Expression and Characterization of a Structural and Functional Domain of the Mannitol-Specific Transport Protein Involved in the Coupling of Mannitol Transport and Phosphorylation in the Phosphoenolpyruvate-Dependent Phosphotransferase System of *Escherichia coli*†

G. T. Robillard,* H. Boer, R. P. van Weeghel,‡ G. Wolters, and A. Dijkstra

The Institute BIOSON, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

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ABSTRACT: The mannitol-specific transport protein in *Escherichia coli*, EII^{mtl}, consists of three structural and functional domains: a hydrophilic EIII-like domain (the A domain); a hydrophobic transmembrane domain (the C domain); and a second hydrophilic domain (the B domain) which connects the A and C domains together. The A domain contains the first phosphorylation site, His554, while the B domain contains the second phosphorylation site, Cys384. The phosphoryl group which is needed for the active transport of mannitol is sequentially transferred from P-enolpyruvate via the two phosphorylation sites to mannitol bound to the substrate binding site. In this paper, the expression, purification, and initial characterization of the B domain, IIB^{mtl}, are described. Oligonucleotide-directed mutagenesis was used to produce an amber stop codon (TAG) and *Hin*dIII restriction site in a flexible loop between the B and A domains in the subcloned gene fragment coding for $IIBA^{mtl}$ (van Weeghel et al., 1991c). The gene fragment coding for IIB^{mtl} was then subcloned behind strong promoters, located in two different expression/mutagenesis vectors, which directed the expression of the 15.3-kDa polypeptide in *Escherichia coli*. The domain was purified from *E. coli* crude cell extracts by using Q-Sepharose Fast Flow, S-Sepharose Fast Flow, and hydroxylapatite column steps. This purification procedure resulted in 1 mg of pure IIB^{mtl}/g of cell, wet weight. The purified B domain was analyzed *in vitro* for its catalytic activity with membranes containing the phosphorylation site mutant form of EII^{mtl}, C384S, and with the transmembrane domain, IIC^{mtl}. The B domain, together with purified IIA, was able to restore the P-enolpyruvate-dependent phosphorylation activity of the membrane-bound C domain. Steady-state mannitol phosphorylation kinetics at saturating EI, HPr, and IIA^{mtl} yielded an apparent K_m of P-IIB^{mtl} for IIC^{mtl} of 200 μM and an apparent V_{max} of 71 nmol of mtl-P min^{-1} mg of membrane protein)$^{-1}$. This V_{max} value is comparable to that of wild-type EII^{mtl} measured under the same experimental conditions.

The uptake and phosphorylation of the carbohydrate substrate mannitol in *Escherichia coli* are catalyzed by the mannitol-specific transport protein enzyme II (EII^{mtl})[1] of the phosphoenolpyruvate-dependent phosphotransferase system (PTS)[1] [for reviews, see Postma and Lengeler (1985), Meadow et al. (1990), and Lolkema and Robillard (1992)]. The mannitol transport protein is localized in the cytoplasmic membrane. The cytoplasmic portion of the enzyme possesses two active-site residues, His554 and Cys384, which become phosphorylated during turnover (Pas et al., 1988; Pas & Robillard, 1988a,b). Sequence comparisons of several sugar transport proteins (Saier et al., 1988; Ebner & Lengeler, 1988) and extensive biochemical, genetic engineering, and kinetic studies have revealed that EII^{mtl}, like many of the other enzyme II's or enzyme II/III pairs of the PTS, consists of three structural and functional domains. There is a hydrophilic enzyme III-like domain, IIA^{mtl}, containing the essential His554 as the first phosphorylation site, a second hydrophilic domain, IIB^{mtl}, which contains the Cys384 as the second phosphorylation site, and a hydrophobic transmembrane domain, IIC^{mtl}, which is able to bind and transport the mannitol (Grisafi et al., 1989; Stephan & Jacobson, 1986; Stephan et al., 1989; White & Jacobson, 1990; van Weeghel et al., 1991a,b,c; Lolkema et al., 1990, 1991a,b). The phosphoryl group derived from P-enolpyruvate is transferred via enzyme I and HPr to His554 on the A domain, which in turn phosphorylates Cys384 located on the B domain. This latter active-site residue is involved in the phosphorylation of mannitol bound to its binding site on the C domain. Recent kinetic studies have established that the transmembrane C domain constitutes the mannitol translocator. Dephosphorylated EII^{mtl} and the subcloned IIC^{mtl} are able, under certain circumstance, to catalyze a slow facilitated diffusion of mannitol (Lolkema et al., 1990). Furthermore, evidence has been accumulating that, in terms of the catalytic mechanism, the translocation and phosphorylation activities are separable functions localized on different domains. Lolkema et al. (1991a,b) have demonstrated that

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* Correspondence should be addressed to this author.

‡ Present address: Mount Sinai Hospital, 600 University Ave., Toronto, Ontario, Canada, M5G 1X5.

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[1] Abbreviations: NEM, *N*-ethylmaleimide; PTS, phosphoenolpyruvate-dependent phosphotransferase system; EII^{mtl}, mannitol-specific enzyme II; IIA^{mtl}, A domain of the mannitol-specific enzyme II; IIB^{mtl}, B domain of the mannitol-specific enzyme II; IIC^{mtl}, C domain of the mannitol-specific enzyme II; $IIBA^{mtl}$, BA domain of the mannitol-specific enzyme II; EI, enzyme I of the phosphoenolpyruvate-dependent carbohydrate transport system; DTT, dithiothreitol; ISO, inside-out; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NMR, nuclear magnetic resonance; OPA, *o*-phthalaldehyde; FMOC, 9-fluorenylmethyl chloroformate; PMSF, phenylmethanesulfonyl fluoride; DOC, deoxycholate; dPEG, decylpoly(ethylene glycol); CAT, chloramphenicol acetyltransferase.

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phosphorylation of the enzyme modulates the activity of the translocator domain; phosphorylation of Cys384 causes at least a 1000-fold stimulation of the translocation rate by a conformational coupling between the B and C domains. Studies of the structures and activities of the individual domains should, therefore, provide important insights into how the individual domains modulate the transport and phosphorylation activities.

In order to carry out such structure–function studies, several different expression-coupled mutagenesis phagemid systems were constructed (van Weeghel et al., 1990, 1991a,b,c). These expression vectors enabled us to make site-directed mutations for easy subcloning of gene fragments coding for different EII^{mtl} domains, IIA^{mtl}, $IIBA^{mtl}$, and IIC^{mtl}, to overexpress these domains, and to produce several important phosphorylation site mutants which were used in kinetic studies (van Weeghel et al., 1991a; Lolkema et al., 1991b) and in complementation assays with expressed domains (van Weeghel et al., 1991b,c). This report describes the mutagenesis, overexpression, and purification of IIB^{mtl}, together with kinetic evidence that this domain is enzymatically active.

MATERIALS AND METHODS

Materials. Restriction endonucleases, DNA polymerase Klenow fragment, T4 DNA ligase, and T4 polynucleotide kinase were obtained from Boehringer Mannheim. M13KO7 helper phage was purchased from Pharmacia. Q-Sepharose Fast Flow and S-Sepharose Fast Flow were from Pharmacia, and hydroxylapatite was from Bio-Rad. Nitrocellulose filters were from Schleicher & Schuell. The four synthetic oligonucleotides (R1, R7, R19 amber, and R23 obtained from Eurosequence BV Groningen) were prepared on an Applied Biosystems Model 380B DNA synthesizer, completely deprotected, and used unpurified. Enzyme I and HPr were purified as described (Dooijewaard et al., 1979; Van Dijk et al., 1990). D-[1-^{14}C]Mannitol (59 mCi/mmol; 1 mCi = 37 MBq) was purchased from the Radiochemical Centre Amersham. The nonionic detergent decyl-PEG was obtained from B. Kwant (Department of Chemistry, University of Groningen). IgG–horseradish peroxidase conjugate and reagents for color development were purchased from Bio-Rad. Phenylmethanesulfonyl fluoride (PMSF) was obtained from Serva. Other biochemicals were of analytical grade.

