JOURNAL OF BACTERIOLOGY, Mar. 1996, p. 1525–1531 Vol. 178, No. 6
0021-9193/96/$04.00+0
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Identical Transcriptional Control of the Divergently Transcribed *prtP* and *prtM* Genes That Are Required for Proteinase Production in *Lactococcus lactis* SK11

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Received 25 September 1995/Accepted 8 January 1996

We have investigated transcriptional regulation of the divergently transcribed genes required for proteinase production (*prtP* and *prtM*) of *Lactococcus lactis* SK11. Their promoters partially overlap and are arranged in a face-to-face configuration. The medium-dependent activities of both *prtP* and *prtM* promoters were analyzed by quantitative primer extension studies and β-glucuronidase assays with *L. lactis* MG1363 cells harboring transcriptional gene fusions of each promoter with the promoterless β-glucuronidase gene (*gusA*) from *Escherichia coli*. High-level production of *prtP*- or *prtM*-specific mRNAs was found after the growth of cells in media with low peptide concentrations, while increases in peptide concentrations resulted in an approximately eightfold decrease in mRNA production. Furthermore, *prtP* and *prtM* promoters exhibited similar efficiencies under different growth conditions. Deletion analysis of the *prt* promoter region showed that all the information needed for full activity and regulation of the *prtP* and *prtM* promoters is retained within a 90-bp region which includes both transcription initiation sites. An inverted repeat sequence positioned around the *prtP* and *prtM* transcription initiation sites was disrupted by either deletion or insertion of a small DNA sequence to analyze their effects on the activities of both *prtP* and *prtM* promoters. The mutations affected the activities of these promoters only marginally at low peptide concentrations but resulted in 1.5- to 5-fold derepression at high peptide concentrations. These results indicate that the expression of both *prtM* and *prtP* genes is controlled in an identical manner via a control mechanism capable of repressing transcription initiation at high peptide concentrations.

Lactococci are gram-positive bacteria used in the production of a great number of fermented milk products. *Lactococcus lactis* strains possess efficient proteolytic and transport systems to release and incorporate essential or growth-stimulating peptides and amino acids from milk proteins (19, 29). The cell envelope-associated serine proteinase (PrtP) is a key enzyme in the proteolytic system, as it performs the initial steps in the degradation of caseins that are essential for the rapid growth of lactococci in milk. In cooperation with intracellular peptidases, PrtP produces peptides and amino acids from casein that contribute to flavor development in fermented milk products (34).

Biochemical and genetic studies have shown that the proteinases of several *L. lactis* strains are highly related but differ in their caseinolytic specificities (14, 15). The complete nucleotide sequences of plasmid-located proteinase genes from *L. lactis* Wg2 (16), SK11 (36), and NCDO763 (13) have been determined. Comparison of the deduced amino acid sequences and further analysis revealed small differences that explained the variations in their caseinolytic specificities (8, 35). The proteinase genes of these strains have similar organizations, and further genetic studies have shown that two divergently transcribed genes, *prtP* and *prtM*, are required for the production of the active serine proteinase (5, 14, 15). The structural *prtP* gene encodes the proteinase precursor, with a size of approximately 200 kDa, that has homology to the subtilisin family of serine proteases (28, 36). The *prtM* gene encodes a *trans*-acting maturase, a lipoprotein of 33 kDa, that is required for the activation of the inactive proteinase precursor (9, 37).

