS162	WS160 <i>modB247::Tn10</i>	WS160 $\times$ (P1)VJS720
WS168	WS160 $\Delta modF-Km$	WS160 $\times$ P1(SE1978)
WS169	WS160 $\Delta modEF-Km$	WS160 $\times$ P1(SE1980)
WS178	WS161 <i>modB247::Tn10</i>	WS161 $\times$ (P1)VJS720
WS192	WS161 $\Delta modB$ <i>pcnB80</i>	This study
Phages		
P1	Tn9 Cm ^R <i>clr-100</i>	Laboratory collection
λ RZ5	λ 'bla' <i>lacZ</i> <i>lacY</i> ⁺	Laboratory collection
λ WS1	λ <i>bla</i> ⁺ $\Phi(P_{hyc}lacZ)$ <i>lacY</i> ⁺	This study
Plasmids		
pVJS1	<i>narX</i> ⁺ <i>L</i> ⁺	V. Stewart‡
pAG1	pTrc99a- <i>modE</i> ⁺ , Amp ^R	Grunden <i>et al.</i> (1996)
pAH69	<i>narX-narK</i> intergenic region, Amp ^R	This study
pFGH100	pUC19, <i>hypA</i> ⁺ <i>hycA</i> ⁺ <i>hycB</i> ⁺ <i>hycC</i> ⁺	This study
pHYCP1	pACYC177, <i>hyc</i> promoter (<i>Pst</i> I– <i>Bst</i> EII)	This study
pHYC1	pACYC177, <i>P_{hyc}lacZ</i>	This study
pHYC3	pBR322, <i>P_{hyc}lacZ</i>	This study
pWS40	pUC19, <i>P_{hyc}</i> (–199 to –56)	This study
pWS31	pAG1, Cm ^R , <i>modE</i> ⁺	This study
pWS33	pAG1, Cm ^R , <i>modE</i> -G133D	This study
pWS34	pAG1, Cm ^R , <i>modE</i> -252	This study
pWS35	pAG1, Cm ^R , <i>modE</i> -216	This study
pZ1918	' <i>lacZ</i> , Amp ^R	Schweizer (1993)

* Dept Microbiology and Immunology, State University of New York Health Science Center, USA.

† Dept Microbiology and Molecular Genetics, University of California, Los Angeles, USA.

‡ Sections of Microbiology, Genetics and Development, Cornell University, USA.

into the wild-type strain carrying one of the various λ constructs.

Plasmid constructions. Standard published procedures (Grunden *et al.*, 1996; Maniatis *et al.*, 1982) or suppliers' recommendations were used for plasmid constructions and manipulations. Plasmid pFGH100, which carries the entire intergenic region of the *hyc* and *hyp* operons as well as the *hycAB* genes, was constructed by removing a 2.3 kb *Pst*I–*Ban*II fragment from plasmid pSE22 (Sankar *et al.*, 1985) and ligating into *Pst*I/*Ban*II-digested pUC19. For construction of plasmid pWS40, which carries the *hyc* operon promoter (–199 to –56 with respect to the transcription start site), a segment of *hyc*-upstream DNA was amplified by PCR using two primers, 5' CCGGATCCGTTATTTTCGAGCATATC 3' (primer 1) and 5' CCCTGCAGTTTAAAGCTAAAGATGAA 3' (primer 2). This amplified DNA, after digestion with *Pst*I and *Bam*HI, was cloned into pUC19 digested with *Pst*I and *Bam*HI, yielding plasmid pWS40.

Construction of Φ (P_{hyc} lacZ). For construction of a *lacZ* fusion with the *hyc* promoter, a 0.5 kb *Pst*I–*Bst*EII fragment from plasmid pFGH100 containing the intergenic region between the *hyc* and *hyp* operons was removed and cloned into a 3.0 kb *Pst*I–*Bst*EII fragment of plasmid pACYC177, yielding plasmid pHYCP1. A 3.2 kb *Sma*I fragment containing a promoterless *lacZ* gene from plasmid pZ1918 (Schweizer, 1993) was cloned into the *Bst*EII site of pHYCP1 after digestion with *Bst*EII and filling in the 3' recessed ends with the Klenow fragment of DNA polymerase I (Klenow). In this construct, pHYC1, the promoterless *lacZ* gene is located 6 bp upstream of the 'A' in the ATG translation start codon for *hycA*. The *hyc* promoter–*lacZ* fusion (P_{hyc} lacZ) was removed from pHYC1 as a 4.8 kb *Psp*1406I–*Sma*I fragment and cloned into the 4.2 kb *Clal*–*Eco*RV fragment of pBR322, yielding pHYC3. P_{hyc} lacZ was transferred from pHYC3 by recombination *in vivo* to λ RZ5 in the construction of λ WS1 using procedures described by Rosentel *et al.* (1995).

Construction of Δ modF and Δ modEF mutants. For the construction of Δ modF, a 1.3 kb *Mfe*I fragment from within the *modF* gene was removed from pRM25, which carries the entire *modEF* operon and part of the *galE* gene (Grunden *et al.*, 1996), and the remaining DNA was self-ligated to give pRM53. A 1.3 kb kanamycin-resistance gene cartridge was removed with *Eco*RI from pUC4K (Pharmacia Biotech) and cloned into the *Mfe*I site in pRM53 (pRM54). A 3.4 kb *Pvu*II–*Sca*I fragment which contained only the *modF*–Km deletion and flanking *E. coli* chromosomal DNA was obtained from pRM54 and self-ligated. This DNA, which lacks the origin of replication from the plasmid and the *bla* gene, was transformed into *E. coli* strain RK4353 and kanamycin-resistant clones were selected. The kanamycin-resistance gene, which was cotransducible with *gal* at >90% by phage P1, was transduced into strain BW545 and the transductant, strain SE1978, was used in further experiments.

For construction of Δ modEF–Km, a 4 kb *Acc*I–*Hind*III fragment containing the entire *modEF* operon and part of *modA* and *galE* was removed from pFGH15 (Grunden *et al.*, 1996) and cloned into pUC19, also digested with *Acc*I–*Hind*III, to give pRM51. A 2.2 kb *Afl*II–*Mfe*I fragment containing most of the *modEF* operon was removed from pRM51 and replaced with a 1.3 kb (*Hinc*II fragment) kanamycin-resistance gene cartridge from pUC4K (pRM52). A 3.4 kb *Pvu*II fragment which contains the kanamycin gene and flanking DNA but not the origin of replication or the *bla* gene was obtained, self-ligated and transformed into strain RK4353. Kanamycin-resistant clones were selected and tested for *modE* mutation.

