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The *ptsH*, *ptsI*, and *crr* Genes of the *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System: a Complex Operon with Several Modes of Transcription

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The *ptsH*, *ptsI*, and *crr* genes, coding for three of the proteins of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) (HPr, enzyme I, and enzyme III^{Glc}, respectively) have been studied by determination of their nucleotide sequence and analysis of their expression. The three genes constitute an operon, but analysis of the *ptsH*, *ptsI*, and *crr* transcripts by Northern (RNA) blotting revealed the existence of three major mRNA species. One encompassed the three cistrons, a second one the *ptsH* gene and part of the *ptsI* gene, and the third one only the distal gene *crr*. The short *crr* transcripts were initiated inside the *ptsI* open reading frame at points which were identified by S1 mapping. Expression of the genes was studied in vivo by using operon and protein fusions between the *lacZ* gene and the *ptsH*, *ptsI*, or *crr* gene on IncW low-copy-number plasmids. The present study showed that (i) the *ptsH*, *ptsI*, and *crr* genes exhibited high basal expression, (ii) transcription of the *ptsH* and *ptsI* genes was stimulated threefold by the cyclic AMP-cyclic AMP receptor protein complex and also by growth on glucose, but only in the presence of an active enzyme II^{Glc}, (iii) *crr*-specific expression was not sensitive to the complex or to growth on glucose, and (iv) under the growth conditions tested, the major part of *crr* transcription was initiated from internal promoters.

Transport of a large number of glucose-related carbon sources is mediated by the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS). Glucose is transported vectorially into the cell with concomitant phosphorylation to glucose-6-phosphate (glucose-6-P), which is immediately funneled into intermediary metabolism. Glucose and related carbohydrates, such as fructose, mannose, amino-hexoses, and hexitols (all termed PTS carbohydrates), are phosphorylated through the PTS phosphorylation cascade, which is mediated by a series of cytoplasmic phosphoprotein carriers (for review, see reference 34). An essential feature of the PTS system is that the phosphoryl donor molecule is PEP, not ATP. The first two steps of the cascade are (i) phosphoryl transfer from PEP to enzyme I (encoded by the *ptsI* gene) and (ii) phosphorylation of HPr by P~enzyme I (HPr is encoded by the *ptsH* gene). Both steps are common to all PTS carbohydrates. Two different pathways arise at this point: for several PTS carbohydrates, the P~HPr protein transfers its phosphoryl moiety to the PTS carbohydrate during its transport, via the action of a membrane-bound protein (enzyme II) specific to the carbohydrate (enzymes II are named according to their high-affinity substrate, although they sometimes have broad substrate specificities). In the case of some PTS carbohydrates, another cytoplasmic phosphoprotein, termed enzyme III, is necessary to allow phosphoryl transfer from P~HPr to the carbohydrate during its translocation via enzyme II. This has been shown to be the case for glucose, mannose, fructose, and glucitol (45). For example, the glucose enzyme III (enzyme III^{Glc}, encoded by the *crr* gene) is necessary for glucose transport and phosphorylation through enzyme II^{Glc}. However, glucose can also be transported through enzyme II mannose (enzyme II^{Man}). Enzyme III^{Glc} has been extensively studied because it appears that its state of phosphorylation determines the extent of PTS-mediated regulation (39) of the uptake and catabolism of several non-PTS carbohydrates (32) as well as on cyclic AMP (cAMP) synthesis (17). This system is therefore of central interest in bacteria. The phosphorylation of enzyme III^{Glc} depends on the level and activity of both enzyme I and HPr and therefore also on the level of expression of both the *ptsH* and *ptsI* genes.

This prompted us to investigate *ptsH*, *ptsI*, and *crr* gene structure and expression at the molecular level. These *pts* genes are clustered at min 52 on the *Escherichia coli* chromosome. It was shown long ago that *ptsH* and *ptsI* are cotranscribed (41), and it was claimed that *crr* did not form part of this operon (5). We have shown that the *ptsH*, *ptsI*, and *crr* genes constitute an operon, the *pts* operon (10). In order to characterize the features controlling expression of these genes further, the complete nucleotide sequence of the operon was determined. The work described here shows that transcription of the DNA region carrying these three genes is much more complex than was originally assumed. Transcription start points were identified by S1 mapping, and the nature of mRNA species was determined by Northern (RNA) blotting of total RNA extracts from wild-type cells. This showed that in addition to the polycistronic mRNA encompassing the entire *pts* operon, short transcripts expressing specifically the *ptsH* and the *crr* genes exist. Such a transcriptional organization suggested the existence of complex regulatory features, which we investigated by using gene fusions between the *ptsH*, *ptsI*, or *crr* gene and the *lacZ* gene. This allowed analysis of the variations in expression of *pts* genes under various environmental conditions. The *ptsH* and *ptsI* genes exhibit high basal expression, but there is a threefold stimulation of their transcription either mediated by the cAMP-cAMP receptor protein (CAP) complex or when glucose is used as a carbon source. The *crr* gene also exhibits high basal expression which is not regulated either by cAMP-CAP or by growth in the presence of glucose. This

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TABLE 1. *E. coli* strains used

Strain	Genotype	Origin, source, or reference
7118	Δ(*lac-proAB*) *thi supE* (F′ *proAB lacI*^{q} *lacZΔM15*)	Yanisch-Perron et al. (47)
LM1	F$^-$ *argG6 crr galT his-1 manA manI metB nagE rpsL thi*	Lengeler et al. (25)
CY313	*zcf-229*::Tn*10 trp-45 his-68 tyrA2 thi-1 lacY1 gal-6 malA1 xyl-7 mtl-2 rpsL125 tonA2* λ$^-$ *tsx-70 supE44*	Garwin et al. (18) via B. Bachmann
ZSC103	*lacZ82* or *lacZ827 ptsG22 glk-7 rha-4 rpsL223*	Curtis and Epstein (6) via B. Bachmann
TP2000	F$^-$ *xyl argH1* Δ*cya* Δ*lacX74*	Roy and Danchin (36)
TP2100	F$^-$ *xyl argH1 ilvA* Δ*lacX74*	Roy et al. (37)
TP2110	F$^-$ *xyl argH1 ilvA* Δ*lacX74 recA srl*::Tn*10*	De Reuse et al. (12)
TP2010	F$^-$ *xyl argH1* Δ*lacX74* Δ*cya recA srl*::Tn*10*	Roy and Danchin (36)
TP2339	F$^-$ *xyl argH1* Δ*lacX74* Δ*cya* Δ*crp39*	Roy et al. (37)
TP2506	*lacZ82* or *lacZ827 ptsG22 glk-7 rha-4 rpsL223 zcf-229*::Tn*10*	Phage P1 (CY313) transductant of strain ZSC103
TP2503	F$^-$ *xyl argH1 ilvA*	Phage P1 (wild type) transductant of strain TP2100
TP2504	F$^-$ *xyl argH1 ilvA zcf-229*::Tn*10 ptsG22*	Phage P1 (TP2506) transductant of strain TP2503

is consistent with Northern blot analyses indicating that *crr* is mainly expressed from its specific internal promoters.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* strains used in this work are listed in Table 1. Bacteriophage P1 transduction was used in the construction of strains when required. All strains used for the β-galactosidase assays (TP2110, TP2010, TP2339, TP2503, and TP2504) are derivatives of the same original strain (TP2000) and therefore have closely related genetic backgrounds.

