The *cis*-Acting Regulatory Element of the *mvaAB* Operon of *Pseudomonas mevalonii*[†]

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DNA upstream of the transcription start site of the *mvaAB* operon of *Pseudomonas mevalonii*, which encodes 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.88) and HMG-CoA lyase (EC 4.1.3.4), contains a *cis*-acting regulatory element which functions in the response to mevalonate. The regulatory element resides within a 36-bp region located from 48 to 84 bp upstream of the transcription start site of *mvaA*. This location was inferred from the β -galactosidase activities of *P. mevalonii* harboring plasmid-encoded *mvaA-lacZ* fusions induced by mevalonate and by DNA gel retardation and competition assays. While protein from *P. mevalonii* grown on mevalonate produced a band shift, protein from cells grown on succinate gave no band shift, even when mevalonate was added. The operator contains three 10-bp direct repeats with the consensus sequence TGGGTACAGT, which may be important for regulation of the *mvaAB* operon.

Mevalonate (3,5-dihydroxy-3-methylpentanoate) was initially isolated as a growth-stimulating factor for *Lactobacillus acidophilus* (23). In eukaryotes, mevalonate is converted to diverse isoprenoids, which include sterols, ubiquinone, isopentenyl adenine, and the farnesyl substituents of certain proteins. Several bacteria can utilize mevalonate as the sole source of carbon for growth (4, 10, 13, 23). Growth of *Pseudomonas mevalonii* on mevalonate induces synthesis of an active transport system specific for *R*-mevalonate (12) and an NAD⁺-dependent 3-hydroxy-3-methylglutaryl (HMG) coenzyme A (CoA) reductase that converts *R*-mevalonate to *S*-HMG-CoA (13). Following HMG-CoA lyase-catalyzed cleavage of HMG-CoA to acetoacetate and acetyl-CoA (22), acetoacetate forms acetyl-CoA (21). *P. mevalonii* HMG-CoA reductase (13, 14) and HMG-CoA lyase (22) have been purified and characterized, and the genes *mvaA* (3) and *mvaB* (1) of the *mvaAB* operon that encode these enzymes have been cloned and sequenced. *mvaA*, which encodes HMG-CoA reductase, is the promoter-proximal gene. The promoter of the *mvaAB* operon has a -12, -24 consensus sequence (27) which in other bacteria is recognized by RNA polymerase holoenzymes which use factor σ^{54} (9). Gene transcription of this class of promoters has been proposed to be under positive control (16). We report here that a 36-bp region 48 bp upstream of the transcription start site of *mvaAB* contains a *cis*-acting element which responds to induction by mevalonate.

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MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophage, and culture conditions. Plasmids and phage M13 were propagated in *Escherichia coli* JM101 (8, 17). *P. mevalonii* was grown on ionic medium (12) containing the indicated sources of carbon and nitrogen. *E. coli* CSH26 harboring plasmid RP4 (2, 11) was grown on M9 medium (17) supplemented with 1 mM proline,

50 μ g of kanamycin per ml, and 25 μ g of tetracycline per ml. Derivatives of phage M13mp19 and plasmids pMC1403 and pKT231 have been previously described (2, 7, 18). Additional molecular biology methods are described by Maniatis et al. (17) and/or Davis et al. (8).

Construction of plasmid pWR449. A 762-bp *Pst*I-*Pvu*I fragment extending from positions -449 to +313 (base pairs are numbered relative to the transcription initiation site at +1) of *mvaAB* was inserted into the *Sma*I site of pMC1403 to give pYW449. That the *mvaA-lacZ* junction was fused in frame was confirmed by sequencing. The *Eco*RI-*Sal*I fragment of pYW449, which contained both the *mvaAB* operator-promoter and the *mvaA-lacZ* fusion gene, was then inserted into the *Eco*RI-*Sal*I sites of broad host range plasmid pKT231 to give plasmid pWR449.

Construction of *mvaAB* operator-promoter deletions fused to *lacZ*. The 0.76-kb *Eco*RI-*Bam*HI fragment of pYW449 was inserted into M13mp19, yielding M13mp19-449. Nested deletions were then prepared with an IBI Cyclone kit. Transformants were screened to determine approximate insert sizes, and selected deletions were also sequenced. *mvaAB* operator-promoter deletions were fused to *lacZ* by the strategy described above for construction of pWR449. pWR expression plasmids are named for the number of base pairs present upstream of the transcription initiation site.