Bacterial Strains, Plasmids, and Growth Conditions. E. coli strains ASL-1 [F$^-$, *lac*Y1, *gal*T6, *xyl*-7, *thi*-1, *his*G1, *arg*G6, *met*B1, *rps*L104, *mtl*A2, *gut*A50, *gat*A50 (MAL$^+$) recA], LJ1008-11-3 [C600; F$^-$, *lac*Y, *gal*T6, *xyl*-7, *thi*-1, *his*G1, *arg*G6, *met*B1, *rps*L104, *mtl*A2, *gut*A50, *gat*A50, *rec*A, (Mal$^+$)] (Van Weeghel et al., 1990), and JM101 [Δ(*lac-pro*AB), *thi*; F′,*tra*D36, *pro*AB, *lac*I^{q}ZΔM15] (Yanish-Perron et al., 1985) were used for plasmid propagation and as host strains for protein expression. *E. coli* strains BMH 71-18 MutL, [Δ(*lac-pro*AB), *thi*, *sup*E; F′ *lac*I^{q}ZΔM15, *pro*A$^+$B$^+$] (Carter et al., 1985) and HB2154 [Δ(*lac-pro*AB), *thi*, *ara*, *phx*, *mut*L::Tn10 (Tc, 10 μg/mL); F′, *pro*A$^+$B$^+$, *lac*I^{q}Z$^-$ΔM15] (Kramer et al., 1984) were both used for oligonucleotide-directed mutagenesis. *E. coli* BW313 [*dut*, *ung*, *rel*A, *spo*T1, F′*lys*A] was used to produce uracil-containing single-stranded DNA for the Kunkel mutagenesis. Phagemid pMcCIIIi was obtained as described (van Weeghel et al., 1991c) and used as the parental vector in the Kunkel mutagenesis procedure (Kunkel, 1985). The expression phagemid pMcCl was obtained as described (van Weeghel et al., 1991b). Strains harboring different plasmids or phagemids were grown in 2 × TY broth (16 g of Bacto-tryptone, 10 g of yeast extract, and 5 g of NaCl in 1 L) supplemented with the appropriate antibiotics. McConkey indicator plates (agar base, Difco) containing 1% D-mannitol were prepared as indicated by the supplier.

Site-Directed Mutagenesis and Plasmid Constructions. Standard recombinant DNA techniques were performed according to Sambrook et al. (1989). Oligonucleotide-directed mutagenesis using single-stranded pMcCIIIi and pMcCII DNA was done according to the Kunkel mutagenesis protocol, by using single-stranded uracil-containing DNA (Kunkel, 1985). The mismatch oligomer R19amber, 5′-GAACAG-GTTAAGCTTGGACTAGTCAAAGCT-3′, was constructed to introduce an amber stop codon and a new *Hin*dIII restriction site in vector pMcCIIIi. Plasmid pMcCIIIi, which directed the overexpression of the complete $IIBA^{mtl}$, was used as the starting plasmid for vector constructions (van Weeghel et al., 1991c). This particular expression vector contained the B domain in a translational fusion behind the strong λ P_R promoter (Davison et al., 1987). Plasmid DNA was prepared from 18 colonies and digested with *Hin*dIII restriction enzyme. The mutant plasmid pMcCIIIam, containing the new *Hin*dIII site, gave two fragments of approximately 700 bp upon restriction analysis. The parental vector pMcCIIIi showed only the expected 1.4-kb fragment (Figure 1). Subcloning of the *Hin*dIII fragment containing IIB^{mtl}, fused to the ribosomal binding site (RBS) and ATG start codon of the *cro* gene downstream of the P_R promoter, was done into the parental phagemid pMc5-8 (Stanssens et al., 1989) to delete the A domain. The *Hin*dIII fragments were excised from vector pMcCIIIam using *Hin*dIII restriction enzyme, gel-purified, and ligated into the vector pMc5-8 linearized with the same enzymes. The ligation mixture was transformed into JM101 and plated on 2 × TY plates containing chloramphenicol (25 μg/mL). The orientation of the correct insert in the recombinant plasmid pMcCII was identified by digesting isolated plasmid DNA with *Hin*dIII and *Sna*BI (Figure 1). Vector pMcCII was subsequently transformed into different *E. coli* strains, ASL-1, LJ1008-11-3, and JM101, to test the constitutive expression of the B domain from the strong P_R promoter. Due to this *Hin*dIII cloning procedure, part of the thermolabile *c*I857 gene in pMcCII was deleted, which would result in constitutive expression of IIB^{mtl} from the unrepressed promoter. In order to test IIB^{mtl} expression from another strong promoter, DNA coding for the B domain was cloned downstream of the P_{trc} promoter situated in pMcCI (Figure 1). This promoter was used before to overexpress the A domain to very high levels in *Escherichia coli* (van Weeghel et al., 1991b).

Plasmid pMcCII was also used to transform *E. coli* BW313 in order to produce and isolate uracil-containing single-stranded DNA (ss-UDNA). In combination with the mutagenic oligomer R23, 5′-GAGGATCCATGGAACCT-CC-3′, the ss-UDNA was used to generate a unique *Nco*I site around the ATG initiation codon of IIB^{mtl} (Figure 2). The ligation mixture was transformed into competent *E. coli* cells after the site-directed mutagenesis reaction had been completed. After selection on 2 × TY plates containing chloramphenicol, plasmid DNA was prepared from randomly picked colonies and tested for the presence of a unique *Nco*I restriction site. A new *Nco*I was generated by changing nucleotides −1 (T → C) and −2 (G → C) upstream of the ATG codon (Figure 2D). The resulting plasmid, pMcCIINcol containing the *Nco*I mutation, was digested with the restriction enzymes *Nco*I and *Hin*dIII. The excised gene fragment encoding IIB^{mtl} was gel-purified and ligated into pMcCI DNA that had been linearized