Growth media were either rich medium LB or synthetic medium M63 supplemented to 0.4% with a carbon source, thiamine (10 μg/ml), the required amino acids (100 μg/ml), and 0.1% Casamino Acids (29). cAMP was added at 3 mM final concentration and methyl-α-glucoside at 0.1%. Antibiotic concentrations were ampicillin, 50 μg/ml, and kanamycin, 25 μg/ml. Screening for the ability to synthesize β-galactosidase was performed on MacConkey plates containing 1% lactose, on M63 lactose (0.4%) plates, or on LB plates containing 40 μg of X-gal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside) per ml. Strains harboring mutations in *ptsG* are difficult to distinguish from the wild type because they are capable of growth on glucose, which can be taken up via the enzyme II^{Man}. For this reason, a medium containing 1% glucose, 0.2% lactose, and X-gal (40 μg/ml) was used (44). Wild-type colonies were white on the medium, while the *ptsG* mutant (TP2504) gave blue colonies. The blue color indicates that X-gal is taken up and hydrolyzed by β-galactosidase in these strains even though they are grown on a medium containing glucose, since the absence of the *ptsG* transport system results in the suppression of a major part of catabolite repression. PEP-dependent phosphorylation of [^{14}C]methyl-α-glucoside was tested on toluene-treated cells of strain TP2110 (*ptsG*$^+$) and strain TP2504 (*ptsG*) by the method of Bouvet and Grimont (3). The *ptsG* transport system is strongly impaired in strain TP2504, which exhibited only 1% of the PEP-dependent phosphorylation activity of the *ptsG*$^+$ strain TP2110.

β-Galactosidase assays. β-Galactosidase was assayed by the method of Pardee et al. (33); 1 U was defined as the amount of enzyme that converted 1 nmol of substrate per min at 28°C. The values indicated in Tables 2, 3, and 4 correspond to experiments in which 10 samples were withdrawn during exponential growth at 37°C in synthetic medium M63 supplemented as indicated in Materials and Methods and in the tables. β-Galactosidase synthesis rates are expressed in units per milligram (dry weight) of bacteria, deduced from the optical density at 600 nm (1 mg [dry weight] per ml is estimated to be 3.7 optical density units at 600 nm). Experiments to compare growth in different media were always conducted with identical strains (i.e., the same genetic background and the same plasmid).

Plasmid constructions. The various plasmids used are illustrated in Fig. 2 and 4. Two plasmids carrying operon fusions were constructed. Plasmid pDIA3241, a fusion with *ptsH*, was constructed by insertion of the *Sal*I-*Sma*I restriction fragment of pDIA3206 into plasmid pDIA3240 (12), and plasmid pDIA3242, a fusion with *ptsI*, was constructed by insertion of the *Sal*I-*Eco*RI restriction fragment of pDIA3206 into pDIA3240. Four protein fusions were constructed. Plasmid pDIA3226, a fusion with *ptsI*, was constructed by insertion of a *Sal*I-*Eco*RI restriction fragment of pDIA3206 between the *Sal*I site and one of the *Eco*RI restriction sites of pDIA3231 (12). Plasmid pDIA3247, a fusion with *ptsH*, was constructed by endonucleolytic cleavage of pDIA3226 with *Xho*I and *Bam*HI, followed by treatment with mung bean nuclease of the *Xho*I and *Bam*HI restriction sites to create the blunt ends necessary for plasmid recircularization, to remove the DNA corresponding to *ptsI* and to allow conservation of an open reading frame (ORF) between *ptsH* and *lacZ*. Plasmid pDIA3238, a fusion with *crr*, was constructed by insertion of a *Sal*I-*Bam*HI restriction fragment of a derivative of pDIA3206 containing a *Bam*HI linker inserted at the *Xmn*I restriction site located at the end of the *crr* gene into the *Sal*I-*Bam*HI restriction sites of pDIA3231. Plasmid pDIA3239 was constructed by deletion of the *Sal*I-*Xho*I restriction fragment of plasmid pDIA3238.

Determination of nucleotide sequence. DNA sequencing was performed by the dideoxy chain termination method of Sanger et al. (42) with [α-^{35}S]thio-dCTP (2). Single-stranded DNA templates were derivatives of the M13 phage vector, tg130 and tg131 (24), or vector PTZ18 (Pharmacia T.M.) carrying large restriction fragments of the *pts* region. The *Sma*I-*Eco*RI restriction fragment containing most of the *ptsI* gene was cloned in one orientation in vector tg131. The other orientation containing the entire *ptsI* gene was obtained by cloning the *Hpa*I-*Sma*I restriction fragment in vector tg131. The *Bgl*II-*Hpa*I restriction fragment containing the end of the *ptsI* gene and overlapping the *Eco*RI restriction site was cloned in vector PTZ18. The *Hpa*I restriction fragment containing the *crr* gene was cloned in both orientations in vector tg131. The *Eco*RI-*Xmn*I restriction fragment was cloned in vectors tg130 and tg131. Nested deletions extending into the large DNA inserts cloned in vector tg131, from the end proximal to the universal primer-binding site of

tg131, generated sequential series of overlapping clones and were performed as described by Dale et al. (7).

RNA extraction. Strain TP2110 containing plasmid pDIA3206 was used for in vivo RNA extraction as described by Hagen and Young (20), treated with RNase-free DNase, and used for the S1 nuclease and reverse transcriptase mapping experiments. Strain TP2110 was used for in vivo RNA preparation for the Northern blotting experiments. RNA was extracted as described by Mazel et al. (28).

Reverse transcriptase and S1 nuclease mapping experiments. Reverse transcriptase mapping of the *crr* transcription start points was performed as described by Debarbouillé and Raibaud (9). The probe was a double-stranded *Xmn*I-*Rsa*I fragment 5′ end labeled at the *Rsa*I site with [γ-^{32}P]ATP by T4 polynucleotide kinase by using the exchange reaction. Mapping with S1 nuclease was performed as described previously (11) with some modifications. The probe was the double-stranded *Nru*I-*Hpa*I fragment 5′ end labeled at the *Hpa*I site as described. A 10-μg amount of RNA was added to 10,000 Cerenkov cpm of the labeled probe. Then, the DNA-RNA mix was incubated at 95°C and cooled gradually to 20°C, allowing the heteroduplex to form.