Until now, a limited number of studies have dealt with the regulation of proteinase production in lactococci (3, 7, 11, 12, 20, 22). The results of these investigations showed that proteinase production is mainly dependent on medium composition. High levels of production were found in milk, while proteinase production in peptide- and amino acid-rich laboratory media was low. Recently, we analyzed medium-dependent regulation of proteinase gene expression by using transcriptional fusions of the *L. lactis* SK11 *prtP* and *prtM* promoters with the β-glucuronidase gene (*gusA*) from *Escherichia coli* (21). β-Glucuronidase production directed by either the *prtP* or *prtM* promoter is controlled at the transcriptional level by the peptide content of the medium and, to a lesser extent, by the growth rate. The levels of expression of both promoters in media that contained relatively low concentrations of peptides were high, while at increased peptide concentrations, their expression was repressed. In addition, the presence of specific dipeptides, such as prolylleucine or leucylproline, in inducive growth media negatively affected expression of the *prtP-gusA* fusion. Together, the results suggested that a regulation mechanism, encoded on the host chromosome, controls the transcriptional initiation of both the *prtP* and *prtM* promoters, most probably in identical fashion.

To elucidate the nature of this transcriptional control mechanism and the contribution of *cis* sequences, the *prt* promoter region was investigated in more detail. Here we describe quantitative primer extension analyses of the *prtP* and *prtM* promoters at different peptide concentrations, followed by deletion

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and mutation analyses of the *prt* promoter region. Together, the results point to the existence of a (common) repressor protein that negatively controls transcription initiation of both promoters at increased concentrations of specific peptides in the medium.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* MC1061 (4) and JM83 (33) were grown in L-broth-based media. *L. lactis* MG1363 (plasmid free, Lac^-, and Prt^-) was grown in glucose-M17 broth (GM17; Merck, Darmstadt, Germany) unless otherwise stated. For the assay of β-glucuronidase production, *L. lactis* cells were grown in whey-permeate medium (6) containing 1.9% (wt/vol) β-glycerophosphate supplemented with various concentrations of Casitone (0.1 to 2% [wt/vol]; Difco Laboratories, Detroit, Mich.) after precultivation in GM17. When needed, media contained chloramphenicol (10 μg/ml), erythromycin (5 μg/ml), ampicillin (50 μg/ml [*E. coli*]), or 0.5% (wt/vol) glucose.

DNA methodology, reagents, and enzymes. The isolation of plasmid DNA from *E. coli* and standard molecular techniques were performed by the methods of Sambrook et al. (26). The isolation of plasmid DNA from *L. lactis* and transformation of *L. lactis* strains were described previously (32, 36). Nucleotide sequence analysis of double-stranded plasmid DNA was performed by the dideoxy chain termination method (27). Oligonucleotides were synthesized on a Cyclone DNA synthesizer (Biosearch, San Rafael, Calif.). All enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.). *para*-Nitrophenyl-β-D-glucuronic acid was purchased from Clontech Laboratories, Inc. (Palo Alto, Calif.).

Plasmid constructions. Schematic representations of plasmid deletion and insertion derivatives are shown in Fig. 1. The construction of plasmids pNZ544 and pNZ554, in which the *gusA* gene is under the control of the *prtP* and *prtM* promoters, respectively, has been described before (21). Deletions upstream or downstream of the *prtP* promoter (pNZ544Δ190, pNZ544Δ260, and pNZ554Δ190) were achieved by using a strategy including PCR amplification. In each case, mutagenic primers specific for the desired DNA region and containing additional *Sal*I or *Eco*RI restriction sites were used in combination with primers specific for either the *gusA* gene or a vector sequence just upstream of the 0.35-kb promoter fragment in pNZ544 (21). PCR amplifications were performed as described previously (18) with a Thermocycler 60 (Biomed, Amstelstad, The Netherlands). Amplification was done in 30 successive cycles of melting the DNA at 93°C for 1 min, annealing at 50°C for 2.5 min, and elongation at 72°C for 2.5 min. The resulting PCR products were purified, digested with *Sal*I and *Eco*RI, cloned into pNZ544, and linearized with *Sal*I and *Eco*RI to replace the wild-type promoter fragment. The deletion in pNZ544Δ110 was achieved by replacing the 0.35-kb *Sal*I-*Eco*RI fragment of pNZ544 with its internal 0.25-kb *Sal*I-*Ssp*I fragment, after linearization of the plasmid with *Sal*I and *Eco*RI, and treating the *Eco*RI site with Klenow enzyme. Plasmids pNZ5441 and pNZ5442 were obtained by the insertion of a 6- or 10-bp DNA linker, respectively, into the *Ssp*I site downstream of the *prtP* promoter (at position +10). Since this site is not unique within pNZ544, this construction required an extra cloning step in pPL344, a pUC19 derivative carrying the 0.35-kb *Sal*I-*Eco*RI fragment. Plasmid pNZ5445 was obtained by PCR using a mutagenic primer with a 10-bp insertion immediately downstream of the *Ssp*I site. All constructs were verified by restriction enzyme and DNA sequence analyses of relevant regions.