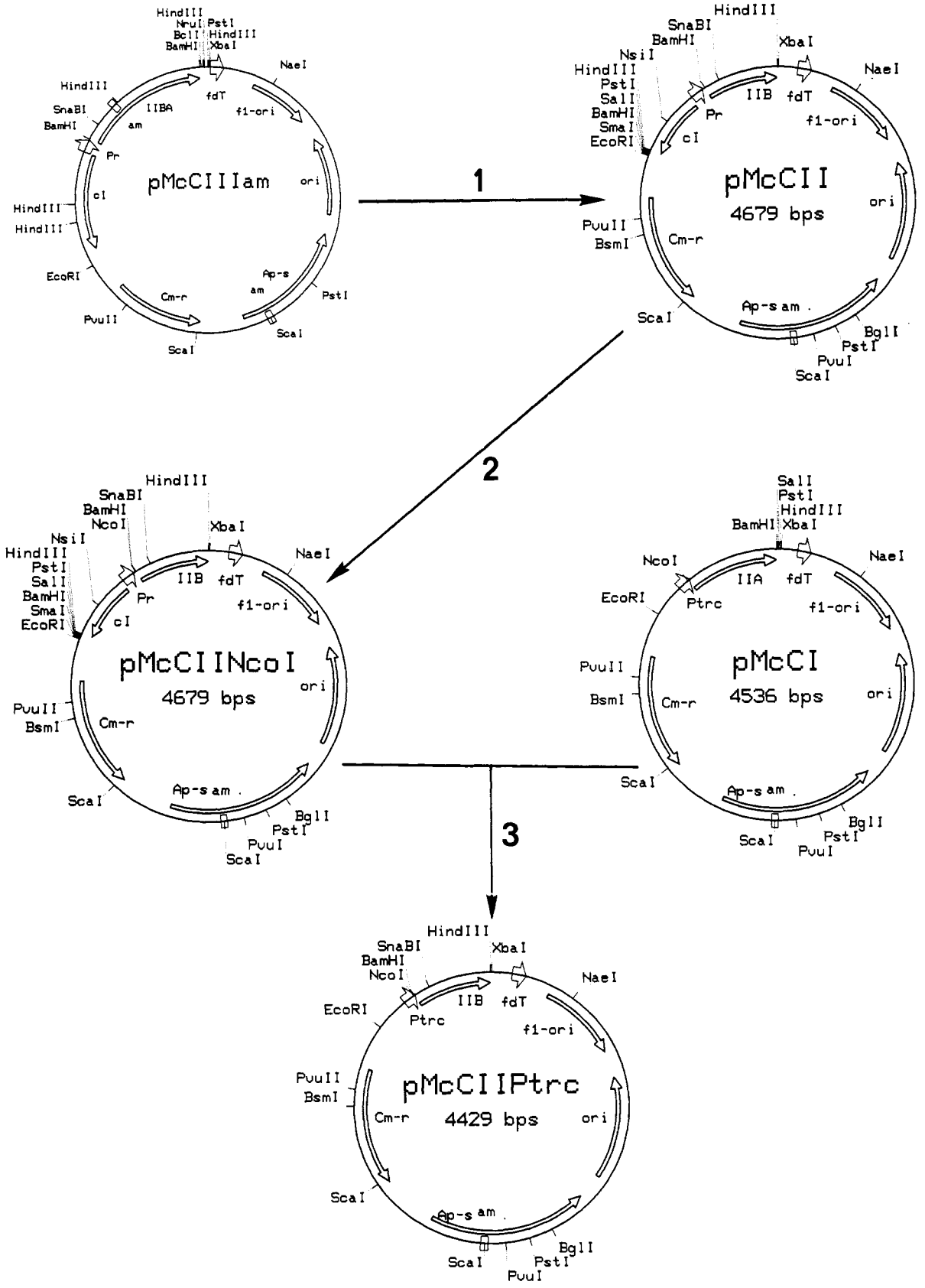

FIGURE 1: Construction of IIB^{mtl} expression vectors. (1) Site-directed mutagenesis was used in combination with primer R19am to introduce an amber stop codon (TAG) and a *Hin*dIII site in pMcCIIIi, the parental plasmid for expression of the $IIBA^{mtl}$ domain. The resulting vector, pMcCIIIam, was used to construct the vector pMcCII for IIB^{mtl}. (2) Single-stranded pMcCII and R23 were used to create a unique *Nco*I site with an internal ATG initiation codon. (3) The mutant vector was named pMcCIINcoI and digested with the restriction enzymes *Nco*I and *Hin*dIII. The excised DNA fragment was used to construct a fusion between the IIB^{mtl} domain-encoding region and the strong P_{trc} promoter in pMcCI, which resulted in the recombinant expression plasmid $pMcCIIP_{trc}$.

with the same restriction enzymes. The resulting recombinant vector, $pMcCIIP_{trc}$, now contained IIB^{mtl} as a translational fusion with the *lac* RBS and ATG start codon just behind the P_R promoter. The expression/mutagenesis vectors $pMcCIIP_{trc}$

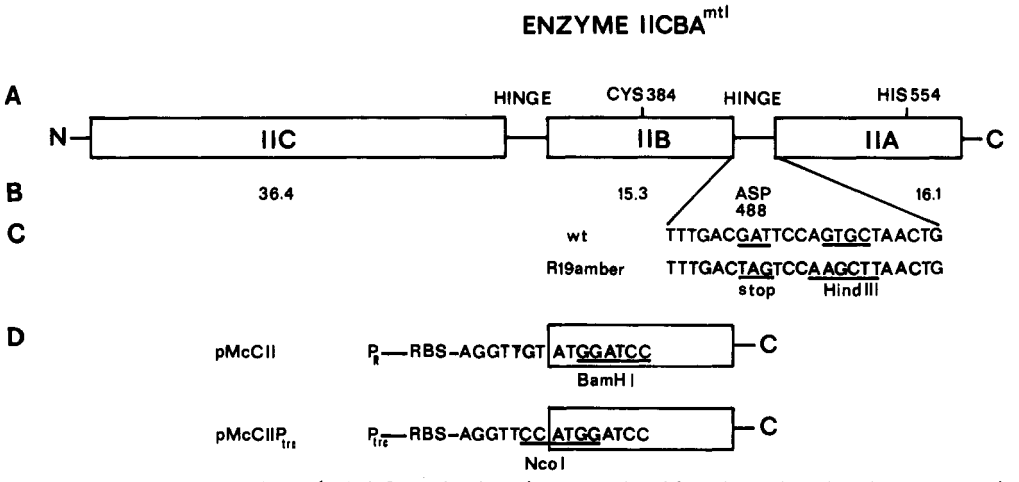

FIGURE 2: Schematic domain organization of EII^{mtl}. (A) Organization of structural and functional domains along the EII^{mtl} polypeptide chain connected by hinge regions. The two sites of phosphorylation are indicated by His554 on IIA^{mtl} and Cys384 on IIB^{mtl}. (B) Calculated molecular weights ($\times 10^{-3}$) of the different domains. (C) The flexible hinge region downstream of IIB^{mtl} was used to create an amber stop codon (TAG) and a *Hin*dIII site. (D) Translational in-frame fusion of the IIB^{mtl} DNA sequence with the P_R promoter in vector pMcCII, and with the P_{trc} in vector pMcCIIP$_{trc}$.

and pMcCII were both used to obtain high levels of expression of IIB^{mtl} in *E. coli*. The actual efficiency of mutagenesis obtained with the Kunkel method was 50%.

The entire gene encoding the B domain in pMcCII was sequenced by the dideoxynucleotide sequencing method (Sanger et al., 1977) using synthetic primers complementary to appropriate regions along the gene. The sequence was identical to that of Lee and Saier (1983) for the same portion of the gene.

ISO Membrane Vesicles. ISO vesicles were prepared from *E. coli* cells (ASL-1 and LJ1008-11-3) expressing the two EII^{mtl} phosphorylation site mutants (EII^{mtl}–C384S and EII^{mtl}–H554A) or the transmembrane C domain as described (van Weeghel et al., 1991a,c). The cells were pelleted by centrifugation, resuspended in buffer (25 mM Tris-HCl, pH 7.6, and 1 mM DTT at 4 °C), and lysed by passage through a French pressure cell at 10 000 psi. The membrane fraction was prepared as previously described (van Weeghel et al., 1990). Aliquots of 50–100 μL were stored at –80 °C.

Protein Concentration. Protein concentrations of the purified IIB^{mtl} or pooled peak fractions after column chromatography were determined by the method of Bradford (1976) using BSA as a standard. The protein concentration, in terms of the number of phosphorylatable active sites, was determined by the pyruvate-burst method (Robillard & Blaauw, 1987). The concentration of EII–C384S (0.68 μM), EII–H554A (2.8 μM), and IIC^{mtl} (3 μM) in the ISO vesicles was determined from mannitol binding data measured with flow dialysis as previously described (Lolkema et al., 1990).

Purification of EII^{mtl}–C384S. ISO vesicles of *E. coli* ASL-1 expressing EII^{mtl}–C384S were resuspended after ultracentrifugation in 25 mM Tris-HCl, pH 8.4, 5 mM DTT, and 1 mM NaN_3 to a concentration of 1 mL/g of starting cells. The vesicles solution was added dropwise to an extraction buffer at 25 °C consisting of 20 mM Tris-HCl, pH 8.4, 50 mM NaCl, 1 mM NaN_3, 0.5% deoxycholate, and 1 mM PMSF. A ratio of 1 mL of vesicle solution per 19 mL of extraction buffer was used. The solution was gently stirred at 25 °C for 30 min and centrifuged at 150000*g* for 45 min.