Northern blotting experiments. Northern transfer and hybridization were performed as described by Damerval et al. (8). Total RNA samples (10 μg) were denatured for 3 min at 68°C in a loading buffer containing 50% formamide, 6% formaldehyde, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-NaOH, 1 mM EDTA, 10% glycerol, 100 μg of ethidium bromide per ml, and 0.025% bromophenol blue. These samples were then fractionated on a 1.4% agarose gel containing 6% formaldehyde, 50 mM HEPES-NaOH, and 1 mM EDTA (pH 7.8). The gel was soaked in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) twice for 20 min each before transfer to nylon filters. Prehybridization and hybridization were performed at 50°C in 5× SSC–1× Denhardt solution–0.1% sodium dodecyl sulfate (SDS)–50 mM NaH_2PO_4-Na_2HPO_4 (pH 6.5)–100 ng of heterologous DNA per ml in the presence of 50% formamide. Filters were washed twice for 5 min each in 2× SSC–0.1% SDS and twice for 5 min each in 0.1× SSC–0.1% SDS before autoradiography. The double-stranded DNA probe was labeled with [α-^{32}P]dCTP by the multiprime DNA labeling system (16). Single-stranded DNA probes were prepared from M13 recombinant phage DNA by the prime cut method (1). Relative amounts of mRNA hybridizing to the probes were deduced from densitometric analysis of autoradiographs.

Reagents and enzymes. DNA polymerase I "Klenow" fragment, T4 DNA ligase, S1 nuclease, reverse transcriptase, T4 polynucleotide kinase, mung bean nuclease, and restriction enzymes were used as recommended by the suppliers (Boehringer Mannheim, Pharmacia, or Amersham). DNA fragments were analyzed by standard gel electrophoresis (0.5 to 1% agarose; Tris-borate buffer) and ethidium bromide staining (26). Agarose type II was from Litex (Denmark), ampicillin, kanamycin, *o*-nitrophenyl-β-D-galactopyranoside, methyl-α-glucoside, and X-gal were from Sigma, and other chemicals were from Merck. Methyl-α-glucoside was tested for the presence of contaminating glucose. The supplier (Sigma) detected, in gas chromatography, a single peak corresponding to methyl-α-glucoside. Purity of methyl-α-glucoside was confirmed by the enzymatic determination of glucose (with the Boehringer-Mannheim kit), which revealed that contamination by glucose was at the most 0.015%. DNA fragment extractions were performed with low-melting-point agarose (Litex). [α-^{35}S]thio-dCTP, [α-^{32}P]dCTP, and [γ-^{32}P]ATP were obtained from Amersham. The cyclone I biosystem kit of International Biotechnologies Inc. was used for generation of deletions in the *pts* inserts cloned in M13 vector tg131. The Amersham multiprime DNA labeling system kit was used for labeling the probe used for the Northern blotting experiments.

RESULTS

Nucleotide sequence of the *ptsH*, *ptsI*, and *crr* genes. The nucleotide sequence of a 2,481-base-pair (bp) *Xho*I-*Hpa*I DNA fragment carrying the *ptsI* and *crr* genes was determined by means of the sequencing strategy described in Materials and Methods and added to that of the 892-bp fragment carrying *ptsH* which we had determined previously (11). The complete nucleotide sequence of the *ptsH-ptsI-crr* operon is displayed in Fig. 1. The sequenced DNA fragment originated from the 11.5-kilobase (kb) *E. coli* DNA insert of plasmid pDIA3206 (Fig. 2) known to carry the whole *pts* operon (10). Three large ORFs were identified in the DNA sequenced. Nucleotide 417 to nucleotide 671 corresponds to the *ptsH* gene as previously described (11), nucleotide 719 to nucleotide 2443 corresponds to the *ptsI* gene, since its 5′ end corresponds to the N-terminus of enzyme I (11), and nucleotide 2487 to nucleotide 2993 corresponds to the *crr* gene by amino acid homology (see below). The first 30 nucleotides of the sequence in Fig. 1 have been shown by Lévy (S. Lévy, doctoral dissertation, University of Paris, 1987) to correspond to the 3′ end of the *cysK* gene. The deduced amino acid sequences of the gene products are shown in Fig. 1. The *ptsH-ptsI-crr* operon promoter was identified previously (11). Located after the termination codon of the *crr* gene, between nucleotides 3016 and 3040, there is a palindromic sequence TGGCGCC CAACGGCGGCA, followed by a run of seven T residues, typical of a *rho*-independent terminator (22). The translation start of each of the three genes is an ATG codon preceded by a ribosome-binding site having standard consensus features (43), except for the *ptsI* gene, which seems rather poor (Fig. 1). All three ORFs are terminated by single TAA stop codons. The *ptsH* and *ptsI* genes are separated by an intercistronic region of 44 nucleotides; the *ptsI* and *crr* genes are separated by 40 nucleotides. Neither of these two intercistronic regions contains prominent sequences showing homologies with either promoter consensus sequences (21) or *rho*-independent termination sites. The sizes of the proteins, as predicted from the nucleotide sequence, were 9.109, 63.489, and 18.099 kilodaltons (kDa) for HPr, enzyme I, and enzyme III^{Glc}, respectively. The enzyme III^{Glc} sequence is almost identical to the sequence found for the corresponding *Salmonella typhimurium* gene (31).

The His residue at position 15 has been shown (46) to be the PEP-dependent phosphorylation site of HPr of *S. typhimurium*. By analogy, the His residue located at the homologous position in HPr of *E. coli* can be proposed as the phosphoryl carrier residue. Enzyme I also receives its phosphoryl group from PEP on a His residue (30). A tryptic peptide isolated from phosphoenzyme I of *E. coli* and containing its active center (M. Dörschug, Ph.D. dissertation, University of Bochum, Bochum, Federal Republic of Germany, 1985) corresponds exactly to the amino acid sequence deduced from the nucleotide sequence from residue 187 to residue 195. The His residue in position 189 located in this region is the phosphoryl carrier of phosphorylated enzyme I. Enzyme III^{Glc} of *E. coli* has also been shown to be phosphorylated at a His residue, and a peptide