The *modEF* deletion was transduced from one of these clones into strain BW545 and this transductant (strain SE1980) was used in further experiments.

Enzyme assays. Cells were grown under anaerobic conditions and harvested for enzyme assays as described previously (Lee *et al.*, 1995; Rosentel *et al.*, 1995). β -Galactosidase activity in whole cells from late-exponential to early-stationary phase of growth was determined with chloroform/SDS-permeabilized cells as described previously (Rosentel *et al.*, 1995). Specific activities [expressed as nmol min^{–1} (mg cell protein)^{–1}] are the mean of at least three independent experiments and varied by less than 15%. One unit of activity is defined as 1 nmol min^{–1} (mg cell protein)^{–1}.

Purification of ModE. ModE was purified as previously described (Grunden *et al.*, 1996).

DNA electrophoretic mobility-shift experiments. DNA mobility-shift experiments were performed essentially as described previously (Grunden *et al.*, 1996). All solutions used for the experiments were made with 'nanopure' deionized water without further treatment to remove trace levels of contaminating molybdate. A 281 bp *Mlu*I–*Bst*EII fragment from pFGH100 was used as the target DNA for the electrophoretic mobility-shift experiment. This fragment, which also contains the upstream binding site for FhlA, was labelled with [³²P]dNTPs by filling in the recessed 3' end of the *Bst*EII site with Klenow. ModE was pre-incubated with binding buffer for 2 min at 37 °C before the labelled DNA was added. Binding reactions, which also included 320 nM poly(dI-dC)-poly(dI-dC) (Pharmacia Biotech) were incubated for 30 min at 37 °C. Gels were pre-run at 100 V for 60 min at room temperature and run for 60 min at 100 V with buffer recirculation after loading the samples. The gels were dried and exposed to X-ray film. Sodium molybdate was added at a final concentration of 0.1 mM to the 5% polyacrylamide gel, circulating TBE buffer and the binding reactions, as needed.

DNase I footprinting experiments. DNase I protection experiments were performed essentially as described by Revzin (1993). For experiments involving *hyc* promoter DNA and ModE, a 396 bp DNA fragment containing the *hyc* operator/promoter DNA was obtained after digestion of pFGH100 with *Pst*I and *Sal*I and purification using a 10–30% sucrose gradient (Sankar, 1987). The *Sal*I end of the DNA was labelled with ³²P by filling in with Klenow and all four α -³²P-labelled dNTPs (DuPont-NEN). Unincorporated dNTPs were separated from the labelled fragment using a G-25 spin column (Pharmacia Biotech). Protein present in this eluent (heat-inactivated Klenow) was precipitated using 2.5 M potassium acetate and removed by centrifugation as previously described (Crouse & Amorese, 1987). The labelled DNA was precipitated using 2 vols 100% ethanol and recovered by centrifugation. The DNA was resuspended in TE buffer and stored at 4 °C at approximately 50 000 c.p.m. μ l^{–1}.

To allow binding of ModE to the DNA, a mixture of buffer (10 mM Tris/HCl, pH 7.9, 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT), ModE and labelled DNA (9.3 nM; 50 000 c.p.m.) was prepared in a volume of 19 μ l and incubated at 37 °C for 20 min. Molybdate was included in this reaction at a final concentration of 0.1 mM, as needed. Unprotected DNA was hydrolysed by the addition of DNase I (Sigma; 90 μ g μ l^{–1}) to the final reaction mixture (20 μ l). After 4 min incubation at room temperature, DNase I activity was stopped by addition of 5 μ l 200 mM EDTA to each reaction and the protein was hydrolysed by inclusion of 1 μ g Proteinase K (Sigma; 15 min at 37 °C). Remaining trace amounts of proteins were removed by 2.5 M ammonium acetate precipitation. After addition of 1 μ g

yeast tRNA, nucleic acids were precipitated with 2 vols 100 % ethanol. Each sample was resuspended in 4 μ l formamide loading buffer (Revzin, 1993) and loaded on an 8 % polyacrylamide denaturing gel for electrophoresis at 50 W for 100 min. The gel was dried and exposed to pre-flashed X-ray film. The exact locations of the ModE-protected regions in the *hyc* DNA were determined by comparison to sequence generated from plasmid pWS40 DNA using primer 1 used in the construction of plasmid pWS40.

For DNase I footprinting of *nar* DNA, a 400 bp fragment containing the *narX*–*narK* intergenic region was removed from plasmid pVJS1 (Stewart & Parales, 1988) as a *HincII*–*DraI* fragment and cloned into *HincII*-digested pUC19 (pAH69). A 335 bp *AccI* fragment (–97 bp from the *narX* translation start to +90 bp from the *narK* translation start) was isolated from pAH69 and labelled with [α - 32 P]dATP (using Klenow) to probe the *narXL* coding strand. The DNA concentration in this experiment was 5 nM. Since the *AccI* site corresponding to the *narXL* non-coding strand contained the sequence 5' GTCGAC 3', this site was not labelled by Klenow in the presence of [32 P]dATP alone.

Materials. Biochemicals were purchased from Sigma. Other organic and inorganic chemicals were from Fisher Scientific and were analytical grade. Restriction endonucleases and DNA modifying enzymes were purchased from Promega and New England Biolabs.

RESULTS

Molybdate is needed for optimum expression of *P_{hyc}lacZ* and *narG*–*lacZ*

In preliminary experiments, optimum expression of the *hyc* operon was reported to require molybdate in addition to formate, the substrate for FHL (Rosentel *et al.*, 1995). Since the requirement of molybdate for *hyc* expression could not be demonstrated in wild-type *E. coli* due to the presence of a high-affinity transport system for molybdate and contamination of normal medium components with trace levels of molybdate, a

modB (molybdate transport) mutant (strain WS162) was constructed. This strain also contains λ WS1, which carries a *hyc* promoter–*lacZ* fusion (*P_{hyc}lacZ*) in the λ *att* site. As expected, production of β -galactosidase from the *P_{hyc}lacZ* fusion required FhlA, formate, and the alternative sigma factor, σ^{54} (data not shown). Anaerobic cultures of strain WS160 (wild-type) produced about 2000 units of β -galactosidase activity when grown in LBG or LBG + Mo (Table 2). Strain WS162, the *modB* mutant, produced only about 12 % of the level of activity of the parent strain when grown in LBG medium without molybdate supplementation and the β -galactosidase activity increased to the wild-type levels with molybdate addition to the growth medium. Although the requirement for molybdate can be demonstrated in cells grown in glucose minimal medium, the level of expression of *hyc*–*lacZ* was significantly lower even in the *mod*⁺ parent strain as compared to the LBG cultures.