(*A*) *Hexylagarose Chromatography.* The supernatant was loaded at a rate of 1–2 mL/min onto a hexylagarose column (20 × 2.5 cm) equilibrated with extraction buffer lacking PMSF and washed with the same buffer until the OD_{280} was equal to that of the washing buffer. The column was eluted with a linearly decreasing gradient in DOC which was, at the same time, a linearly increasing gradient in dPEG. This was achieved by starting with 200 mL of 20 mM Tris-HCl, pH 8.4, 1 mM DTT, 1 mM NaN_3, and 50 mM NaCl containing 0.25% DOC and gradually diluting it with 200 mL of the same buffer solution containing 2% dPEG instead of DOC. EII^{mtl}–C384S was detected by a complementation assay for mannitol phosphorylation with membrane vesicles containing EII^{mtl}–H554A (van Weeghel et al., 1991a) as follows. Assay mixtures (90 μL) containing 25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 0.35% dPEG, HPr, EI, EII^{mtl}–H554A vesicles, and aliquots of the hexylagarose fractions were incubated for 5 min at 30 °C. Then 10 μL of 330 μM [^{14}C]-mtl was added and the incubation continued for 10 min at the same temperature. The reaction mixture was then loaded onto Dowex columns, and the [^{14}C]mtl-P was eluted and counted as described (Robillard & Blaauw, 1987).

(*B*) *Q-Sepharose Chromatography.* The active fractions eluting from the hexylagarose column at approximately 1% dPEG were pooled and loaded directly onto a Q-Sepharose column (1.5 × 20 cm) equilibrated in 20 mM Tris-HCl, pH 8.4, 1 mM DTT, 1 mM NaN_3, and 0.25% dPEG. The column was washed with the same buffer, at pH 8, containing 150 mM NaCl until the OD_{280} was reduced to background and then eluted with a linear gradient of 150–400 mM NaCl in the same buffer. The EII^{mtl}–C384S activity was detected as above, and the active fractions were pooled and frozen until use.

Expression and Purification of the IIB^{mtl} Domain. The expression levels were analyzed in three different strains of *E. coli* harboring the vector pMcCII or pMcCIIP$_{trc}$: ASL-1, LJ1008-11-3, and JM101. The cells were cultured in 2 × TY broth. The expression of IIB^{mtl} directed from pMcCII was constitutive because the *c*I repressor is lacking in all these strains. The expression from plasmid pMcCIIP$_{trc}$ was also constitutive in ASL-1 and LJ1008-11-3, but had to be induced with 1 mM IPTG in JM101, because of the presence of *lac*I$_q$ repressor.

LJ1008-11-3/pMcCII was used for the production of IIB^{mtl} because use of the plasmid pMcP$_{trc}$CII in LJ1008-11-3 or in JM101, with induction, did not result in higher levels of expression (see Results). A 50-mL overnight culture was used to inoculate 1 L of 2 × TY medium in the presence of chloramphenicol (25 μg/mL). The cells were grown for 16

h at 37 °C, after which they were harvested at 20000*g* (10 min) at 4 °C. Under these culture conditions, 5 g of cells (wet weight) per liter of medium was routinely obtained. The cell pellets were resuspended in buffer (20 mM Tris-HCl, pH 9.5, 1 mM EDTA, 1 mM PMSF, and 1 mM DTT) at 10 mL/g wet weight of cells and lysed by passing through a French pressure cell at 10 000 psi. After centrifugation of the suspension at 150000*g* for 60 min, the pellets containing cell debris and membrane fragments were discarded, and the supernatant was tested for IIB^{mtl} activity in a complementation assay with EII^{mtl}–C384S (see Materials and Methods) or analyzed by SDS–PAGE for expression.

The first purification step involved a Q-Sepharose column (7 × 2.5 cm) equilibrated with buffer A (20 mM Tris-HCl, pH 9.5, and 1 mM DTT). Freshly prepared DTT was used during all purification steps. The IIB^{mtl}-containing supernatant was applied to the column at a flow rate of 60 mL/h. The column was washed with the same buffer until the 280-nm absorbance of the effluent reached the base line. Elution was achieved with a linear gradient of 0–300 mM NaCl in buffer A at a flow rate of 30 mL/h. The total volume of the elution gradient was 300 mL. Fractions were tested for activity and analyzed by SDS–polyacrylamide gel electrophoresis using Coomassie Blue staining. Fractions containing IIB^{mtl} were pooled and dialyzed overnight against buffer B (20 mM KP_i, pH 6.0, and 1 mM DTT) and loaded, at a rate of 60 mL/h, onto an S-Sepharose column (6 × 2.3 cm) which was equilibrated with buffer B. The column was washed with 3–4 column volumes of buffer B. Finally, IIB^{mtl} was eluted with a linear gradient of 0–500 mM NaCl in buffer B at a flow rate of 60 mL/h. The total volume of the gradient was 300 mL. Fractions of 8 mL were collected and analyzed by SDS–PAGE for purity. Those fractions containing IIB^{mtl} were pooled, dialyzed overnight against buffer C (10 mM KP_i, pH 7.0, and 1 mM DTT), and applied to the hydroxylapatite column (8 × 1 cm, 30 mL/h) equilibrated with the same buffer C. After the column was washed with 4–5 column volumes of buffer, elution was started using a gradient of 10–100 mM KP_i at a flow rate of 30 mL/h (80 mL). SDS–gel electrophoresis of the fractions was used to verify the IIB^{mtl} elution profile. Those fractions containing IIB^{mtl} were pooled and concentrated by pressure dialysis (Amicon, UM-10 membrane) and Centricon filtration (cutoff M_r 10 000).

Protein Characterization. Analytical SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of total cell extracts or column fractions was performed on 15% gels according to Laemmli (1970). Protein bands either were stained with Coomassie Blue or were electroblotted onto nitrocellulose filters (Towbin et al., 1979). The filters were used for immunodetection of IIB^{mtl} with rabbit polyclonal antibodies elicited against native EII^{mtl} as described (Pas et al., 1987). Isoelectrofocusing (IEF), to test the purity and homogeneity of the IIB^{mtl} domain, was done on a Pharmacia Phast system with silver staining. The N-terminal amino acid sequence was determined with an Applied Biosystems Model 477A protein sequencer (pulse-liquid sequenator), connected on-line with a 120A PTH analyzer (Hewick et al., 1981). The amino acid composition was determined using an HP Aminoquant with an automatic two-step precolumn derivatization with OPA and FMOC (Schuster, 1988). Hydrolysis was performed in the gas phase in 5.7 N HCl at 166 °C for 2 h.

Complementation Assays for IIB. Assays were carried out as previously described by van Weeghel et al. (1991b). The assay mixtures routinely contained 25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 0.35% decyl-PEG, 3.6 μm HPr, 0.22 μM EI, 20–33 μM [^{14}C]mannitol, and inside-out (ISO) vesicles containing EII^{mtl}–C384S or IIC^{mtl}. Crude cell extracts and column fractions containing IIB^{mtl} were assayed for their ability to restore the activity of membrane vesicles containing the EII^{mtl}–C384S mutant. Reactions were started by the addition of 5 mM P-enolpyruvate. [^{14}C]Mannitol phosphate production was measured according to the procedure of Robillard and Blaauw (1987). The details of specific experiments are provided in the text and figure legends.

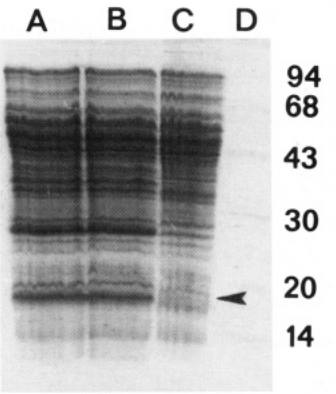

FIGURE 3: Expression of IIB^{mtl}. Coomassie-stained SDS–PAGE analysis of total cell extracts of *E. coli* LJ1008-11-3 cells harboring the IIB^{mtl} expression plasmid pMcCII in lanes A and B and without the plasmid in lane C. Lane D, positions of molecular mass standards given in kilodaltons. The arrow at 16.0 kDa indicates expressed IIB^{mtl}. Lanes A and B were scanned to determine the intensity of the 16-kDa band relative to the total protein. This was done by digitizing the photo of the gel using a CDC camera and determining the intensities with IMAGIC, software for electron micrograph image processing. Processing involved averaging 10 tracks taken in the lengthwise direction across lane A and then integrating. The same was done for lane B. The intensity of the 16-kDa band in lanes A and B was approximately 7% of the total integrated intensity over the whole gel.