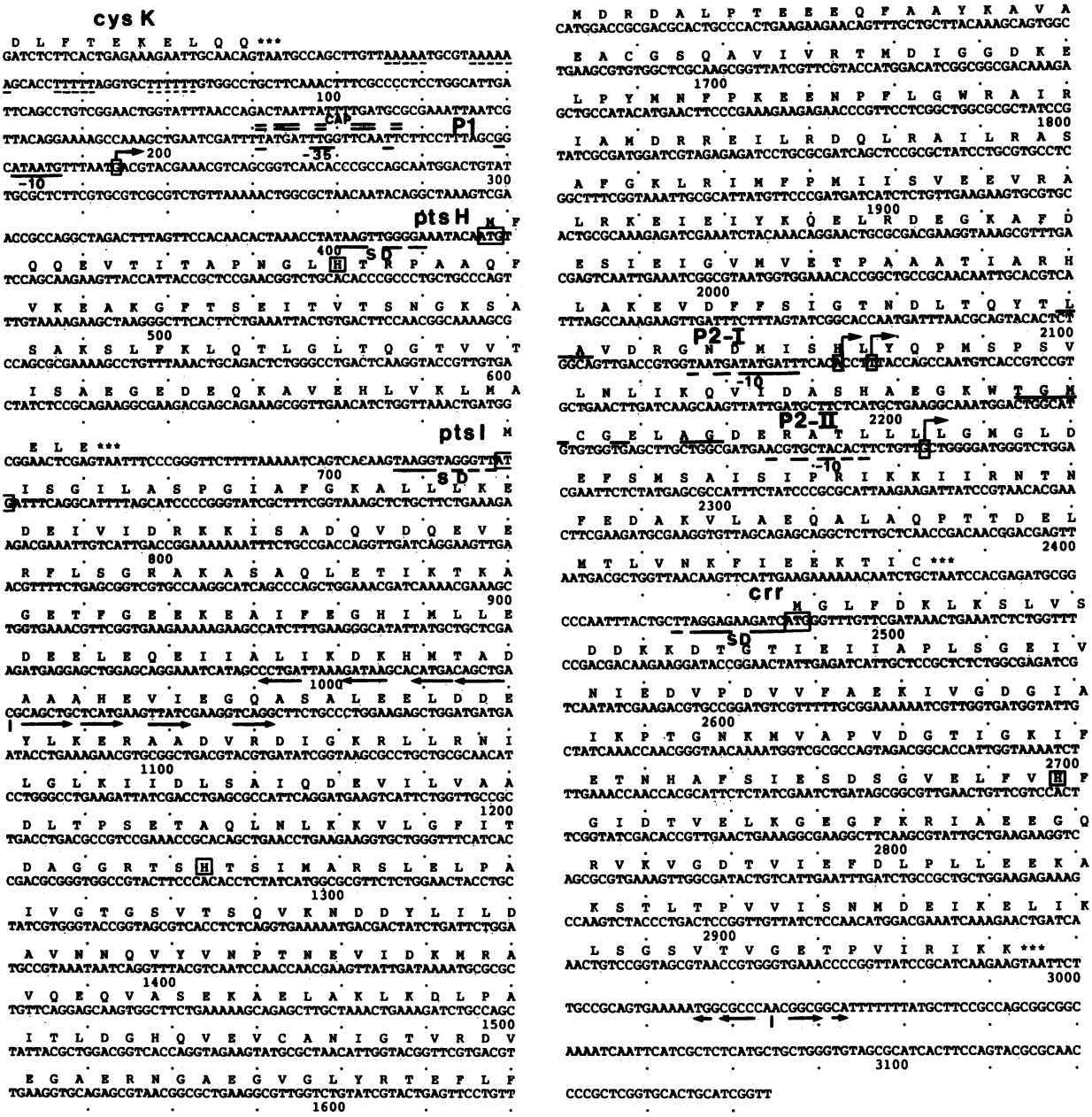

FIG. 1. Nucleotide sequence of a 3,147-bp region containing the end of the *cysK* gene, the *ptsH*, *ptsI*, and *crr* genes, and surrounding regions. Deduced amino acid sequences are indicated. The start codons are boxed, and the end codons are shown by stars. Promoter consensus sequences (−35 and −10) are underlined, and the base at which the transcription starts is boxed, with an arrow indicating the direction of transcription. Nucleotides showing palindromic symmetry are indicated by opposing arrows, and the center of symmetry is shown by a vertical line. The Shine-Dalgarno (SD) regions are underlined. Nucleotides showing homology with the *nif* promoter consensus are overlined. The putative CAP site is double overlined. An A+T-rich region is underlined with a dashed line. His residues carrying the phosphoryl residue in P~HPr, P~enzyme I, or P~crr are boxed.

containing the active center of phosphorylated enzyme III^{Glc} of *E. coli* was obtained (14). The amino acid sequence of this peptide was identical to the amino acid sequence deduced from the *E. coli crr* sequence from residue 90 to residue 95. Therefore, the His residue at position 91 is very likely to be the phosphoryl-carrying residue.

Transcription of the *ptsH-ptsI-crr* operon. In a previous publication (10), we demonstrated that the *ptsH*, *ptsI*, and *crr* genes are part of a polycistronic operon. The promoter of the operon (P1) was located after identification of its transcription start point by S1 nuclease mapping (11) (Fig. 1). There was other evidence, from deletion mapping, that the distal gene *crr* could also be transcribed under control of a second promoter region (P2) (11). Plasmid pDIA3206 (Fig. 2), which carries the three genes, was used to generate deletions of the region carrying P1 and the *ptsH* gene. These deletions extended to the *Xho*I, *Kpn*I, *Bgl*II, and *Nru*I restriction sites located in the *ptsI* gene. These deleted plasmids retained the ability to complement a *crr* mutant (strain LM1) for growth on glucose, whereas the plasmid

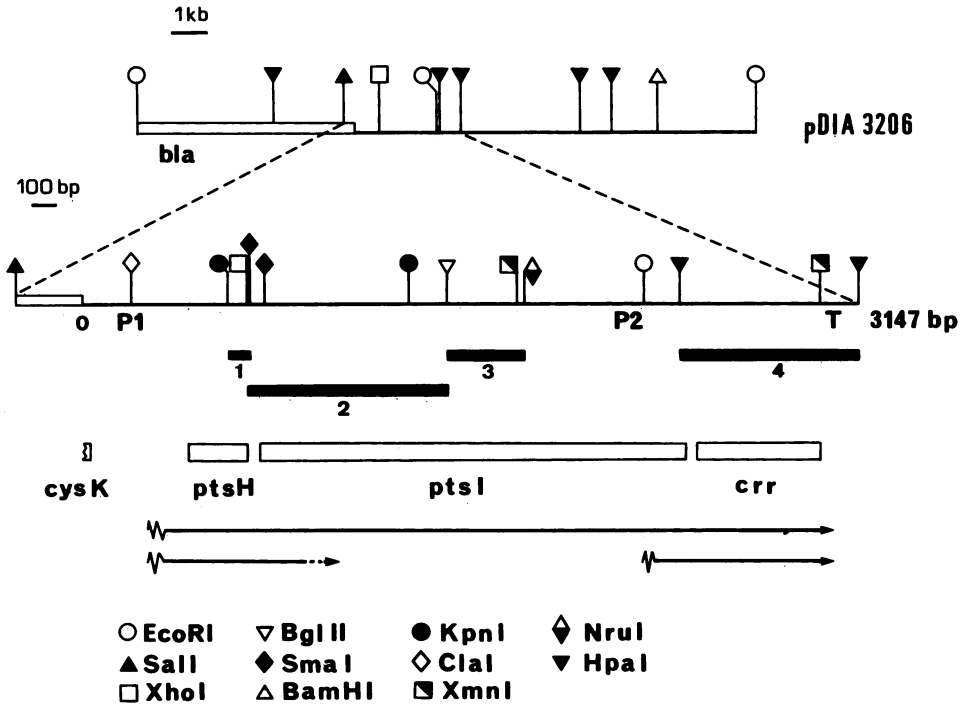

FIG. 2. Restriction map of plasmid pDIA3206. The restriction map of the sequenced DNA region encompassing the *ptsH*, *ptsI*, and *crr* genes is shown underneath. The open boxes represent the vector DNA (pHC79). *bla* indicates the gene conferring ampicillin resistance. Thin lines represent bacterial DNA inserts. Black boxes beneath the map correspond to the probes used in the Northern blotting experiments. The genes are represented by open boxes under the corresponding DNA fragments. A notch at the end of a box indicates that the gene is truncated. Arrows beginning with zigzag lines represent transcripts of the *pts* operon. The dashed line at the end of an arrow indicates that the precise end of the transcript is not known. P1, First promoter; P2, region carrying P2-I and P2-II promoters; T, terminator.

deleted to the *Eco*RI site did not complement this *crr* mutant. This suggested that the promoter region specific for the *crr* gene (P2) is located somewhere between the *Nru*I and *Eco*RI restriction sites within the *ptsI* ORF.