A *modB* mutant carrying λ –*narG*–*lacZ* (strain SE2202) also produced only about 14 % of the parent level of β -galactosidase activity when grown in rich medium without added molybdate (Table 2). This level of activity (2700 units) was about twofold higher than the 1300 units of β -galactosidase activity produced by a *narL* mutant, strain SE2213, lacking the transcriptional activator, NarL. Strain SE2202 grown in LBG + Mo produced parental levels of β -galactosidase activity. However, when the *modB* mutant was grown in glucose minimal medium, the activity was 31 % of the parent level; this is in contrast to the less than 5 % of the corresponding parent level of *hyc*–*lacZ* expression in an isogenic strain (strain WS162) grown in the same medium. The 3000 units of β -galactosidase activity produced by strain SE2202 grown in glucose minimal medium represents a sixfold higher response to nitrate (compared to about 450 units of activity when grown without nitrate). This moderate increase in *narG*–*lacZ*

Table 2. Molybdate dependence of *P_{hyc}lacZ* and *narG*–*lacZ* expression

Values in parentheses represent the percentage of activity in the *modB* mutant in comparison to the values obtained with the corresponding wild-type parent strain grown in the same medium. ND, Not determined.

Strain	Relevant genotype	<i>β</i> -Galactosidase activity*			
		LBG	LBG + Mo	Glu-min	Glu-min + Mo
<i>hyc-lacZ</i> derivatives					
WS160	Wild-type	2030	2020	830	960
WS162	<i>modB</i>	250 (12)	1730 (86)	< 50 (< 5)	1010 (105)
<i>narG-lacZ</i> derivatives†					
SE2176	Wild-type	20000	19500	9700	10000
SE2202	<i>modB</i>	2700 (14)	21000 (108)	3000 (31)	8500 (85)
AH179	<i>modB cys</i>	ND	ND	1400 (14)	2900 (29)

* Expressed as nmol min^{–1} (mg cell protein)^{–1}.

† Growth media used for these strains also contained 30 mM sodium nitrate.

expression in the *modB* mutant, strain SE2202, was observed even in the absence of molybdate supplementation of the glucose minimal medium. Inclusion of molybdate in the growth medium did increase the level of *narG-lacZ* expression to about 85% of the parent level. This molybdate dependence of *narG-lacZ* expression was observed only when nitrate was also present in the medium and is associated with the increase in expression of the *nar* operon in response to nitrate. In the absence of nitrate, the cells grown in either medium produced very low levels of activity (about 450 units of β -galactosidase activity) which was not influenced by the *modB* mutation or molybdate.

The differences in the response to nitrate by strain SE2202 grown in LBG or glucose minimal medium without added molybdate (14% compared to 31% of the parent value respectively) may be attributable to the presence of sulfate transport proteins in the cells grown in the glucose minimal medium, which are absent in cells grown in LBG (Kredich, 1996). Sulfate transport components are known to effectively transport molybdate which is present as a contaminant in the glucose minimal medium constituents (Rosentel *et al.*, 1995). Since removal of the contaminating anion, molybdate, is difficult to accomplish, cystine, known to suppress the sulfate-transport pathway in *E. coli* (Kredich, 1996), was added to the glucose minimal medium and the levels of β -galactosidase activity were determined. Strain SE2202 grown in glucose minimal medium with cystine ($50 \mu\text{g ml}^{-1}$) and nitrate produced about 1700 units of β -galactosidase activity (18% of the parent value), compared to about 3000 units of activity obtained with cells grown without cystine. Introducing a mutation in the *cysTUA* operon (defective in sulfate transport; Kredich, 1996) into strain SE2202 (strain AH179) reduced the level of nitrate response to that observed with LBG/nitrate-grown cells (14% of the parent level; Table 2). Addition of 0.1 mM molybdate to the glucose minimal medium with nitrate did not fully support *narG-lacZ* expression by the *modB cys* double-mutant strain AH179 to the wild-type or to the *modB* mutant level. It is possible that in the *modB cys* double mutant, other anions, such as sulfate, present in the glucose minimal medium compete effectively with molybdate in the transport of molybdate by the only other known molybdate transporter, the non-specific anion-transport system (Rosentel *et al.*, 1995). These results show that the cells grown in glucose minimal medium have alternative pathways for molybdate transport/metabolism which complicated the analysis of the experimental results obtained with *narG-lacZ*.

Molybdate requirement is independent of the need for formate in *hyc* expression

To evaluate whether the requirement for molybdate in *hyc-lacZ* expression is indirect and is related to the need for formate for activation of *hyc* operon, the levels of β -galactosidase activity produced by the *modB* mutant, strain WS162, grown with various formate concen-

Fig. 1. Effect of formate concentration in the growth medium on the expression of $P_{hyc}lacZ$ in a *modB* mutant. Strain WS162 was grown in LBG medium with and without molybdate (1 mM) supplementation.

trations were determined (Fig. 1). The β -galactosidase activity of the culture not supplemented with molybdate increased from 250 units to 700 units between the formate concentrations of 2 and 20 mM. Even at 50 mM formate, which partially inhibited growth, the maximum level of β -galactosidase activity produced by the culture was less than 40% of the values obtained with the parent, strain WS160, grown without formate supplementation of the growth medium (2000 units; Table 2). Addition of molybdate alone increased the level of expression of *hyc-lacZ* in strain WS162 to about 1700 units (Fig. 1). Formate further increased the level of expression of *hyc-lacZ* in this strain by an additional 1000 units, even in the presence of molybdate. The requirement for molybdate for optimum expression of *hyc-lacZ* in strain WS162, which produced formate internally, suggests that the need for molybdate is independent of the need for formate in the expression of *hyc-lacZ*. However, optimum expression of the *hyc* operon requires both formate and molybdate.