^{31}P NMR Measurements. IIB^{mtl} was phosphorylated *in situ* in the NMR tube using a buffer containing 25 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 5 mM DTT, 20 mM P-enolpyruvate, 4 μM EI, 5 μM HPr, and 10 μM IIA. The measurements were performed with a VARIAN VXR300 NMR spectrometer operating at 121.4 MHz using a 5-mm bore probe. The spectra were recorded at 25 °C with a 5000-Hz sweep width, a 17-μs 90° flip angle, and a repetition time of 5 s.

RESULTS

Construction of Expression Vectors. Figure 1 shows the construction of the IIB^{mtl} expression vectors. The gene fragment coding for the B domain could be generated from plasmid pMcCIIIi by site-directed mutagenesis using an oligonucleotide that introduced an amber stop codon at position Asp488 (GAT → TAG) of the amino acid sequence and a *Hin*dIII restriction site at position 1605 of the *mtl*A DNA sequence. Asp488 is located in a proposed flexible loop which connects the A domain with the B domain as shown in Figure 2.

The expression vectors pMcCII and $pMcCIIP_{trc}$ were transformed into various *E. coli* strains to test the expression of the B domain. Figure 3 shows that efficient and stable expression of IIB^{mtl} was achieved in LJ1008-11-3 cells. A 16-kDa protein band accounting for approximately 7% of the total intensity is clearly visible in cells harboring the recombinant phagemid pMcCII, as shown in duplicate in lanes A and B. A control preparation of LJ1008-11-3 without the

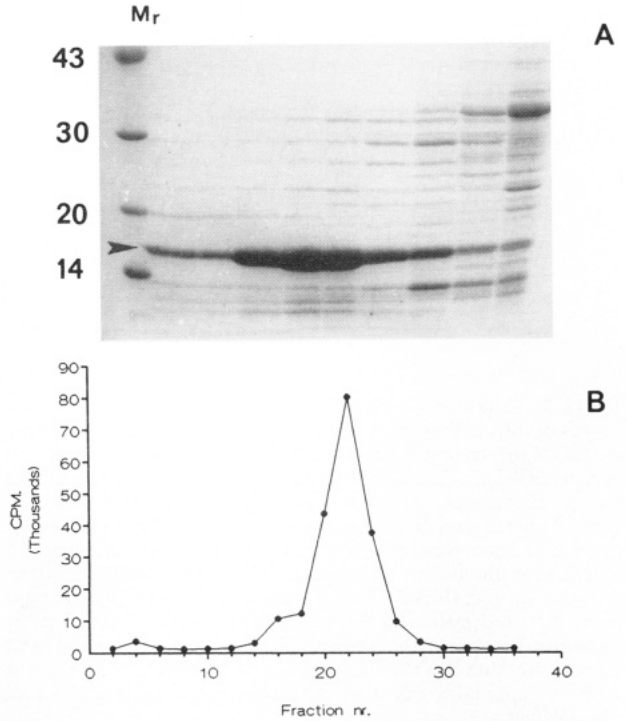

FIGURE 4: Elution and activity profile of IIB^{mtl} from Q-Sepharose. (A) Coomassie Blue stained 15% polyacrylamide gel of Q-Sepharose column fractions (10, 14, 20, 22, 24, 26, 28, 30, and 32) containing IIB^{mtl} eluted during a linear NaCl gradient. The position of IIB^{mtl} is indicated by an arrow. (B) Activity profile of different column fractions, some of which correspond to the fractions shown in panel A. The activities were measured in a complementation assay with inside-out vesicles containing the EII–C384S mutant. Assay mixtures containing 25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 0.35% decyl-PEG, 3.6 μM HPr, 0.22 μM EI, 34.4 nM EII–C384S, and aliquots of the Q-Sepharose fractions were incubated for 5 min at 30 °C. Then 10 μL of 330 μM [^{14}C]mtl was added, and the incubation was continued for 10 min at the same temperature. The reaction mixtures were then loaded onto Dowex columns, and the [^{14}C]mtl-P was eluted and counted as described (Robillard & Blaauw, 1987).

plasmid did not show any IIB^{mtl} expression (Figure 3, lane C). The highly expressed protein of 26 000 daltons is most likely the chloramphenicol acetyltransferase (CAT) which confers resistance to chloramphenicol. Expression of IIB^{mtl} from plasmid pMcCII in *E. coli* ASL-1 did not result in an improved expression level. The IPTG-inducible expression system $pMcCIIP_{trc}$ also did not lead to higher levels of IIB^{mtl} relative to constitutive expression. Expression of IIB^{mtl} was completely lost when the whole expression cassette encoding IIB^{mtl} was subcloned in the wrong orientation in pMc5-8, without the essential transcription terminators, fdT_1 and fdT_2, to produce the correct mRNA transcripts.

Purification of IIB^{mtl}. Five grams, wet weight, of cell material from a 1-L culture of LJ11008-11-3/pMcCII grown for 16 h was used for the purification of IIB^{mtl}. It was found that freshly prepared DTT was necessary to keep the protein active. Insufficient DTT led to complete and irreversible inactivation, most likely due to oxidation of the active-site Cys384. This sensitivity to inactivation is higher for IIB^{mtl} than for intact, purified EII^{mtl}. The Q-Sepharose column was used at pH 9.5 because we had observed, during the purification of IIA^{mtl}, that the CAT protein was strongly bound to the resin at this pH and did not elute during the NaCl gradient. The B domain eluted with 150–200 mM NaCl. The activity profile determined using the EII^{mtl}–C384S complementation assay is presented in Figure 4 along with the SDS–gel electrophoresis pattern of selected fractions. Fractions containing IIB^{mtl} were pooled. Table I shows that 55% of the starting activity was recovered in the pool. The purity was judged to be 70–80% as shown in Figure 5A, lane B.

Table I: Purification Scheme of the IIB^{mtl} Domain

sample with IIB^{mtl}	total[b] protein (mg)	purification[c]	% recovery
LJ1008-11-3[a]	414	1.0	100
Q-Sepharose	15	15.3	55
S-Sepharose	5.7	24.3	33.5
hydroxylapatite	5.4	23.5	30.7

[a] Crude cell extract after removal of the cell debris and membranes of LJ1008-11-3. [b] Protein concentrations were determined by the method of Bradford as described. [c] The activity was determined using the EII–C384S mutant complementation assay described under Materials and Methods. Assay mixtures contained 25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 0.35% decyl-PEG, 3.6 μM HPr, 0.22 μM EI, and inside-out (ISO) vesicles containing 34.4 nM EII–C384S. Aliquots containing IIB^{mtl} were added and incubated for 5 min at 30 °C, and the phosphorylation reaction was started by addition of 30 μM [^{14}C]mannitol. Samples were taken at various times and analyzed for mtl-P to determine the phosphorylation rates (Robillard & Blaauw, 1987).

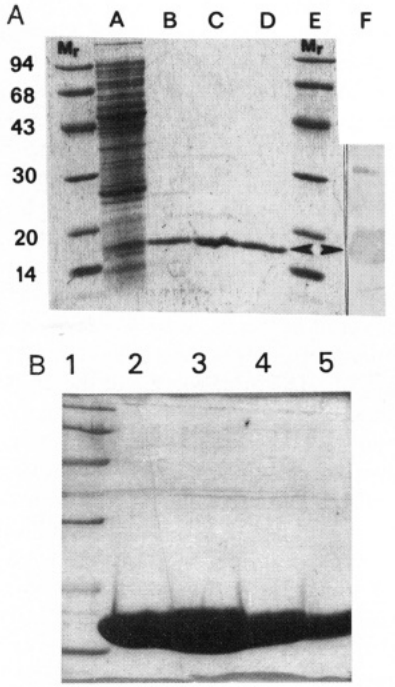

FIGURE 5: Coomassie Blue stained 15% SDS–polyacrylamide gels of IIB^{mtl} at various stages of purification. (A) Lane A, crude cell extracts after high-speed centrifugation to remove cell debris and membrane fragments; lane B, pooled peak fractions from Q-Sepharose column chromatography; lane C, pooled peak fraction from S-Sepharose column chromatography; lane D, pooled fractions from hydroxylapatite column chromatography; lane E, molecular mass markers (in kilodaltons); the position of IIB^{mtl} (16.0 kDa) is indicated by an arrow; lane F, immunochemical detection of purified IIB^{mtl} with polyclonal antibodies raised against native EII^{mtl}. (B) Various concentrations of purified IIB^{mtl}. Lane 1, molecular mass markers. Lanes 2–5, 45, 118, 39, and 17 μg of purified IIB^{mtl}, respectively.