Determination of the initiation sites of the *crr*-specific mRNAs. Preliminary identification of the *crr*-specific transcripts was performed by reverse transcriptase mapping with an *Xmn*I-*Rsa*I DNA fragment corresponding to the 3′ end of the *crr* gene as the primer. This experiment revealed (data not shown) the existence of two transcripts having 5′ ends located between the *Nru*I and *Eco*RI sites in the *ptsI* ORF. In order to determine precisely the 5′ ends of the *crr*-specific transcripts, S1 nuclease mapping was performed with the double-stranded *Nru*I-*Hpa*I restriction fragment labeled at the *Hpa*I site as the probe. The DNA-mRNA hybrids were digested with S1 nuclease and analyzed by electrophoresis on a DNA sequencing gel together with M13mp8 sequence reactions, which allowed measurement of the length of the protected DNA fragments. As can be seen in Fig. 3B, three bands were revealed. Two bands, a and b, differed in length by 3 bp and probably correspond to transcripts having 5′ ends at an A residue at position 2133 (band a) and at a T residue at position 2137 (band b). Located 5 bp upstream from the A residue, a TGATATGAT sequence showed strong homology with the TGTTATAAT consensus sequence of the −10 region of a promoter, as defined by Hawley and McClure (21). No sequence correctly spaced with respect to the −10 region showed strong homology with the −35 region consensus sequence (Fig. 1). Band c corresponded to a transcript having at its 5′ end a G residue at position 2263. Located 6 bp upstream, a TGCTACACT sequence also showed significant homology with the −10 region consensus sequence, but as with the longer transcripts, there was no notable sequence homology in the −35 region with the consensus sequence.

The pentanucleotide CTGGC occurred three times in this region 29 bp upstream of the longer transcripts' A start site and at 23 bp and 44 bp upstream of the shorter transcript start site. This sequence is part of the motif characteristic of *nif* promoters, CTGGYAYRNNNNTTGCA (19), but the three examples here showed only limited homology to the second part of the consensus (overlined in Fig. 1). We are attempting to define a role for these pentanucleotides in *crr* expression. The P2 promoter region thus appears to be composed of two promoters, P2-I and P2-II, where P2-I directs initiation of transcription at two distinct sites.

Northern blotting of the PTS operon transcripts. In order to visualize the transcripts accounting for the expression of *ptsH*, *ptsI*, and *crr*, Northern blotting experiments were performed. Four different probes, each covering part of only one of the three genes, were used and are illustrated in Fig. 2. Probe 1, a *Kpn*I-*Sma*I single-stranded DNA fragment, detected the *ptsH* transcripts. Probe 2, a *Xho*I-*Bgl*II double-stranded DNA fragment, detected the *ptsI* transcripts. Probe 3, a *Bgl*II-*Nru*I single-stranded DNA fragment, also hybridized with the *ptsI* transcripts. Probe 4, an *Hpa*I single-stranded DNA fragment, detected the *crr* transcripts.

As can be seen in Fig. 3A, all four probes hybridized with an mRNA of approx. 2.95 kb. This length is consistent with that predicted for a long polycistronic mRNA initiated at the P1 promoter, covering the entire PTS operon (the *ptsH*, *ptsI*, and *crr* genes) and ending at the terminator structure located after the *crr* gene.

Probe 1 (*ptsH*) revealed a second intense band of approx.

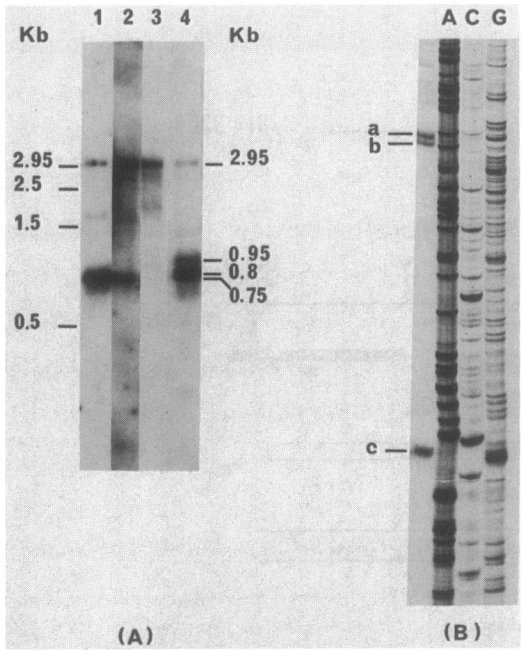

FIG. 3. (A) Northern blotting analysis with total-RNA extracts of strain TP2110. The procedures are described in Materials and Methods. Lane 1, Hybridization with probe 1, a fragment of *ptsH*. Lanes 2 and 3, Hybridization with probes 2 and 3, two fragments of *ptsI*. Lane 4, Hybridization with probe 4, the *crr* gene. On the left side, the positions and lengths of rRNA size markers are indicated. On the right side, sizes of the transcripts deduced from their relative migration rates are indicated. (B) S1 nuclease mapping of total-RNA extracts of strain TP2110 containing pDIA3206. The probe was the *Nru*I-*Hpa*I fragment labeled at the *Hpa*I site. The procedures are described in Materials and Methods. Location of the transcription start points was deduced from the lengths of the protected bands of the DNA probe, bands a, b and c. Their lengths were obtained by comparison on a DNA-sequencing gel with the ladders of the A, C, and G reactions of M13mp8 DNA, as shown on the right.

0.75 kb which was also revealed by probe 2 (*ptsI*). Probe 3, covering a DNA region located in the middle of the *ptsI* gene, did not reveal this latter band. This band of approx. 0.75 kb corresponds therefore to an mRNA, covering the *ptsH* gene and the beginning of the *ptsI* gene.

Probe 4 revealed, in addition to the long polycistronic mRNA, the existence of two transcripts. The most intense band corresponded to a transcript of approx. 0.8 kb, which is consistent with the transcription initiation site indicated by band c observed in the S1 mapping experiment and is probably the mRNA transcribed from the P2-II promoter. A slightly weaker band of approx. 0.95 kb was consistent with the transcription initiation sites indicated by the doublet of bands a and b observed in the S1 mapping experiment and is probably the mRNA transcribed from the P2-I promoter. An illustration of the observed transcripts of the *pts* operon is given in Fig. 2.