Enzyme assays. Unless otherwise stated, *L. lactis* strains were grown to mid-log growth phase (A_{600}, 0.7). Cells were harvested, washed twice, and resuspended in GUS buffer (50 mM $NaHPO_4$, 10 mM β-mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100). Cell extracts of lactococcal cells were prepared with a bead beater, as described previously (25). Cell extracts (10 to 50 μl) were added to GUS buffer supplemented with 1 mM *para*-nitrophenyl-β-D-glucuronic acid as the substrate. The initial rates of β-glucuronidase activity were measured at 405 nm in a Cary 1E UV-visible spectrophotometer (Varian, Australia) with a thermostatically controlled cell compartment at 37°C. Protein concentrations were determined by the method of Bradford (2), with bovine serum albumin as the standard.

Primer extension analysis. RNAs were isolated from exponentially growing *L. lactis* cultures by the Macaloid method described by Kuipers et al. (17). Primer extension was performed by annealing 20 ng of oligonucleotides specific for the *gusA* gene to 15 μg of RNA as previously described (17). For the P*prtP*-*gusA* fusion (pNZ544), primer 18745 with sequence, 5′-GGGTTGGGGTTTCTTA CAGGACGTA, complementary to the 5′ end of *gusA* was used. For the P*prtM*-*gusA* fusion (pNZ554), primer 920916 with sequence, 5′-CATTAAGATAATA ATACTGGTCCGGAA, specific for multiple cloning sites just upstream of *gusA* was used. Primer extension products were quantified by using a PhosphorImager and Image Quant software programs (all from Molecular Dynamics).

RESULTS

Primer extension analysis. Previously, we described the construction of plasmids, pNZ544 and pNZ554, which carry transcriptional gene fusions of the promoterless *gusA* gene with the *prtP* and *prtM* promoters, respectively (Fig. 1) (21). In cells of *L. lactis* MG1363 harboring pNZ544 or pNZ554, β-glucuronidase production was gradually inhibited by increasing the concentrations of peptides, such as those present in Casitone, in the growth medium (21). An approximately 10-fold repression of β-glucuronidase activity was observed in the peptide- and amino acid-rich medium GM17 compared with that obtained in whey-permeate medium containing 0.1% Casitone (21). Quantitative primer extension assays were performed (i) to confirm this transcriptional control of the *prtP* and *prtM* promoters and (ii) to determine the location of transcription initiation under different growth conditions. Total RNA was isolated from MG1363 cells harboring pNZ544 or pNZ554 that were grown in GM17 or whey-permeate medium with increasing concentrations of Casitone (0.1 to 2%). Equal amounts of each RNA sample were annealed with antisense probes specific to the *gusA* gene. This experiment was carried out with excess primer to ensure that the intensities of cDNA products reflected the amount of mRNA present in the sample and hence the efficiencies of the promoters. For the *prtP-gusA* fusion (pNZ544) under all conditions, two major bands were visible on the autoradiogram (Fig. 2A); the upper band, with the highest intensity, corresponds to the transcription initiation site of the *prtP* gene determined previously (36), and is designated +1 here (Fig. 1). Although processing cannot be excluded, it is likely that the lower, less intense band is derived from a less efficient secondary start site, positioned 2 nucleotides downstream at position +3. For the *prtM-gusA* fusion (pNZ554), two major primer extension products were also visible under different conditions (Fig. 2B). The corresponding start sites mapped in the proteinase promoter region at positions +8 and +5 (on the opposite strand; corresponding to 240 and 237 nucleotides upstream of the ATG of the *prtM* gene, respectively [37]). For both promoters, a good correlation between the amounts of labeled cDNA products in the primer extension assay and the β-glucuronidase activities obtained previously (21) was observed. The highest labeling intensities were obtained with RNA samples isolated from whey-permeate cultures containing 0.1 to 0.5% Casitone. Increased Casitone concentrations resulted in steady decreases in mRNA levels. The lowest levels of expression were observed for samples from GM17 media (Fig. 2). These data clearly demonstrate that the expression of both *prtP* and *prtM* genes is regulated in an identical way at the transcriptional level via control of transcription initiation.