Mobilization of broad host range plasmids into *P. mevalonii* and assay of β -galactosidase activity. *P. mevalonii* lacks detectable β -galactosidase activity, is permeable to mevalonate, and contains all factors required for recognition of the *mvaAB* operator-promoter. pWR plasmids were therefore mobilized from *E. coli* into *P. mevalonii* by triparental conjugation (2, 11). Assays of β -galactosidase activity were conducted as described by Miller (19) but with correction of the data to a sample volume of 1.0 ml.

Preparation of protein extracts for DNA gel retardation assays. Mid-log-phase cells grown on either *R,S*-mevalonate or succinate were harvested by centrifugation, washed with 0.9% NaCl, suspended in 2.5 ml of 100 mM KCl–1 mM EDTA–1 mM dithiothreitol–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate), pH 8.0, and passed twice through a French pressure cell at 10,000 lb/in². The cell lysate was centrifuged (12,000 $\times$ g, 4°C, 30 min), adjusted to a glycerol concentration of 20% (vol/vol), and stored in

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liquid N₂. Protein was determined with the Bio-Rad Bradford reagent (5).

Probes and competitor DNAs for DNA gel retardation assays. Four probes and seven competitor DNAs were prepared by restriction enzyme digestion of selected M13mp19-449 deletion fragments or by *SalI-HindIII* digestion of pHMGR (15). Following electrophoresis on 2% agarose, appropriately sized fragments were electroeluted in 4.5 mM Tris–4.5 mM borate–0.1 mM EDTA (pH 8.0). After extraction with phenol-chloroform, precipitation with ethanol, and vacuum drying, the amount of DNA present was determined spectrophotometrically at 260 nm. To prepare radioactive probes (specific activity, 2 to 5 cpm/pmol of DNA), DNA fragments were digested with alkaline phosphatase and then labeled with T4 polynucleotide kinase and [γ -³²P]ATP (17).

DNA gel retardation assays. Gel retardation assays were performed essentially as described by Strauss and Varshavsky (25). The best results were obtained with high-ionic-strength buffer (24). Reaction mixtures contained, in a final volume of 20 μ l, 10 mM HEPES (pH 8.0), 150 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 2.5 mM MgCl₂, 10% (vol/vol) glycerol, 5 to 20 μ g of protein, 1,000 cpm (approximately 0.07 ng) of ³²P-end-labeled probe DNA, and 1 μ g of poly(dI-dC). Samples were applied to a 4% polyacrylamide gel (acrylamide-bisacrylamide ratio, 80:1) cast in, and previously electrophoresed in, 1 mM EDTA–2.5% (vol/vol) glycerol–50 mM Tris–380 mM glycine, pH 8.5 (24). Following addition of fresh buffer, samples were subjected to electrophoresis for an additional 2 h. Gels were fixed in 10% methanol–10% acetic acid for 10 min, dried on Whatman 3 MM paper, and exposed to X-ray film.

RESULTS

The region upstream of the transcription initiation site contains a *cis*-acting element whose integrity is required for regulation of the *mvaAB* promoter. We employed β -galactosidase as a reporter enzyme to locate *cis* elements of the *mvaAB* operon responsible for induction by mevalonate. Following exposure to mevalonate, the β -galactosidase activity of cells harboring plasmid pWR449 rose 1,800-fold (Table 1). This indicated that the sequence from –449 to +313 bp contains a *cis*-acting element that is required for regulation of the *mvaAB* operon.

Base pairs –84 to –48 are essential for the response to mevalonate. Operator-promoter deletion constructs were assayed for the ability to respond to mevalonate by assay of β -galactosidase activity. Plasmids containing 118 to 273 bp upstream of the transcription start site responded to mevalonate in a manner comparable to that of the parent plasmid, pWR449. β -Galactosidase activities for plasmids pWR106 and pWR84 were, however, three- to sixfold lower. Plasmids containing less than 84 bp were apparently noninducible (Table 1). The 36-bp fragment extending from –84 to –48 thus appears to be essential for the response to mevalonate.