Expression of the *ptsH*, *ptsI*, and *crr* genes. Standard multicopy fusion vectors such as those constructed by Casadaban et al. (4) could not be conveniently used to monitor *pts* gene expression because of their high copy number. The *ptsI* gene seems to be toxic to high-copy-number vectors. In addition, ColE1 replicon derivatives are known to vary in copy number as a function of the growth medium or of the nature of the host strain, which would complicate interpretation of the quantitative data obtained. Instead, a series of low-copy-number vectors of the IncW incompatibility group were used, which were suitable for protein and operon fusions with *lacZ*, in which the copy number varied neither with the growth conditions used in this paper nor with the host strain (12). Plasmids carrying an operon fusion with the *ptsH* gene (pDIA3241) as well as its counterpart with *ptsI* (pDIA3242) are shown in Fig. 4. The plasmids carrying protein fusions with *ptsH* (pDIA3247), *ptsI* (pDIA3226), and *crr* (pDIA3238 and pDIA3239) are also shown (Fig. 4).

Expression of the *pts* genes as a function of cAMP-CAP. As can be seen from Table 2, the rate of synthesis of β-galactosidase in a Δ*cya* strain (TP2010) harboring the protein fusion pDIA3247 or pDIA3226 and the operon fusion pDIA3241 or pDIA3242 was enhanced threefold when cAMP was included in the growth medium. No enhancement was observed in the isogenic *cya*$^+$ strain TP2110 (Table 2). In the Δ*cya* Δ*crp* double mutant (TP2339), expression of *ptsH* and *ptsI* genes was no longer sensitive to cAMP. These data indicate that *ptsH* and *ptsI* transcription is positively controlled by the cAMP-CAP complex.

Expression of the *crr-lacZ* fusion of plasmid pDIA3239 (deleted of promoter P1) was not significantly changed in the presence of cAMP. Analysis of the effect of cAMP-CAP on the expression of the *crr-lacZ* protein fusion on plasmid pDIA3238 (carrying P1 and P2 promoters) was not possible since strain TP2010 containing plasmid pDIA3238 did not grow in the presence of cAMP. This lethality is presumably a consequence of the stimulation of transcription from P1 in the presence of cAMP-CAP producing toxic amounts of enzyme I, since it was not observed when pDIA3239 (deleted for P1) was used in the same growth conditions.

Expression of the *ptsH*, *ptsI*, and *crr* genes as a function of carbon source. As can be seen in Tables 3 and 4, the rate of synthesis of β-galactosidase in strain TP2110 containing the protein fusion pDIA3247 or pDIA3226 was increased approximately threefold when the sole carbon source of the growth medium was glucose over that in a medium containing glucose-6-P as the sole carbon source. Glucose appeared to exert its stimulating effect at the transcriptional level, since a similar enhancement was also observed with the two operon fusions pDIA3241 and pDIA3242 (Table 3).

The rate of synthesis of β-galactosidase in strain TP2110 containing the protein fusion pDIA3247 or pDIA3226 was compared during growth on glucose, fructose, pyruvate, and glucose-6-P (Table 4). The rate of synthesis of β-galactosidase was the same during growth on glucose-6-P or on pyruvate. A 1.5-fold enhancement of the rate of synthesis was observed during growth on fructose.

As can be seen in Table 4, no significant difference in the rate of synthesis of β-galactosidase of strain TP2110 containing either plasmid pDIA3238 or plasmid pDIA3239 was observed during growth in media containing glucose-6-P, pyruvate, or fructose. During growth on glucose, the rate of synthesis of β-galactosidase of strain TP2110 carrying pDIA3238 was slightly enhanced (1.3-fold), while that of TP2 110 carrying pDIA3239 did not change.

The effect of an analog of glucose, methyl-α-glucoside, on *ptsH-ptsI* expression was then tested. This glucose analog is taken up with high affinity and phosphorylated by the enzyme II^{Glc}-enzyme III^{Glc} system of the PTS. However, methyl-α-glucoside-6-P cannot be further metabolized and therefore cannot be used by the cell as a carbon source. The rate of synthesis of β-galactosidase was measured in a strain grown in a medium containing glucose-6-P or fructose as a carbon source, supplemented or not with 0.1% methyl-α-

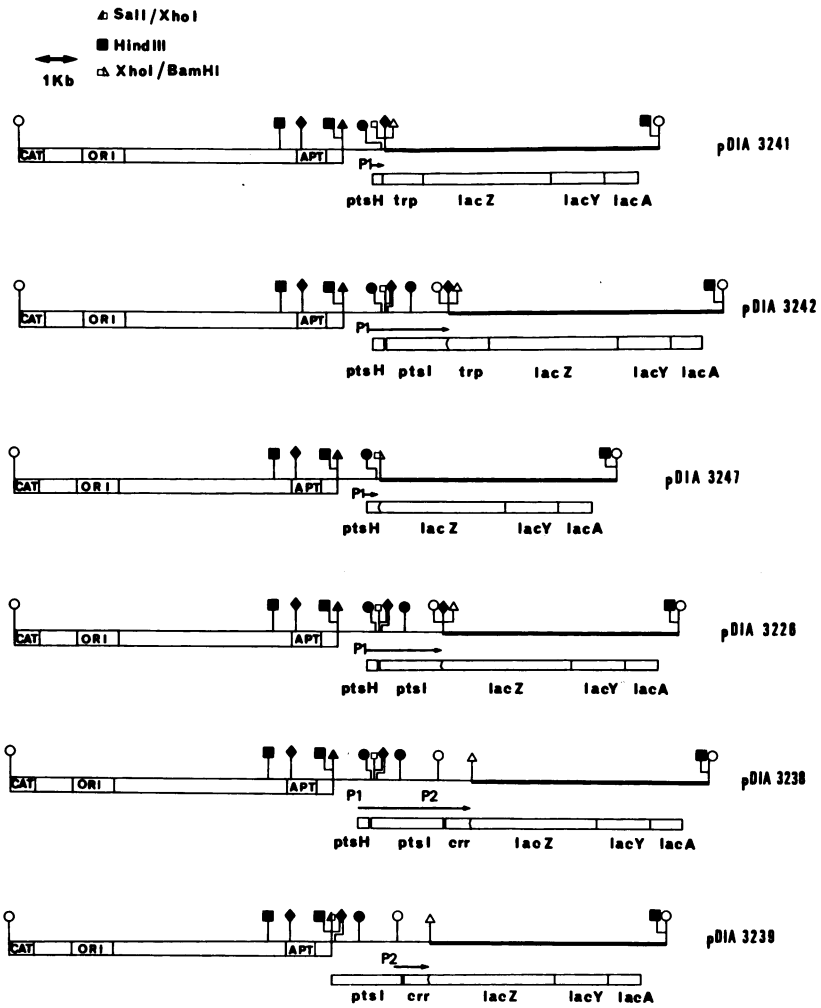

FIG. 4. Restriction map of plasmids pDIA3241 and pDIA3242 carrying operon fusions and plasmids pDIA3247, pDIA3226, pDIA3238, and pDIA3239 carrying protein fusions (for construction, see Materials and Methods). Heavy lines represent the *trp-lacZYA* or *lacZYA* genes. The open box represents vector DNA (pSA206). 'CAT, Chloramphenicol acetyltransferase gene devoid of its promoter; APT, gene conferring kanamycin resistance; ORI, replication origin of the plasmids. The direction of transcription of the *pts* genes is indicated by an arrow. P1 and P2 indicate the positions of the respective promoters. *SalI/XhoI*, Ligation site between *Sal*I and *Xho*I restriction sites; *XhoI/BamHI*, *Xho*I-*Bam*HI ligation site after formation of blunt ends by mung bean nuclease digestion. Other symbols are as in Fig. 2.

glucoside. As shown in Table 4, methyl-α-glucoside stimulated both *ptsH-lacZ* and *ptsI-lacZ* fusions to approximately the same extent as did glucose.