The molybdate-response regulator, ModE, has a role in transcription of $P_{hyc}lacZ$ and *narG-lacZ*

Since the ModE protein is the only known molybdate-responsive DNA-binding protein in *E. coli*, its role in molybdate-dependent activation of transcription of *hyc-lacZ* and *narG-lacZ* was investigated. A *modE* mutant, strain WS161, only produced about 40% of the wild-type levels of β -galactosidase activity, which was not affected by molybdate addition (Table 3). When the ModE⁺ phenotype was restored by introducing pWS31 (*modE*⁺), strain WS161(pWS31) produced β -galactosidase activity at the wild-type levels.

The amount of β -galactosidase activity produced by *modE* strain SE2163 carrying a *narG-lacZ* fusion was also reduced by about 60% (Table 3). Addition of molybdate slightly increased the level of activity. Intro-

Table 3. ModE is essential for optimum expression of P_{hyc} -*lacZ* and *narG-lacZ*

Values in parentheses represent the percentage of activity in the mutants in comparison to the values obtained with the corresponding wild-type strain grown in the same medium. Plasmids pAG1 and pWS31 carry the same *modE*⁺ gene but differ in their antibiotic resistance properties. Strain SE2176 produced about 3000 units of β -galactosidase activity when grown in LBG medium without nitrate.

Strain	Relevant genotype	β -Galactosidase activity*	
		– Mo	+ Mo
<i>hyc</i> – <i>lacZ</i> derivatives			
WS160	Wild-type	2030	2020
WS161	Δ <i>modE</i>	820 (40)	810 (40)
WS161(pWS31)	Δ <i>modE</i> <i>pmodE</i> ⁺	2120 (105)	2320 (115)
WS178	Δ <i>modE modB</i>	<50 (0)	800 (40)
<i>narG</i> – <i>lacZ</i> derivatives†			
SE2176	Wild-type	20000	19500
SE2163	Δ <i>modE</i>	7600 (38)	9000 (46)
SE2163(pAG1)	Δ <i>modE</i> <i>pmodE</i> ⁺	19000 (95)	21000 (108)
SE2200	Δ <i>modE modB</i>	3100 (16)	9000 (46)

* Expressed as nmol min^{–1} (mg cell protein)^{–1}.

† Growth media used for these strains carrying λ PC50 also contained 30 mM sodium nitrate.

Table 4. Effect of mutant ModE proteins on transcription of P_{hyc} -*lacZ* in a *modB* mutant

All strains carry λ WS1 (P_{hyc} -*lacZ*). Strain WS192 also carries a *pcnB* mutation to reduce the copy number of plasmid pWS31 (*modE*⁺) and plasmids carrying *modE* mutations (Grunden *et al.*, 1996).

Strain	Relevant genotype	β -Galactosidase activity*	
		– Mo	+ Mo
WS160	Wild-type	2030	2020
WS192	Δ <i>modE modB</i>	<50	930
WS192(pWS31)	Δ <i>modE modB pmodE</i> ⁺	230	2470
WS192(pWS33)	Δ <i>modE modB pmodE</i> -G133D	1290	1230
WS192(pWS34)	Δ <i>modE modB pmodE</i> -252	1870	2300
WS192(pWS35)	Δ <i>modE modB pmodE</i> -216	1830	1580

* Expressed as nmol min^{–1} (mg cell protein)^{–1}.

ducing a *modE*⁺ plasmid restored the level of β -galactosidase activity produced by strain SE2163 to that of the wild-type. These results suggest that the ModE protein is required for optimum expression of both *hyc* and *narG* operons. The residual 40% of β -galactosidase activity produced by the *modE* mutants was still molybdate-dependent, since introducing a *modB* mutation that prevented the uptake of molybdate from molybdate-unsupplemented medium completely abolished the expression of the *hyc* operon and the nitrate response of the *narG-lacZ* fusion. These results show that besides ModE, another factor is involved in Mo-dependent expression of the *hyc* and *narGHJ* operons. Unpublished results show that the second factor is the MoeA protein (A. Hasona *et al.*, unpublished).

It is possible that the molybdate-bound form of ModE, the known repressor of the *modABCD* operon, is also the active form participating in *hyc* and *narG* expression. To evaluate this possibility, a *modE*⁺ plasmid (pWS31) was introduced into a *modE modB* double mutant, strain WS192, that carries the P_{hyc} -*lacZ* fusion (Table 4). Strain WS192 also carries a *pcnB* mutation to reduce the copy number of the *modE*⁺ plasmid and to minimize plasmid copy number effect (Lopilato *et al.*, 1986). Even in the presence of pWS31, which encodes native ModE protein, expression of P_{hyc} -*lacZ* was completely molybdate-dependent (Table 4).

We have previously described mutant ModE proteins which repressed *modA-lacZ* expression even in the absence of molybdate (Grunden *et al.*, 1996). When

Table 5. Transcriptional activation of $\Phi(P_{hyc}lacZ)$ in various *mod* mutants

Values in parentheses represent the percentage of activity produced by the mutant strain as compared to the wild-type parent, strain WS160. pWS31 carries *modE*⁺. All strains carry λ WS1.

Strain	Relevant genotype	β -Galactosidase activity*	
		LBG	LBG + MoO ₄ ²⁻
WS160	Wild-type	2030	2020
WS161	$\Delta modE$	820 (40)	810 (40)
WS168	$\Delta modF$	1840 (91)	1700 (84)
WS169	$\Delta modEF$	850 (42)	800 (40)
WS162	<i>modB</i> ::Tn10	120 (6)	1840 (91)
WS178	$\Delta modE modB$::Tn10	< 50 (0)	800 (40)
WS160(pWS31)	Wild-type (<i>pmodE</i> ⁺)	2050 (101)	2120 (105)
WS161(pWS31)	$\Delta modE$ (<i>pmodE</i> ⁺)	2120 (104)	2320 (115)
WS169(pWS31)	$\Delta modEF$ (<i>pmodE</i> ⁺)	2710 (133)	2580 (128)

* Expressed as nmol min⁻¹ (mg cell protein)⁻¹.

plasmids encoding these mutant ModE proteins were introduced into strain WS192, $P_{hyc}lacZ$ expression was also independent of added molybdate (Table 4). These results are in agreement with the proposal that ModE-molybdate is the active form in enhancing transcription of the *hyc* operon. Similar results were also obtained with *narG-lacZ modB* mutant strains carrying plasmids encoding mutant ModE proteins (data not shown).