The p*I* of the B domain was determined by isoelectric focusing to be approximately 6.3. The protein bound to S-Sepharose at a pH of 6.0 and eluted at a NaCl concentration of 250 mM. Two minor protein bands with molecular weights of 21 000 and 32 000 could not be removed during this step. The recovery of active IIB^{mtl} was 60% of the IIB^{mtl} present in the Q-Sepharose pool or 30% of the IIB^{mtl} in the starting material (Table I). The purity was estimated to be 90% on the basis of SDS–PAGE analysis of the S-Sepharose pool (Figure 5A, lane C).

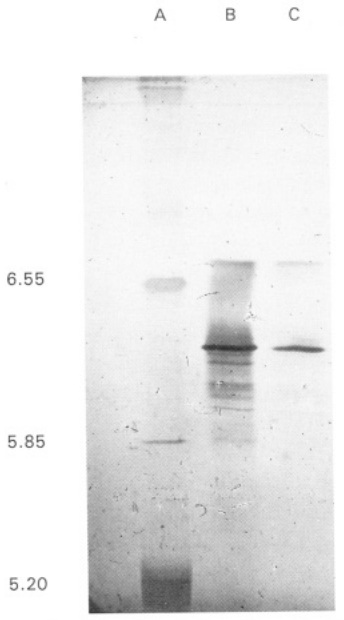

FIGURE 6: Silver-stained isoelectric focusing gel of purified IIB^{mtl}. Protein for this gel was taken from the same stock as that used for the gel in Figure 5B. The isoelectric focusing was done on a Pharmacia Phast system using gels in the pH range 5–8. The gel was silver-stained. Lane A (left), pH markers; the p*I* of each marker is given at the left side of the figure. Lane B (middle), 0.4 mg/mL IIB^{mtl}; lane C (right), 0.15 mg/mL IIB^{mtl}.

The hydroxylapatite column was necessary to eliminate the two remaining contaminants present in the S-Sepharose pool (Figure 5A, lane D). The recovery of IIB^{mtl} from this purification step was 92%. The B domain reacted with polyclonal antibodies raised against native EII^{mtl} (Figure 5A, lane F). Polyclonal antibodies raised against purified IIB^{mtl} reacted on immunoblots with intact EII^{mtl}, IIB^{mtl}, and $IIBA^{mtl}$, but not with IIA^{mtl}.

The purity of IIB^{mtl} was estimated to be more than 99% from the Coomassie blue stained SDS–polyacrylamide gel in Figure 5B in which the protein concentration was varied over a 10-fold range. The total purification procedure resulted in 1 mg of IIB^{mtl}/g of wet weight cells. The final recovery was 30%, and the final purification factor was 23.5 (see Table I). This purification factor implies that IIB^{mtl} constitutes 4–5% of the total protein in crude cell extract. Densitometric analysis of lanes A and B of the gel in Figure 3 shows that the integrated intensity of the band at 16 kDa is approximately 7% of the total protein intensity on the gel. The bulk of this intensity is attributable to IIB^{mtl}; a small fraction will be due to proteins other than IIB^{mtl} because the extract of cells lacking the plasmid also contains some protein at 16 kDa (see lane C, Figure 3).

Characterization of IIB^{mtl}. Figure 6 presents a silver-stained isoelectric focusing gel of purified IIB^{mtl} at 0.4 and 0.15 mg/mL in lanes B and C, respectively. It shows that the preparation consists of one major component. The extra bands in lane B below the main component probably result from deamidation and/or oxidation of the cysteine. An increase in the intensities of these bands has been observed when the sample was kept for longer periods in the unfrozen state.

Gas-phase sequence determination of the first seven amino acid residues, Met1-Asp2-Pro3-Arg4-Arg5-Met6-Gln7-, confirmed the expected translation start of IIB^{mtl} from the *cro*ATG initiation codon. Besides the correct translation product, a shorter N-terminal sequence, Arg5-Met6-Gln7-Asp8-Met9-Lys10-Ala11-, was found. It appears to be the result of proteolytic cleavage between Arg4 and Arg5 in the B domain. Approximately 6% of the total IIB^{mtl} was present in this form; it probably accounts for one of the minor bands on the isoelectric focusing gel (Figure 6, lane B).

Table II: Amino Acid Composition of IIB^{mtl}

amino acid	expected	found[a]
Asx	19	17.4
Glx	12	8.6
Ser	14	11.3
His	4	3.4
Gly	8	9.1
Thr	8	8.5
Ala	14	15
Arg	9	10
Tyr	1	1
Val	10	9
Met	6	6
Phe	2	2.1
Ile	7	5.6
Leu	13	13.2
Lys	8	7.5
Pro	5	5.1

[a] On the basis of an expected molecular mass for IIB^{mtl} of approximately 16 kDa. Cysteine was not determined.

The amino acid composition of IIB^{mtl} is presented in Table II. The agreement between the analysis and the composition expected on the basis of the DNA sequence is very good. Especially noteworthy is the number of phenylalanines. The third residue from the C-terminal end of IIB^{mtl} is a phenylalanine; in addition, there is 1 other phenylalanine approximately 30 residues further toward the N-terminus. Finding a stoichiometry of 2 for phenylalanine in the amino acid composition clearly indicates that there is no significant proteolytic processing at the C-terminus.

Pyruvate burst measurements on purified IIB^{mtl} carried out in the presence and absence of purified IIA^{mtl} demonstrate that IIB^{mtl} can only be phosphorylated in the presence of IIA^{mtl}. The data are presented in Figure 7. A background burst (●) was observed due to the phosphorylation of EI and HPr. The same burst was observed if the reaction mixture also contained 1.7 μM IIB^{mtl} (▲), indicating that IIB^{mtl} could not be phosphorylated in the absence of IIA^{mtl}. If 3.2 μM IIA^{mtl} was added together with HPr and EI, a significant burst due to the phosphorylation of IIA^{mtl} could be seen (■). Finally, carrying out a burst on 1.7 μM IIB^{mtl} plus 3.2 μM IIA^{mtl} resulted in a burst accounting for the sum of the IIA^{mtl} and IIB^{mtl} phosphorylation sites (♦).

For the sake of comparison with P-$IIBA^{mtl}$, ^{31}P NMR measurements were carried out using catalytic amounts of EI, HPr, and IIA^{mtl} and 1 mM IIB^{mtl} as described previously for $IIBA^{mtl}$ (Pas et al., 1991). Only three resonances are visible in the ^{31}P NMR spectrum (Figure 8): one due to phosphocysteine situated at 11.7 ppm, one at 0 ppm due to inorganic phosphate, and one at –3 ppm arising from P-enolpyruvate. No P-His resonance in the vicinity of –6 to –7 ppm was observed. This confirms that the signal reported previously in the region of 11.9 ppm in $IIBA^{mtl}$ is due to the cysteine phosphorylation site on the B domain, and it indicates that the environments of the sites in IIB^{mtl} and $IIBA^{mtl}$ are comparable. The slight difference in chemical shift between the phosphocysteine resonance in IIB^{mtl} and $IIBA^{mtl}$ is probably due to differences in the pH of the samples.