In order to investigate whether the glucose effect was mediated from outside, the effect of melibiose, which is metabolized to glucose and galactose inside the cell, was also tested. As indicated in Table 4, growth in a medium containing melibiose did not significantly change expression of any of the *ptsH-lacZ*, *ptsI-lacZ*, or *crr-lacZ* fusions.

Expression of the *ptsH-lacZ* and *ptsI-lacZ* fusion was examined in a strain carrying a *ptsG* mutation (in a genetic background very close to that of the strains used above, Table 1). Although the *ptsG* strain used was *lac*$^+$ (Table 1), we verified that there was no detectable β-galactosidase expressed from the chromosome. The plasmids pDIA3247 and pDIA3226 produced the same levels of β-galactosidase expression in strains TP2110 (Δ*lac*) and TP2503 (*lac*$^+$) (Table 3). In strain TP2504, deficient in enzyme II^{Glc}, expression of the *ptsH* and *ptsI* genes was not stimulated during growth on glucose (Table 3).

DISCUSSION

Analysis of the expression of the *ptsH*, *ptsI*, and *crr* genes of *E. coli* was performed by three complementary approaches: (i) the nucleotide sequence of a 3.15-kb region carrying these genes as well as adjacent sequences was determined; (ii) transcripts covering the region were identified by S1 mapping and Northern blotting experiments; and (iii) in vivo expression of the *ptsH*, *ptsI*, and *crr* genes was studied by gene fusions with *lacZ* and measurement of β-galactosidase activity.

The nucleotide sequence determination of the whole operon complements the results of an earlier study that allowed identification of the *ptsH* ORF (11). After we completed the nucleotide sequence of the *pts* operon, a study by Saffen et al. (38) provided similar information on a slightly shorter DNA fragment (2.85 kb). The overall nucleotide sequence is identical to ours except for the sequence of a few nucleotides located downstream from the *crr* ORF. Noteworthy features of the sequence have been described in the Results section.

TABLE 2. Effect of cAMP-CAP on β-galactosidase synthesis rate of gene fusions between *ptsH*, *ptsI*, or *crr* and *lacZ*[a]

Strain	Plasmid[c]	Relevant genotype		β-Galactosidase synthesis (U/mg)[b]	
		Strain	Plasmid	Without cAMP	With cAMP
TP2110	pDIA3247	*cya*$^+$	*ptsH-lacZ*	7,900	8,100
TP2110	pDIA3226	*cya*$^+$	*ptsI-lacZ*	1,700	1,600
TP2010	pDIA3247	Δ*cya*	*ptsH-lacZ*	3,900	12,000
TP2010	pDIA3226	Δ*cya*	*ptsI-lacZ*	800	2,500
TP2010	pDIA3238[d]	Δ*cya*	*crr-lacZ*	4,500	Toxic
TP2010	pDIA3239[e]	Δ*cya*	*crr-lacZ*	3,000	3,600
TP2339	pDIA3247	Δ*cya* Δ*crp*	*ptsH-lacZ*	2,700	2,650
TP2339	pDIA3226	Δ*cya* Δ*crp*	*ptsI-lacZ*	700	675
TP2339	pDIA3238[d]	Δ*cya* Δ*crp*	*crr-lacZ*	4,500	4,500
TP2339	pDIA3239[e]	Δ*cya* Δ*crp*	*crr-lacZ*	3,200	3,200
TP2010	pDIA3241	Δ*cya*	*ptsH-trp-lacZ*	850	2,800
TP2010	pDIA3242	Δ*cya*	*ptsI-trp-LacZ*	850	3,050

[a] The carbon source used in these experiments was fructose.
[b] Rates of synthesis are expressed in Pardee units per milligram (dry weight) of bacteria (33).
[c] pDIA3247, pDIA3226, pDIA3238, and pDIA3239 carry protein fusions; pDIA3241 and pDIA3242 carry operon fusions.
[d] Plasmid pDIA3238 carries a *crr-lacZ* fusion preceded by P1 and P2 promoters.
[e] Plasmid pDIA3239 carries a *crr-lacZ* fusion preceded by P2 promoters.

Codon usage seems to correspond to that in highly expressed genes of *E. coli* (23), consistent with the high expression of the *pts* operon genes.

The transcription pattern of the operon is complex. As illustrated in Fig. 2, there are three prominent transcripts. The 2.85-kb mRNA encompassing the whole operon is initiated at the P1 promoter and ends at the postulated terminator located after the *crr* gene. Two short transcripts are specific for *ptsH* and *crr*. The *ptsH*-specific transcript also expresses the 5′ end of the *ptsI* gene and is quite abundant, corresponding to 80% of the total RNA hybridizing with the *ptsH* probe in the Northern blotting experiment. S1 mapping of the site of initiation of *ptsH* transcription (11), as well as identification of the *cysK* ORF, located upstream from *ptsH* (S. Lévy, Ph.D. dissertation), strongly argue in favor of P1 as the only transcription start site for both the 2.85-kb and 0.75-kb transcripts covering the *ptsH* ORF. It is not known at present whether expression of the short *ptsH* transcript results from premature transcription termination or from RNA maturation. A possible signal for either event is the long palindromic structure present in the presumed 3′-end region of the transcript (Fig. 1, between positions 990 and 1052). Precise mapping of the 3′ end of this transcript is in progress.

Expression of the *crr* gene also involves multiple transcripts. The *crr* gene is expressed from the 2.85-kb transcript, initiated at P1, and from three short transcripts initiated in the P2 region. The S1 mapping experiment as well as the Northern blot revealed two species of mRNAs specific for the *crr* gene. The most abundant is the product of transcription from promoter P2-II; the other species is the product of transcription from promoter P2-I, which initiates transcription at two distinct positions. Relative intensities of the bands revealed by the *crr* probe on the Northern blot (Fig. 3A, probe 4) showed that approx. 85% of *crr* expression is due to transcription from the P2 region. The observation that the corresponding promoters do not fit the standard consensus used in *E. coli* argues in favor of specific controls operating in the P2 region. Experiments with *lacZ* fusions were carried out to confirm and clarify these observations.