modE is the first gene in the *modEF* operon, which is divergent from the *modABCD* operon on the *E. coli* chromosome (Grunden *et al.*, 1996; Walkenhorst *et al.*, 1995). Expression of $P_{hyc}lacZ$ in $\Delta modE$ and $\Delta modEF$ deletions and $\Delta modE modB$ double-mutant strain with and without pWS31 carrying *modE*⁺ was analysed (Table 5). Although the amount of β -galactosidase activity produced by the *modE* deletion, strain WS161, was reduced to 40% of the parent level, the *modF* deletion had a minimal effect on the expression of *hyc-lacZ* (strain WS168). The level of expression of *hyc-lacZ* in a *modEF* deletion (strain WS169) was similar to that of the *modE* mutant alone. Introducing a plasmid containing only *modE*⁺ (pWS31) into the *modEF* deletion strain restored the expression to levels higher than the parent strain with the same plasmid. Apparently, the higher than expected level of β -galactosidase activity produced by strain WS169(pWS31) is a result of the high copy level of *modE*⁺ and the absence of ModF.

An alternative explanation for the decrease in *hyc-lacZ* expression in a *modE* mutant could be that in the absence of ModE, the transport of molybdate is derepressed and the high intracellular molybdate is indirectly influencing the level of *hyc-lacZ* expression. To evaluate the possibility, a *modE modB* double mutant (strain WS178) was constructed. This double mutant was completely dependent on molybdate for expression, and even in the presence of molybdate

(1 mM) the level of β -galactosidase activity produced by the strain was the same as that of a *modE* mutant, strain WS161 (Table 5). These results show that the decrease in transcription of *hyc-lacZ* in a *modE* mutant can not be attributed to the presence or absence of ModF, whose function is not known, or to unregulated expression of the molybdate transport system encoded by *modABC*.

Effect of *modE* mutation on *narK-lacZ* expression

The regulation of *narK*, which codes for a nitrate-extrusion system, is reported to be similar to that of the *narG* operon (Bonney & DeMoss, 1992; Kolesnikow *et al.*, 1992; Noji *et al.*, 1989; Rowe *et al.*, 1994). Since *narG* expression required the ModE protein, the effect of the *modE* mutation on *narK-lacZ* expression was investigated and the results are presented in Table 6. Under our experimental conditions, wild-type strain with $\lambda\Phi(narK-lacZ)$ (strain AH121) produced about 1300 units of β -galactosidase activity when grown without nitrate. This level was increased by about fourfold by inclusion of nitrate in the growth medium. The molybdate concentration of the medium had no effect on the amount of β -galactosidase activity produced by strain AH121, probably due to the presence of the native high-affinity molybdate transport system (data not presented). In a *modE* mutant (strain AH79), this nitrate-dependent increase was reduced to about 40% of the parent level. Similar results were obtained when the cells were grown in glucose minimal medium. Plasmid pAG1, producing ModE⁺ protein, fully restored the nitrate-dependent enhancement in strain AH79. The residual nitrate-dependent expression of *narK-lacZ* (about 40% of the *modE*⁺ parent level) in a *modE* mutant, strain AH79, was similar to the level of activity produced by the *modE* mutant, strain SE2163, carrying *narG-lacZ* (46% of the corresponding parent level) and was molybdate-dependent.

Table 6. Effect of *modE* mutation on nitrate-dependent increase in expression of various *nar-lacZ* fusions

Cells were grown in LBG + Mo and the sodium nitrate concentration (where present) was 30 mM. Values in parentheses represent the percentage of activity in the various strains in comparison to the values obtained with the corresponding wild-type parent strain grown in the same medium.

Strain	Relevant genotype	β -Galactosidase activity*	
		– NO ₃ [–]	+ NO ₃ [–]
<i>narG</i> – <i>lacZ</i> derivatives			
SE2176	Wild-type	2900	19500
SE2163	Δ <i>modE</i>	2500 (86)	9000 (46)
SE2163(pAG1)	Δ <i>modE</i> <i>pmodE</i> ⁺	2400 (83)	21000 (108)
SE2213	<i>narL</i>	2800 (97)	1300 (7)
<i>narK</i> – <i>lacZ</i> derivatives			
AH121	Wild-type	1300	5500
AH79	Δ <i>modE</i>	1100 (85)	2200 (40)
AH79(pAG1)	Δ <i>modE</i> <i>pmodE</i> ⁺	1100 (85)	5570 (101)
AH107	<i>narL</i>	920 (71)	970 (18)
<i>narX</i> – <i>lacZ</i> derivatives			
AH122	Wild-type	950	2000
AH78	Δ <i>modE</i>	900 (95)	1000 (50)
AH78(pAG1)	Δ <i>modE</i> <i>pmodE</i> ⁺	800 (80)	1800 (90)
AH106	<i>narL</i>	920 (96)	970 (49)

* Expressed as nmol min^{–1} (mg cell protein)^{–1}.

ModE protein is required for expression of *narXL* operon

The *narXL* operon encodes the two regulatory proteins needed for activation of the *narG* and *narK* operons (Darwin & Stewart, 1996; Egan & Stewart, 1990; Kalman & Gunsalus, 1990; Schroder *et al.*, 1994; Walker & DeMoss, 1993). Since the expression of *narG* and *narK* is influenced by ModE, the effect of mutations in *modE* on *narX-lacZ* expression was investigated. The wild-type strain with $\lambda\Phi$ (*narX-lacZ*) (strain AH122) produced about 1000 units of β -galactosidase activity when grown without nitrate and this activity increased by about twofold in the presence of nitrate (Table 6). This nitrate-dependent enhancement of *narX* expression was absent in a *modE* mutant, strain AH78, and as expected, also in a *narL* mutant, strain AH106. Introducing a plasmid containing the *modE*⁺ gene into the *modE* mutant (strain AH78) restored the nitrate response to that of the wild-type. These results suggest that the requirement of ModE for optimum transcription of *narG-lacZ* and *narK-lacZ* is indirect and is mediated through the control of the *narXL* operon.