Measurement of the Activity of IIB^{mtl} with IIC^{mtl} and IIA^{mtl}. The activity of the purified B domain can be demonstrated by its ability to catalyze mannitol phosphorylation in the presence of the A and C domains. The mannitol phospho-

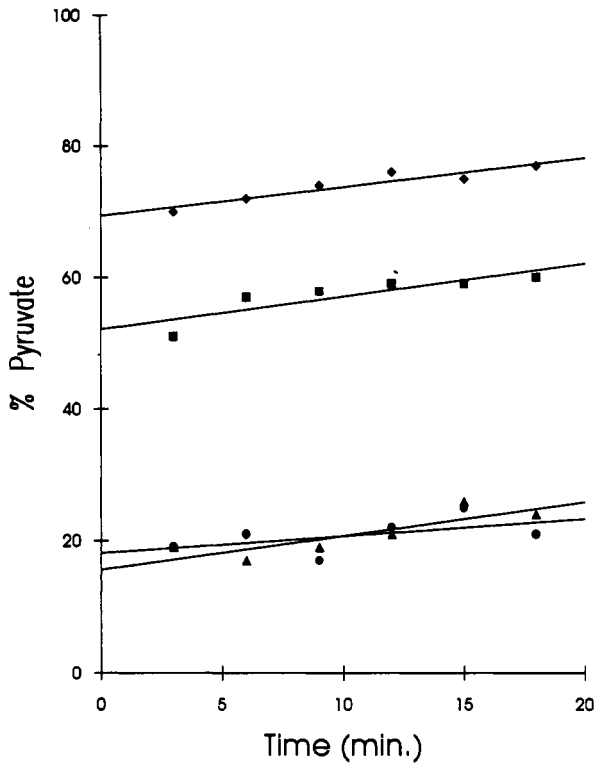

FIGURE 7: Pyruvate burst measurement of the phosphorylation of IIB^{mtl}. Reaction mixtures of 190 μL were prepared containing 25 mM Tris-HCl, pH 7.6, 5 mM DTT, 5 mM $MgCl_2$, 0.08 μM EI, 0.7 μM HPr, and (●) buffer, but no IIA^{mtl} or IIB^{mtl}, (▲) 1.7 μM IIB^{mtl}, (■) 3.2 μM IIA^{mtl}, or (♦) 1.7 μM IIB^{mtl} plus 3.2 μM IIA^{mtl}. The reaction mixtures were incubated at 30 °C for 10 min, and then, at t = 0, 20 μL of 0.1 mM [^{14}C]P-enolpyruvate was added. At the indicated times, 25-μL portions from the reaction mixtures were loaded onto Dowex columns and processed following published procedures (Robillard & Blaauw, 1987). 100% pyruvate is equivalent to 9.5 μM pyruvate.

rylation activity of solubilized membranes expressing IIC was measured with increasing concentrations of IIB^{mtl} in the presence of saturating concentrations of P-enolpyruvate, EI, HPr, and IIA^{mtl} and 33 μM mannitol. The data in Table III demonstrate that IIB^{mtl} plus IIA^{mtl} could restore the phosphorylation activity of IIC^{mtl} and that the process is saturable with respect to IIB^{mtl}. Although sufficient IIB^{mtl} was not used to measure through the K_M, the dependence of the mannitol phosphorylation rate on the IIB^{mtl} concentration resulted in linear Lineweaver–Burk plots with an extrapolated apparent K_M for P-IIB^{mtl} of approximately 200 μM and an apparent V_{max} of 71 nmol of mtI-P min^{-1} (mg of membrane protein)$^{-1}$ in duplicate sets of experiments.

Measurements of the Activity of IIB^{mtl} with EII^{mtl}–C384S. The simplest indication of the catalytic activity of IIB^{mtl} was its ability to restore activity to EII^{mtl}–C384S in the complementation assay. The function of IIB^{mtl} would be to provide the phosphorylation site necessary to catalyze the transfer of the phosphoryl group from the phospho-A domain to mannitol bound to the C domain. We have demonstrated previously that the B domain of $IIBA^{mtl}$ was able to phosphorylate mannitol in the same complementation assay with the EII^{mtl}–C384S mutant (van Weeghel et al., 1991c). The data of the *in vitro* P-enolpyruvate-dependent complementation assay, as shown in Figure 4B, clearly suggest that IIB^{mtl} is active and able to complement the EII^{mtl}–C384S mutant in this mannitol phosphorylation reaction as well. These data, however, have been accumulated with membrane preparations where the state of EII^{mtl} is difficult to define. Stephan and Jacobson (1986) have provided evidence for a sensitive proteolysis site in the linker between the B and C domains. Consequently, free IIC^{mtl} may be present in the membranes, and it, rather than intact EII^{mtl}–C384S, may be responsible for the complementation activity measured with IIB^{mtl}. IIB^{mtl} complementation has been carried out with partially purified EII^{mtl}–C384S to address this issue.

EII^{mtl}–C384S was purified by hexylagarose and Q-Sepharose chromatography as stated under Materials and Methods. Its elution characteristics are similar to those of native EII^{mtl} on these gels. The Q-Sepharose step is especially important for the present experiment because IIC^{mtl}, by virtue of its high isoelectric point and its very hydrophobic nature, should not bind to this resin. As a result, a preparation of EII^{mtl}–C384S can be obtained which should lack IIC^{mtl}. IIB^{mtl} complementation activity measurements were carried out exactly as stated under Materials and Methods for the purification of EII^{mtl}–C384S, but in this case purified IIB^{mtl} (5–10 μM) replaced the ISO membrane vesicles containing EII^{mtl}–H554A as a source of the active B domain. The presence of IIB^{mtl} resulted in mannitol phosphorylation rates with EII^{mtl}–C384S comparable to those observed with EII^{mtl}–H554A. These data indicate that the complementation activity of isolated IIB^{mtl} with EII^{mtl}–C384S is due to the isolated B domain's ability to replace the defective B domain even though the defective B domain is covalently attached to the A and C domains.

DISCUSSION

EII^{mtl} can be divided into three structural and functional domains, which can be stably expressed, and biochemically and kinetically studied (Stephan & Jacobson, 1986; Stephan et al., 1989; Grisafi et al., 1989; White & Jacobson, 1990; van Weeghel et al., 1991a,c; Lolkema et al., 1990; Pas et al., 1991). Primary sequences of the EII single species or EII/EIII pairs indicate that several of these proteins are structurally and evolutionarily related (Ebner & Lengeler, 1988; Saier et al., 1988) and that they all consist of a hydrophobic transmembrane domain (IIC), a hydrophilic EIII-like domain (IIA) containing the P-HPr phosphoryl group accepting site, and another hydrophilic domain (IIB) containing the second phosphorylation site. The *E. coli* cellobiose-specific and sorbose-specific phosphotransferase systems are the only ones in which IIB occurs as a separate protein (Parker & Hall, 1990; Reizer et al., 1990; Wehmeier et al., 1992). In all other systems, it is found fused either to the A domain or to the C domain or both. We have recently expressed and characterized the A domain, the complete cytoplasmic BA domain, and the mannitol-transporting C domain (van Weeghel et al., 1991b,c). The data in this paper demonstrate that IIB^{mtl} can exist as an enzymatically active protein which has the capacity to restore the *in vitro* mannitol phosphorylation activity together with IIA^{mtl} and IIC^{mtl} in a fashion analogous to the cellobiose-specific PTS.