Inducer specificity of the *mvaAB* operator-promoter. Although intact cells respond to mevalonate, the physiologic inducer might be a mevalonate catabolite rather than mevalonate per se. Since intact cells are impermeable to CoA thioesters, the anions of the free acids of the mevalonate catabolites HMG-CoA, acetoacetyl-CoA, and acetyl-CoA were tested for the ability to induce transcription of *mvaA-lacZ* fusion plasmid pWR449. The experiment, conducted essentially as described in Table 1, footnote a, used the ammonium salts of the indicated compounds. β -Galactosi-

TABLE 1. Ability of promoter deletions to respond to mevalonate by inducing production of β -galactosidase^a

Plasmid ^b	Miller U of β -galactosidase activity with the following inducer:		Fold induction
	None	Mevalonate	
pWR449	12	21,600	1,800
pWR273	66	66,800	1,010
pWR172	48	58,800	1,230
pWR118	42	51,900	1,240
pWR106	108	37,400	345
pWR84	58	11,800	202
pWR48	200	325	1.6
pWR18	430	295	<1

^a Cultures of *P. mevalonii* harboring the indicated plasmids were grown, with shaking at 30°C, in 250-ml sidearm flasks containing 15 mM ammonium succinate and 50 μ g of kanamycin per ml in 15 ml of ionic medium. When cultures had attained densities of approximately 250 μ g of dry cells equivalent per ml (100 Klett units), water or ammonium *R,S*-mevalonate (final concentration, 8.3 mM) was added and shaking was continued. Cells removed during the early stationary phase (up to 3 h after addition of the inducer) were assayed for β -galactosidase activity. The data are mean values for duplicate or triplicate (pWR18) experiments.

^b pWR expression plasmids are named for the number of base pairs present upstream of the transcription initiation site.

dase activities, expressed in Miller units, were 7 with no inducer, 15,000 with 8.3 mM *R,S*-mevalonate, 308 with 5.8 mM acetoacetate, 13 with 8.3 mM 3-hydroxy-3-methylglutarate, and 16 with 12.3 mM acetate. Thus, only mevalonate and acetoacetate induced expression of β -galactosidase. The 44-fold induction produced by acetoacetate was, however, only 2% of that produced by mevalonate.

Band shift experiments indicate that a protein(s) binds to the *cis*-acting element. The in vivo experiments described above implicated a 36-bp segment of DNA extending from –84 to –48 bp as important for the response to mevalonate. Since *trans*-acting proteins may bind to this region, gel retardation experiments were next conducted. A ³²P-end-labeled 325-bp DNA fragment containing the 36-bp region was mixed with extracts of cells grown on either mevalonate or succinate and incubated at room temperature for 20 min. Portions were then subjected to native polyacrylamide gel electrophoresis, and the resulting patterns were detected by autoradiography. While cytosol from mevalonate-grown cells produced a band shift (Fig. 1, lane X), cytosol from succinate-grown cells, either with or without mevalonate, gave no band shift (data not shown). Mevalonate-grown cells thus appear to contain a factor(s), either absent from or inactive in succinate-grown cells, that can bind to the 325-bp probe DNA.

To investigate the specificity of the DNA-protein interaction, unlabeled competitor DNA fragments which contained or lacked the 36 bp of interest were incubated with the 325-bp probe DNA in the presence of cytosol from mevalonate-grown cells. All DNA fragments that contained a portion of the 36-bp region competed with probe DNA for protein binding (Fig. 1, lanes A, B, F and G). DNA fragments that failed to compete (Fig. 1, lanes C and D) or competed only partially (Fig. 1, lane E) lacked the 36-bp region.

Gel retardation experiments using shorter probes confirmed the specificity of the DNA-protein interaction (Fig. 2). While probe B, which extends from –84 to +52 bp, forms DNA-protein complexes similar to those formed by the 325-bp DNA probe, probes shorter than B at their 5' end

FIG. 1. Competition data support the binding specificity of the 36-bp DNA fragment. The 325-bp region extending from -273 to +52 served as the labeled probe for band shift analysis. (a) Shown is a portion of the *mvaAB* operon. This includes the region upstream of *mvaA* (white), the 36-bp region (black), the *mvaA* gene (shaded), and the region downstream of *mvaA* (cross-hatched). Also shown are the lengths and relative positions of the radioactive probe (probe) and competitor DNAs (A through G), which contained the following numbers of base pairs: A, 224; B, 170; C, 576; D, 331; E, 361; F, 136; and G, 158. (b) All lanes contained the radioactive DNA probe. Cytosol, derived from mevalonate-grown cells, was present in all lanes except Y. Lanes A through G contained the indicated competitor DNAs, present in approximately 2,000-fold molar excess over the radioactive probe. Lanes X and Y lacked competitor DNA. The experiment was conducted as described in Materials and Methods.

either did not form (E) or only partially formed (C or D) DNA-protein complexes (Fig. 2).