ModE binds to *hyc* promoter DNA

Since genetic and physiological experiments suggested that ModE is involved in the regulation of both P_{*hyc*} *lacZ* and *narX-lacZ*, direct binding experiments consisting of ModE protein and promoter DNA were carried out. Electrophoretic mobility of a 396 bp *hyc* promoter fragment was retarded in the presence of ModE, but

Fig. 2. DNA electrophoretic mobility-shift experiment with purified ModE protein and *hyc* promoter DNA. (a) Without molybdate; (b) with molybdate (0.1 mM) added to binding reactions and electrophoresis buffer. Lane 1, no protein added; lane 2, 85 nM ModE; lane 3, 130 nM ModE; lane 4, 170 nM ModE; lane 5, 212 nM ModE; lane 6, 255 nM ModE; lane 7, 340 nM ModE. The arrow indicates the DNA-protein complex.

only in the presence of molybdate (Fig. 2b). The minimal concentration of ModE-molybdate required for the mobility shift was about 250 nM and this was about a 10-fold higher concentration of ModE than was required for binding to the *modA* operator (Anderson *et al.*, 1997; McNicholas *et al.*, 1997). These results show that the ModE-molybdate complex is capable of binding to *hyc* upstream DNA and efficient binding of ModE to *hyc* promoter DNA requires molybdate.

DNase I-footprinting experiments involving ModE and *hyc* promoter DNA revealed that ModE-molybdate protects a 27 bp region in this DNA from DNase I

Fig. 3. DNase I hydrolysis of *hyc* promoter DNA in the presence of ModE with or without molybdate. Lanes 3–5 have 0.1 mM molybdate in the binding reaction; lanes 6–8 have no added molybdate. Lane 1, sequencing ladder (only the C track is shown); lane 2 DNA alone (9.3 nM); lanes 3 and 6, 1.77 μM ModE; lanes 4 and 7, 3.54 μM ModE; lanes 5 and 8, 7.08 μM ModE. The numbers represent the position of the bases with respect to the *hyc* transcription start site. The vertical line indicates the ModE–molybdate protected area of the DNA.

hydrolysis (Fig. 3). The centre of the ModE-protected region is 94 bases upstream of the centre of the DNA protected by the transcriptional activator, FhlA, from DNase I hydrolysis (Fig. 4a; Schlensog *et al.*, 1994). The 29 bp ModE-protected *modA* operator contains three copies of a pentamer sequence 5' TAYAT 3' (Y, pyrimidine; Fig. 4b). Besides these pentamer sequences a tetramer sequence, 5' GTTA 3', can also be detected at nine locations within an 80 bp region in the *modA* operator DNA. Within the *hyc* promoter region protected by ModE–molybdate, both the tetramer and pentamer sequences can be detected (5' GTTA-7 bp-CATAT 3'; Fig. 4a). These two sequences are separated by seven bases, which is the same distance between the two pentamer sequences (regions 1 and 2) in the *modA* operator (Fig. 4b). An inverted repeat, 5' CAATAATTG 3' (–153 to –145), and another sequence, 5' TAACTATTG 3' (–173 to –165) containing the 5' GTTA 3' tetramers, are also found near the ModE-recognition/binding sequence in the *hyc* promoter DNA. The role of these tetramer sequences is not known. This comparison shows that there are significant

Fig. 4. ModE–molybdate-protected regions of *hyc* promoter DNA (a) and *modABCD* operator DNA (b). The bases enclosed by a shaded box are protected by ModE from DNase I. The FhlA- and IHF-protected regions in the *hyc* promoter DNA are from Schlensog *et al.* (1994). Transcription start sites for *hyc* and *hyp* are indicated by arrows. Putative ModE-binding motifs are shown as white on black. 'GTTA' sequences present in the two sequences are underlined. The data on the *modA* operator DNA are from Grunden (1996).

similarities as well as differences in the protected regions of DNA between the *modA* operator and *hyc* promoter.

The amount of ModE required for protection of *hyc* promoter DNA from DNase I was $> 1 \mu\text{M}$; this is about sevenfold higher than the amount of the transcriptional activator, FhlA, required for protection of *hyc* promoter DNA from DNase I hydrolysis (Schlensog *et al.*, 1994). The same preparation of ModE–molybdate protected *modA* operator DNA completely from DNase I hydrolysis at a concentration of less than 3 nM (data not shown). The higher concentration of ModE protein required for protection of the *hyc* promoter DNA *in vitro* may reflect the role of ModE as a secondary activator rather than a required primary regulator of transcription.

ModE–molybdate binds to DNA in the *narXL*–*nark* intergenic region

Since the ModE protein is required for nitrate-dependent expression of *nar* operons and also protects *hyc* promoter DNA from DNase I, the ability of ModE to bind to various *nar* promoter sequences was determined. In an electrophoretic mobility-shift experiment, ModE–molybdate failed to shift the mobility of *narGHJ* promoter DNA. ModE–molybdate did shift the electro-

Fig. 5. DNase I hydrolysis of the *narX*-coding strand in the *narX*-*narK* intergenic region, in the presence of ModE protein with or without molybdate. Lanes 3–7, with 0.1 mM molybdate in the binding reaction; lanes 8–12, without added molybdate. Lanes 1 and 2, sequencing ladder (T and C tracks, respectively); lane 3, DNA alone (5 nM); lanes 4 and 8, 0.5 μM ModE; lanes 5 and 9, 1.0 μM ModE; lanes 6 and 10, 1.5 μM ModE; lanes 7 and 11, 2.0 μM ModE; lane 12, 4.0 μM ModE. The numbers represent the position of the bases in the DNA sequence with respect to the 'A' in the translation start site in *narXL*. The vertical lines indicate the regions of DNA protected by ModE-molybdate.

phoretic mobility of the DNA in the *narK*-*narXL* intergenic region but the amount of ModE-molybdate required for this shift was significantly higher than the amount required for ModE/*modA* operator DNA (data not shown).