The concept of domain complementation arose from the early proposals that phosphotransferase systems which lacked a soluble EIII (IIA) possessed a covalently bound domain which took over the function of the soluble enzyme. Vogler et al. (1988) and Vogler and Lengeler (1988) expanded this concept by showing that EII^{nag} and EII^{bgl}, with C-terminal domains highly homologous to IIA^{glc}, could replace IIA^{glc} in EII^{glc}-dependent glucose phosphorylation and transport. Furthermore, addition of IIA^{glc} to a truncated EII^{nag} lacking a functional IIA^{nag} domain restored the activity of the truncated enzyme. Their experiments provided the first evidence for

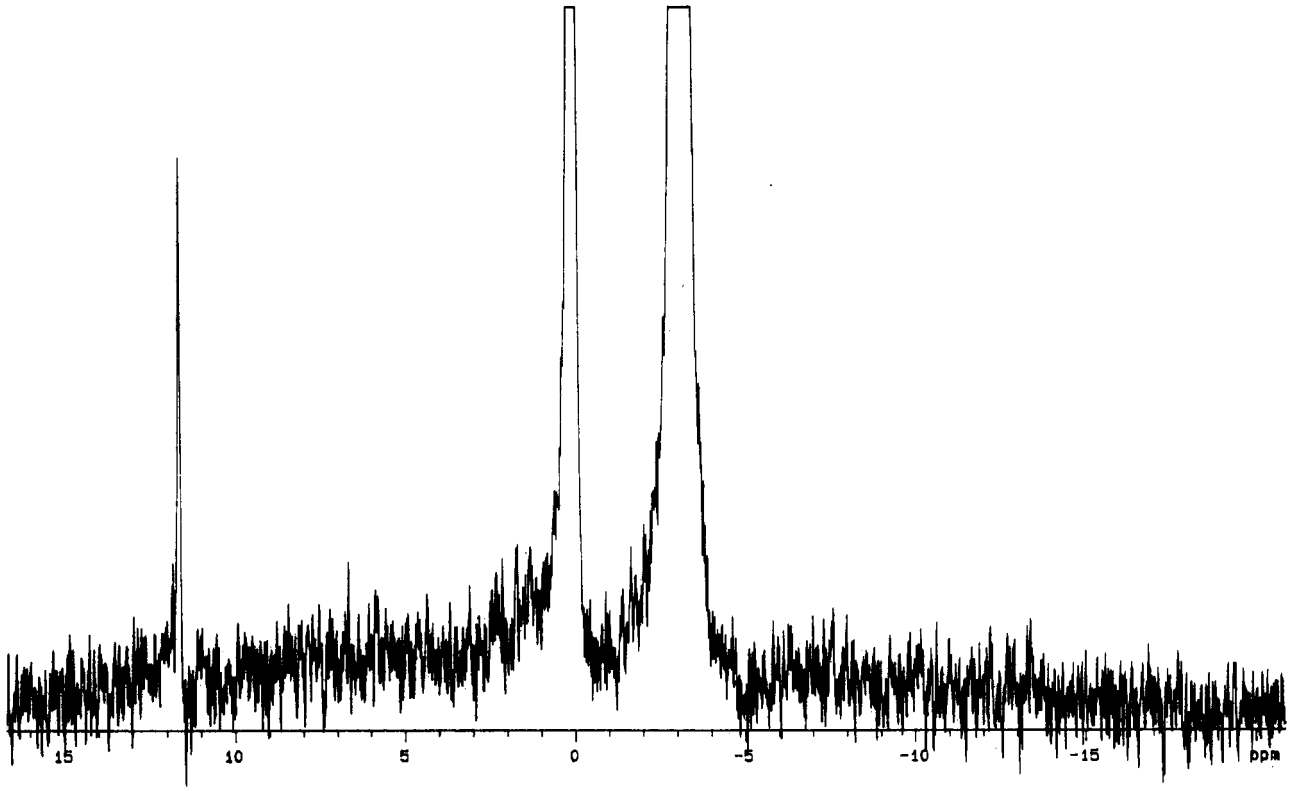

FIGURE 8: ^{31}P NMR spectrum of phosphorylated IIB^{mtl}. The sample in 10% D_2O contained 25 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 5 mM DTT, 4 μM EI, 5 μM HPr, 10 μM IIA^{mtl}, 20 mM P-enolpyruvate, and 1 mM IIB^{mtl}. The spectrum was taken on a Varian VXR 300 at 25 °C, using a 17-μs pulse width and a 5-s repetition rate. The data were processed with a 2-Hz line-broadening. The chemical shifts are reported relative to inorganic phosphate at 0 ppm.

Table III: IIB^{mtl} Complementation Activity in Combination with IIA^{mtl} and Solubilized Membranes Containing the IIC^{mtl} Domain[a]

[IIB^{mtl}] (μM)	activity [nmol of mtl-P min^{-1} mg of membrane $protein^{-1}$]
0	0.3
29	9.4
37	11.3
74	19.2
111	25.4
148	30.0

[a] The assay mixtures contained 25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 0.35% decyl-PEG, 24 μM HPr, 0.35 μM EI, 30 μM IIA^{mtl}, and the stated concentrations of IIB^{mtl}. They were incubated for 5 min at 30 °C. Then 10 μL of 330 μM [^{14}C]mtl was added. Aliquots of the reaction mixture were withdrawn at constant time intervals and loaded onto Dowex columns. The [^{14}C]mtl-P was eluted and counted as described (Robillard & Blaauw, 1987). A control series of assays with 8 μM IIA^{mtl} instead of 30 μM gave nearly the same rates, indicating that the 30 μM IIA^{mtl} used above was saturating.

the A domain of one EII species being able to phosphorylate the B domain of another enzyme. Additional support for this concept was provided by van Weeghel et al. (1991a) and more recently by Weng et al. (1992) by showing that two inactive EII^{mtl} species with mutated phosphorylation sites, one in the A domain and the other in the B domain, became active when combined *in vitro* or *in vivo*. We can safely conclude from these data that the A domain of one EII molecule is able to pass its phosphoryl group on to the B domain of another. Whether this process is transient or requires a stable dimer has not been settled.

Domain complementation provided such a simple procedure for assaying the activity of the isolated A domain in conjunction with membranes containing the inactive EII^{mtl}–H554A mutant that the procedure was adopted for assaying the activity of isolated $IIBA^{mtl}$ (van Weeghel et al., 1991c) and, in this report, for assaying isolated IIB^{mtl}. However, since P-IIB^{mtl} is active with the isolated A and C domains, it was necessary to prove that the complementation activity with EII^{mtl}–C384S was due to replacing the defective B domain in this protein rather than to reaction with a C domain proteolytic fragment in the membrane. The data with partially purified EII^{mtl}–C384S support the view that IIB^{mtl} can replace a defective B domain in the intact enzyme. However, an additional test was also undertaken which gave a conflicting result.

Roossien and Robillard (1984) showed that low concentrations of [^{14}C]NEM rapidly inactivated EII^{mtl} with the concomitant incorporation of 1 mol of ^{14}C and that this inactivation could be prevented by prior oxidation or phosphorylation of the enzyme. Subsequently, Pas and Robillard (1988a) showed that C384 was the residue which was protected by phosphorylation and oxidation. If isolated IIB^{mtl} could functionally replace the inactivated B domain as suggested from the complementation data with EII^{mtl}–C384S, we reasoned that it should be able to restore the activity of an NEM-inactivated EII^{mtl} as well. To test this, purified EII^{mtl} was inactivated under essentially the same conditions used by Roossien and Robillard (1984), and the ability of IIB^{mtl} to complement the inactive enzyme and restore its ability to phosphorylate mannitol was tested. Specifically, 1.5 μM purified EII^{mtl} in 25 mM Tris, pH 7.6, and 5 mM DTT was diluted 100-fold into a buffer containing 25 mM potassium phosphate, pH 7.1, 5 mM $MgCl_2$, 0.25% d-PEG, and 0.4 mM NEM at 30 °C. The NEM inactivation was allowed to proceed for 2 min at 30 °C. The reaction was quenched by diluting an aliquot 10-fold in buffer containing 25 mM Tris-HCl, pH 7.6, 5 mM DTT, and 0.25% d-PEG and storing on ice until assayed. A control sample was treated in an identical manner except that no NEM was present. Both samples were then

assayed for their mannitol phosphorylation activity in the presence and absence of added IIB^{mtl}. Treatment with low concentrations of NEM led to 80% inactivation of the mannitol phosphorylation activity. The presence of IIB^{mtl} at concentrations up to 38 μM was not able to restore the activity of the NEM-treated enzyme.

The differences between the NEM-treated EII^{mtl} and the EII^{mtl}–C384S which could account for these discrepancies may have to do with an additional reaction site for NEM or the additional bulk introduced by the maleimide ring at the position of C384 which prevents the correct approach of the active B domain. An additional reaction site is unlikely. The NEM reaction was carried out under well-defined circumstances where incorporation is known to occur only at C384. The question of additional bulk is presently under investigation using a series of EII^{mtl} site-specific mutants in which C384 has been replaced by amino acids of varying polarity, charge, and size.

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