Indeed, measurements of β-galactosidase synthesis rates in *crr-lacZ* fusions having both P1 and P2 regions (pDIA3238) or only the P2 region (pDIA3239) confirm that the *crr* gene is transcribed from both P1 and P2 but that the major contribution to *crr* expression comes from P2 (80%; Table 4). Cordaro and Roseman (5) may therefore not have detected the slight decrease of enzyme III^{Glc} activity in their Δ*cysK-ptsHI* strains, which were not deleted for the P2 region.

Expression of the *ptsH-lacZ* protein fusion (pDIA3247) is four to five times higher than that of the *ptsI-lacZ* protein fusion (pDIA3226) in all the growth conditions tested here. Mattoo and Waygood (27) also observed, by quantitative sugar phosphorylation assays and immunoelectrophoresis, that there was significantly more HPr than enzyme I in the cell. From our data, it can be deduced that this difference in level of expression is primarily due to the greater quantity of

TABLE 3. Effect of glucose on β-galactosidase synthesis rate of gene fusions between *ptsH* or *ptsI* and *lacZ*[a]

Strain	Plasmid	Relevant genotype		β-Galactosidase synthesis (U/mg)	
		Strain	Plasmid	Glucose-6-P	Glucose
TP2110	pDIA3247	Δ*lac*	*ptsH-lacZ*	5,700	15,000
	pDIA3226	Δ*lac*	*ptsI-lacZ*	1,100	3,300
	pDIA3241	Δ*lac*	*ptsH-trp-lacZ*	2,000	6,100
	pDIA3242	Δ*lac*	*ptsI-trp-lacZ*	2,150	6,050
TP2504	pDIA3247	*lac*$^+$ *ptsG*	*ptsH-lacZ*	4,100	5,150
	pDIA3226	*lac*$^+$ *ptsG*	*ptsI-lacZ*	1,100	1,075
TP2503	pDIA3247	*lac*$^+$	*ptsH-lacZ*	5,600	15,000
	pDIA3226	*lac*$^+$	*ptsI-lacZ*	1,000	3,000

[a] See Table 2, footnotes *b* and *c*.

TABLE 4. Rate of synthesis of β-galactosidase by strain TP2110 carrying *ptsH-lacZ*, *ptsI-lacZ*, and *crr-lacZ* fusions during growth on various carbohydrates[a]

Carbon source	β-Galactosidase synthesis (U/mg)			
	pDIA3247	pDIA3226	pDIA3238	pDIA3239
Glucose-6-P	5,700	1,100	4,000	3,150
Pyruvate	5,600	1,100	3,900	3,750
Fructose	7,700	1,650	4,550	3,350
Melibiose	7,000	1,200	3,000	2,350
Glucose	15,000	3,300	5,500	3,500
Glucose-6-P + methyl-α-glucoside	13,000	3,000	ND[b]	ND
Fructose + methyl-α-glucoside	23,100	5,300	ND	ND

[a] See Table 2 and footnotes for fusion carried by each plasmid and β-galactosidase activity explanation.
[b] ND, Not determined.

ptsH-specific transcript. Far fewer transcripts corresponding to the *ptsI* gene are detected because of the rather frequent occurrence of either premature transcription termination or RNA maturation at a site located within the *ptsI* ORF. However, it cannot be excluded that *ptsI* translation is also somewhat unefficient. Similar expression levels of the two operon fusions (carried by plasmid pDIA3241 and pDIA3242) are certainly due to the fact that in plasmid pDIA3242, the *lacZ* gene is fused to *ptsI* downstream of P2 promoter, and thus pDIA3242 measures transcription corresponding to *crr* rather than *ptsI*.

In addition to confirming the results on the transcriptional organization of this operon, measurements of β-galactosidase synthesis rates provide interesting data on its regulation. The *ptsH*, *ptsI*, and *crr* genes are expressed at high basal levels, but expression of *ptsH* and *ptsI* can be modulated threefold according to environmental conditions. This is in agreement with the observations of Saier and Roseman (40), Rephaeli and Saier (35), and Mattoo and Waygood (27), who measured the HPr and enzyme I levels under various growth conditions. Stimulation of *ptsH* and *ptsI* expression by the cAMP-CAP complex probably occurs by the well-documented mechanism of activation of transcription initiation; the DNA sequence located in the −35 region of the *ptsH* promoter (Fig. 1) shows significant homology with the so-called CAP site consensus (15). Similarly, transcription of the *ptsH* and *ptsI* genes is stimulated threefold during growth on glucose, a PTS carbon source, over growth on pyruvate or glucose-6-P. However, in contrast with the observations made by Rephaeli and Saier (35) or Mattoo and Waygood (27), we observed significantly less stimulation during growth on fructose than on glucose. These differences could be due to species (*S. typhimurium* versus *E. coli*) or strain differences. Neither the cAMP-CAP complex nor growth on glucose or fructose significantly affected *crr* expression from the P2 region (see plasmid pDIA3239 in Tables 2 and 4).

Thus, in the conditions tested, only modulation of transcription from P1 promoter could be demonstrated. Although the mechanism accounting for the positive transcriptional regulation of the *ptsH* and *ptsI* genes during growth on glucose remains puzzling, some important characteristics can be pointed out. First, *ptsH-ptsI* transcription was shown to be stimulated from without by glucose; this carbohydrate therefore behaves as an exogenous inducer. Indeed, no induction occurred during growth on glucose-6-P, which is the intracellular product of glucose uptake via the PTS. The nonmetabolized analog of glucose, methyl-α-glucoside, stimulated *ptsH-ptsI* expression approximatively to the same extent as glucose, showing that glucose itself, and not one of its degradation products, is the inducer. In addition, no stimulation is observed during growth on melibiose, whose catabolism produces intracellular glucose. Although this last result has to be taken with some caution, since the intracellular concentration of glucose during growth on melibiose is not known, it is in good agreement with the results of Rephaeli and Saier (35) showing no induction of HPr and enzyme I during growth on maltose, whose metabolism produces intracellular glucose and glucose-1-P. Dills et al. (13) proposed a model for a mechanism accounting for this exogenous induction. Second, an active enzyme II^{Glc}, which is the PTS permease specific for glucose transport, was found to be necessary for the *ptsH-ptsI* transcriptional stimulation of glucose to occur.

The complex transcriptional organization of the DNA region carrying the *ptsH*, *ptsI*, and *crr* genes reflects the complexity of the regulations affecting their expression. Although these three genes constitute an operon, *crr* expression is not regulated in the same way as the *ptsH-ptsI* genes. It could, however, be of some importance for the cell to maintain a high level of *crr* expression, since its product, enzyme III^{Glc}, regulates adenylate cyclase activity and thus the cAMP level, which is known to regulate, when complexed to CAP, the expression of a great number of genes.

The results discussed here have characterized the different transcripts of this operon and started to define modes of regulation by cAMP-CAP and glucose. Our present work is designed to elucidate the mechanism by which glucose acts from outside the cell to induce *ptsH-ptsI* operon expression.

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