Since the ModE protein interacted with *narX* DNA, the specific location of this interaction was determined by a DNase I footprinting experiment (Fig. 5). For this experiment, DNA covering the entire *narXL*-*narK* intergenic region was used and ModE binding was determined. In the presence of molybdate, ModE protected a 30 bp region of DNA corresponding to -156 to -127 in which +1 is the 'A' in the ATG for *narX* (Fig. 6). The concentration of ModE-molybdate required for protection in these experiments was about 1.5 μM, although DNase I hypersensitive sites can be readily seen at a concentration as low as 500 nM. It has been reported that the concentration of NarL required

Fig. 6. ModE-molybdate-protected regions in the *narX*-*narK* intergenic region of *E. coli*. The bases enclosed by the shaded box are protected by ModE from DNase I hydrolysis. Putative ModE-binding motifs (consensus sequence) are shown in white on black. An asterisk above a base indicates hypersensitivity to DNase I. NarL- and IHF-protected regions are enclosed by rectangles and are presented for comparison. NarL-, IHF- and FNR-binding regions (also enclosed by a rectangle) are from Li *et al.* (1994). Transcription from the *narK1* start site is regulated by nitrate while the *narK2* promoter is constitutive. The transcription start site for *narX* is not known. The bases are numbered with the T in the TAC (inverse of ATG) in the *narX* translation start site as +1.

for protection of the upstream NarL-binding site in the *narG* operon is > 1 μM (Darwin *et al.*, 1996; Walker & DeMoss, 1994) and this is comparable to the values obtained with ModE-molybdate and the *narX*-*narK* intergenic region. ModE-molybdate also protected the same region of DNA when the complementary strand was probed (data not shown). Within the ModE-protected region, the DNA sequence 5' GTTA-7 bp-TACAT 3' was identified. DNase I hypersensitive bases are located outside this ModE-binding sequence motif. These results suggest a conformational change in the DNA due to ModE binding and that ModE-molybdate serves as a molybdate sensor for optimal expression of the *nar* operons by directly interacting with the *narXL* upstream DNA.

DISCUSSION

It is now well established that the *mod* operon transcription is repressed by ModE, which is encoded by the first gene in the divergent *modEF* operon. Repression of *modABCD* is effected by ModE-molybdate binding to the operator region (Anderson *et al.*, 1997; Grunden,

1996; Grunden & Shanmugam, 1997; McNicholas *et al.*, 1997). The results presented in the previous section show that ModE also participates in the regulation of molybdoenzyme synthesis in *E. coli*. Two lines of evidence support this proposal: (1) *modE* mutants produced less than 50% of β -galactosidase activity from either $P_{hyc}lacZ$ or *nar-lacZ*; (2) ModE-molybdate binds to upstream promoter regions of both *hyc* and *narXL* DNA. This requirement for ModE-molybdate is in addition to the appropriate transcriptional activators, FhlA and NarL. However, it should be noted that the *modE* mutants still produced about 40% of the β -galactosidase activity of the *modE*⁺ strain, which was also molybdate-dependent (Table 3). Apparently, a second level of molybdenum-dependent control, besides ModE-molybdate, also exists in the cell. The work by A. Hasona and others (unpublished) identifies the second component of Mo-dependent regulation as the MoeA protein. The MoeA protein was proposed as a molybdenum-activator protein (Hasona *et al.*, 1998); however, neither the chemical nature of the activated molybdenum nor the mechanism by which the MoeA protein contributes to transcriptional activation of *hyc* and *nar* operons is known.

Based on the results presented, a working model is proposed for *hyc* expression, in which ModE-molybdate binds to the DNA upstream of the FhlA-binding site (Fig. 4) and helps to increase the level of transcription activation by FhlA. In a MoeA⁺ strain, the FhlA-formate complex activates the *hyc* operon to a limited extent (by only about 40% of the optimum level in a ModE⁻ mutant) and upon binding of ModE-molybdate to promoter DNA, transcription of the *hyc* operon is enhanced to a higher level. In this proposal, the FhlA protein is still the primary transcriptional activator interacting with the σ^{54} RNA polymerase. The ModE-molybdate, upon binding to DNA, may interact with FhlA, which stabilizes the ModE-molybdate-DNA complex which by itself has a high apparent K_d . In this configuration, the FhlA-formate-ModE-molybdate complex activates transcription of the *hyc* operon to optimum levels.

The ModE protein is also proposed as one of the molybdate sensors in the expression of the *narGHJI*, *narK* and *narXL* operons. The same amount of reduction in nitrate response of the three operons in a *modE* background suggests that the primary site of action of ModE is at the *narXL* operon (Table 6). A lower level of NarL in a *modE* mutant could account for the decrease in expression of the *narGHJI* and *narK* operons. In agreement with this proposed model, ModE did bind to the *narXL* promoter DNA (Fig. 5) but not to *narG* promoter DNA (data not shown). The ModE-protected region in the *narX-K* intergenic region overlaps by 2 bases with one of the two NarL-protected regions (Fig. 6). The second NarL-protected region is centred about 79 bases towards *narK*. Since the contribution of specific DNA in the intergenic region between *narK* and *narXL* in the transcription of the two operons is not known, it is difficult to differentiate the

role of ModE-protected DNA in the activation of either operon. The need for NarXL in the control of *narK* (Bonney & DeMoss, 1992; Kolesnikow *et al.*, 1992) is in agreement with the proposal that the primary target of ModE in the control of *nar* genes is the *narXL* operon. As per this working model, optimum transcription of *narXL* requires NarL-P_i as a primary activator and ModE-molybdate as a secondary activator when *E. coli* is grown under anaerobic conditions with nitrate. Higher intracellular levels of NarX and NarL in the presence of ModE-molybdate, in turn, would increase the response of other *nar* operons to nitrate. In *E. coli* NarQ was found to be interchangeable with NarX in the regulation of the *narGHJI* operon (Kalman & Gunsalus, 1989; Rabin & Stewart, 1993; Schroder *et al.*, 1994). However, expression of *narQ-lacZ* and its cognate response regulator gene, *narP-lacZ*, was found to be independent of ModE-molybdate.

Besides the two operons which were analysed in this study, ModE-molybdate also binds to upstream regions of other operons encoding molybdoenzymes, such as *dmsABC*, as well as to molybdopterin synthesis genes like *moa* (McNicholas *et al.*, 1997, 1998). Although ModE plays a role in the control of the *dmsABC* operon, the ModE protein appears to repress transcription from a downstream promoter, P2 (McNicholas *et al.*, 1998). The close similarity in the sequence in the ModE-molybdate-binding region in the *dmsABC* operon to that of the ModE-repressed operons (*modABCD*) (Fig. 7) also suggests that the *dmsABC* operon is repressed in the presence of excess molybdate. The presence of two promoters and the possible repression by ModE complicates the analysis of the operon encoding the molybdoenzyme DMSO reductase. ModE also binds to the *moa* operon upstream DNA but a need for molybdate in the control of *moa-lacZ* could not be demonstrated (Baker & Boxer, 1991; McNicholas *et al.*, 1997).