The 36-bp DNA fragment contains three 10-bp direct repeats. Structure analysis revealed three 10-bp direct repeats present wholly or partially within the 36-bp region extending from -73 to -43 bp upstream of the translation initiation site. Base pair +1 is the transcription initiation site, not the translation start site as previously numbered (27). The two more highly conserved repeats lie entirely within the 36-bp region (Fig. 3). The third repeat is less conserved, and only its conserved TGGG sequence falls within the 36-bp region.

DISCUSSION

A *cis*-acting regulatory element for the *mvaAB* operon is located within a 36-bp region that extends from 48 to 84 bp upstream of the transcription initiation site. This was shown initially by the ability of mevalonate to induce β -galactosidase activity in *P. mevalonii* strains harboring *mvaA-lacZ* fusion plasmids. Following exposure of intact cells to mevalonate, β -galactosidase activity rose 1,800-fold, a rise comparable to the 200- to 800-fold induction of HMG-CoA reductase typical of cells grown on mevalonate (13). The magnitude of the induction emphasizes the strength of the *mvaAB* operator-promoter, a property which may ultimately

FIG. 2. DNA binding to protein requires the 36-bp element. (a) Shown are the lengths of the five ³²P-labeled probes used for gel retardation analyses. The dark box highlights the 36-bp region extending from -84 to -48 bp. The probes contained the following numbers of base pairs: A, 325; B, 136; C, 111; D, 100; and E, 70. (b) Lanes marked with a plus sign contained cytosol from mevalonate-grown cells, and those marked with a minus sign did not. The experiment was conducted as described in Materials and Methods.

prove useful for controlling the expression of cloned genes in pseudomonads.

Promoters recognized by the σ^{54} holoenzyme typically are subject to positive regulation via *cis*-acting regulatory elements located approximately 100 bp upstream of the transcription start site (6, 16, 20). Since deletion of the -118 to -84 bp segment reduced induction three- to sixfold (Table 1), this region may contain additional regulatory elements that complement that located at -84 to -48 bp.

The ability of cell protein to retard DNA mobility differed both qualitatively and quantitatively for probes deleted to various extents at their 5' ends. As judged by gel retardation analysis, only DNA deleted to bp -18 failed to bind protein (Fig. 2, lane E+). That DNA probes truncated at bp -48 or -59 bound protein was apparent from the single shifted band (Fig. 2, lanes D+ and C+, respectively). Furthermore, the relative intensities of this band in lanes C+ and D+ suggest the presence of a protein-binding site with a major portion present between bp -59 and -48 but extending beyond bp -48. Probes containing the entire 36-bp segment implicated in Table 1 also bound protein strongly but gave two shifted bands, suggesting a second protein-binding site. Whether these putative sites bind the same or different proteins cannot, however, be inferred from these data.

The ability of cytosol from *P. mevalonii* grown on mevalonate to produce a band shift implies the existence of a *trans*-acting protein that binds to the *cis*-regulatory element.

FIG. 3. Sequence of the 36-bp DNA fragment between 48 and 84 bp upstream of the transcription initiation site.

The inability of cytosol from cells grown on succinate to produce a comparable shift, even in the presence of added mevalonate, implies that this putative *trans*-acting factor is itself inducible by mevalonate. Alternatively, succinate-grown cells may lack an unidentified mevalonate metabolite which is the true physiologic inducer. However, a survey of three compounds related structurally or metabolically to mevalonate failed to identify any inducer as effective as mevalonate. Only acetoacetate, a mevalonate catabolite and an inhibitor of both HMG-CoA reductase (13) and mevalonate transport (12), induced β -galactosidase in strains bearing *mvaA-lacZ* fusion plasmids. The level of induction was, however, only 2% of that produced by mevalonate.

Three 10-bp imperfect direct repeats are present partially or totally within the 36-bp fragment. While most prokaryotic operators contain regulatory elements with palindromic sequences (6, 20), *cis*-acting elements which contain direct repeats include *ompF* and *ompC* (26). The direct repeats of the *mvaAB* operator thus may ultimately prove important for induction by mevalonate.

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