Since the ModE-molybdate-binding region in the DNA is known for at least five operons in *E. coli* (*modABCD*, *hyc*, *narX*, *dmsABC* and *moa*; Figs 4 and 6; Anderson *et al.*, 1997; Grunden, 1996; McNicholas *et al.*, 1997, 1998), it is possible to identify a consensus ModE-binding motif in this organism (Fig. 7). The *E. coli modA* operator/promoter DNA protected by ModE-molybdate from DNase I hydrolysis has three pentamer sequences (5' TAYAT 3') (Fig. 7). The pentamer sequences 1 and 2 are separated by seven bases. The K_d for ModE interaction with the *modA* operator DNA was reported to be about 25 nM (Anderson *et al.*, 1997; McNicholas *et al.*, 1997). Tight binding of ModE to DNA to effectively repress transcription may require only the first two pentamer sequences with a spacing of 7 bp, since deleting the third pentamer sequence had a minimal effect *in vivo* on *modA-lacZ* repression (A. M. Grunden, unpublished). Other experiments revealed that the 28271 Da ModE protein binds to DNA as a dimer through the amino acid sequence in the N-terminal part of the protein, which contains a helix-turn-helix motif.

	1	2	3
<i>EcmodA</i> (-)	TCGTATATTTGTCGCC	TACATAACGT	TACAT
<i>AvanfA</i> (-)	GCGTATATAGACATATATATATCGAT		
<i>RcanfA</i> (-)	TCGTATATGGAATCAC	TATATATCGAT	
<i>HimodA</i> (-)	TTCAATATATAATCAAG	TAAATAACGTT	
<i>Ecdms</i> (-?)	TCGATGTATACAAGCC	TATATAGCGAAC	
<i>EcmodA</i> (-?)	TGACGCTATATACATGATT	TACATAGCGAAA	
<i>Echyc</i> (+)	CAGAGGGTTATTTTCGTG	CATATCGCCTC	
<i>EcnaXL</i> (+)	GTGTGGTTAGTCGCTT	TACATCGGTA	
Consensus (negative)	TATAT - 7b- TAYAT		
Consensus (positive)	GTTA - 7b- YAYAT		

Fig. 7. Consensus ModE-molybdate-binding motif in various operons from different organisms. ModE regulates the top four operons negatively (indicated in parentheses). Transcription of *hyc* and *narX* operons are enhanced by ModE (+). *EcmodA*, *E. coli modABCD* (molybdate transport; Grunden & Shanmugam, 1997); *AvanfA*, *Azotobacter vinelandii* alternate nitrogenase (Joerger *et al.*, 1989); *RcanfA*, *Rhodobacter capsulatus* alternate nitrogenase (Kutsche *et al.*, 1996); *HimodA*, *Haemophilus influenzae modABC* (Fleischmann *et al.*, 1995); *Ecdms*, *E. coli* DMSO reductase (McNicholas *et al.*, 1998); *EcmodA*, *E. coli* molybdopterin synthesis (McNicholas *et al.*, 1997); *Echyc*, *E. coli hyc* promoter; *EcnaXL*, *E. coli narXL* promoter. Information on *E. coli modA*, *hyc*, *narX*, *dmsABC* and *moa* is based on DNase I-footprinting data and others are based on DNA sequence. (-?) indicates that the role of ModE is not clearly defined in these two operons but the presence of sequence motifs suggest that it could negatively regulate transcription. The GT sequence flanking the first 5' TATAT 3' sequence in the first three operons is double-underlined to indicate the presence of the 5' GTTA 3' tetramer in these three sequences. Inverted repeat sequence in the *E. coli modA* sequence is indicated by the lines above the sequence. The ModE-molybdate binding motif in the sequences is shown as white on black. Numbers above the *EcmodA* sequence refer to the three pentamer sequences, 5' TATAT 3', 5' TACAT 3' and 5' TACAT 3', respectively. Y in the sequence represents pyrimidine.

The sequence motif, 5' TATAT-7 bp-TACAT 3' (regions 1 & 2), present in the *modABCD* operator DNA (repressed by ModE-molybdate), is also present in the *dmsABC* and *moa* operons. This consensus sequence for ModE-binding in *modA*, *dms* and *moa* (Fig. 7) is a refinement of the consensus sequence proposed for molybdate-sensitive operons from analysis of DNA sequences by Kutsche *et al.* (1996). The operator DNA from three other operons (*AvanfA*, *RcanfA* and *HimodA*) whose control is similar to the *modA* regulation also contains the pentamer sequences 5' TATAT-7 bp-TATAT 3', except *Rhodobacter capsulatus anfa* DNA, in which the spacing is eight bases (Fig. 7; Fleischmann *et al.*, 1995; Joerger *et al.*, 1989; Kutsche *et al.*, 1996). *Haemophilus influenzae* ModE can functionally replace *E. coli* ModE in the repression of *E. coli modA-lacZ* (A. M. Grunden, unpublished) and thus its apparent function in the native organism is also to repress *modABC* operon expression.

The second pentamer sequence found in the *modA* operator DNA is also conserved in the two operons (*hyc* and *nar*; 5' CATAT 3' and 5' TACAT 3', respectively) whose transcription is positively regulated by ModE (Fig. 7). But the first pentamer is replaced by a tetramer sequence, 5' GTTA 3', with the same spacing of 7 bp. The inverted repeat found in the operator regions of the ModE-repressed operons is also absent in the *hyc* and *narXL* operons. The tetramer sequence 5' GTTA 3' is

also present, and repeated several times, in the *E. coli modA* operator region (Fig. 4; Grunden & Shanmugam, 1997) and may play a critical role in the initial recognition of DNA by ModE-molybdate. Taken together, for high-affinity binding of ModE to DNA, as in the case of repression, the two pentamer sequences appear to be critical. Replacing the first pentamer with a tetramer sequence, 5' GTTA 3', apparently reduces the affinity of ModE to DNA, as would be needed for operons whose transcription is only enhanced by ModE-molybdate (*hyc* and *nar*).

In summary, ModE-molybdate is one of two regulatory proteins positively regulating the expression of the *hyc* and *nar* operons in response to molybdate availability. The promoter DNA to which ModE-molybdate is binding in the two operons could be defined as an enhancer element and the ModE-molybdate as a secondary activator (enhancer DNA-binding protein).

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