Deletion analysis of the promoter region. To localize the DNA sequences involved in medium-dependent regulation of the overlapping *prtP* and *prtM* promoters, the regions upstream and downstream of these promoters in pNZ544 were deleted. The construction of plasmid pNZ544Δ190 involved the deletion of 190 bp upstream of the *prtP* promoter, which started at −41 from its transcription start site and stretched out to the *Sal*I site of pNZ544 at −230 (Fig. 1). From pNZ544Δ190, the 70-bp downstream region beginning at +51 of the *prtP* transcription start site and stretching out to the *Eco*RI site at +120 just upstream of the *gusA* gene was also removed to obtain pNZ544Δ260. In both constructs, pNZ544Δ190 and pNZ544 Δ260, the −35 and −10 regions of both *prtP* and *prtM* promoters were not affected by these deletions (Fig. 1). Plasmids were introduced into *L. lactis* MG1363, and β-glucuronidase activities were determined under different conditions (Table 1). The deletion in pNZ544Δ190 had no effect on the activity and regulation of the *prtP* promoter, since the β-glucuronidase activities obtained after growth on whey-permeate medium containing 0.1 and 2% Casitone were similar to those of con-

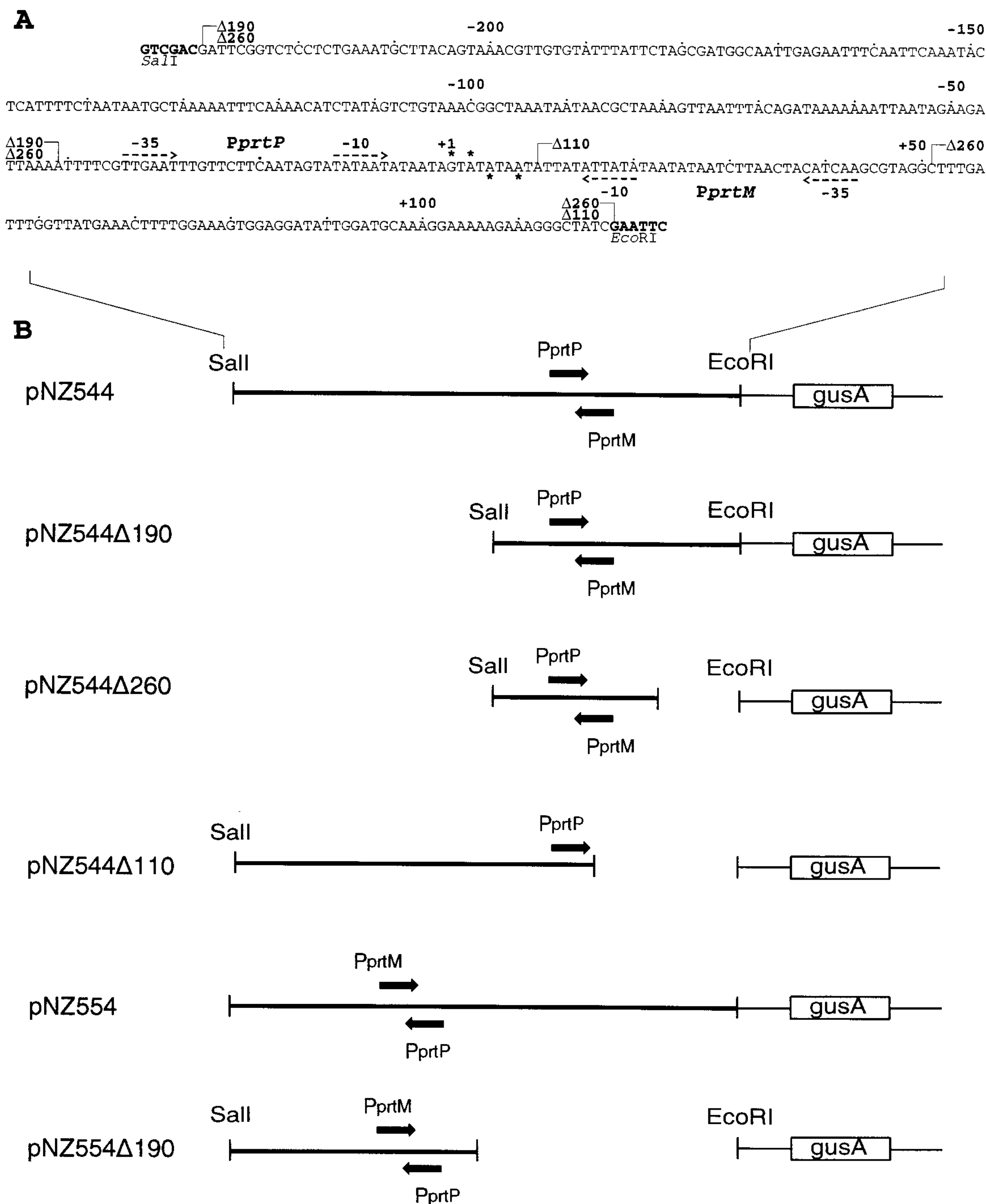

FIG. 1. (A) Nucleotide sequence of the 0.35-kb *Sal*I-*Eco*RI fragment encoding the oppositely orientated *prtP* and *prtM* promoters in plasmids pNZ544 and pNZ554 (21). The −35 and −10 promoter sequences of both promoters are indicated with horizontal arrows over (*prtP*) and under (*prtM*) the nucleotide sequence. Transcription initiation sites are indicated by asterisks (see text for details). The exact positions and extents of the different deletions (Δ110, Δ190, and Δ260) present in pNZ544 and pNZ554 derivatives are given. (B) Schematic representations of transcriptional fusions of *prtP* (pNZ544), *prtM* (pNZ554), and their deletion derivatives to the promoterless *gusA* gene encoding β-glucuronidase. Promoter regions are indicated by bold lines, while promoters are represented by solid arrows.

trol pNZ544. The induction ratios calculated for pNZ544Δ190 and the wild type were 5.3 and 5.6, respectively (Table 1). Primer extension analysis using RNA derived from cultures of this deletion variant confirmed these findings. A 4.2-fold difference in the intensities of cDNA bands in low- and high-concentration Casitone media was found, which is in good correlation with the data obtained in the β-glucuronidase assay (Fig. 3A; Table 1). Furthermore, the location of transcription initiation was not changed by this deletion. In contrast, the β-glucuronidase levels for pNZ544Δ260 in both media were about 50-fold lower than the control values and therefore did not allow accurate estimation of the induction ratio (Table 1). However, primer extension analysis of this construct showed that neither the transcription initiation site nor regulation of the *prtP* promoter was affected, as both the labeling intensities of cDNA bands in media with high and low levels of peptides

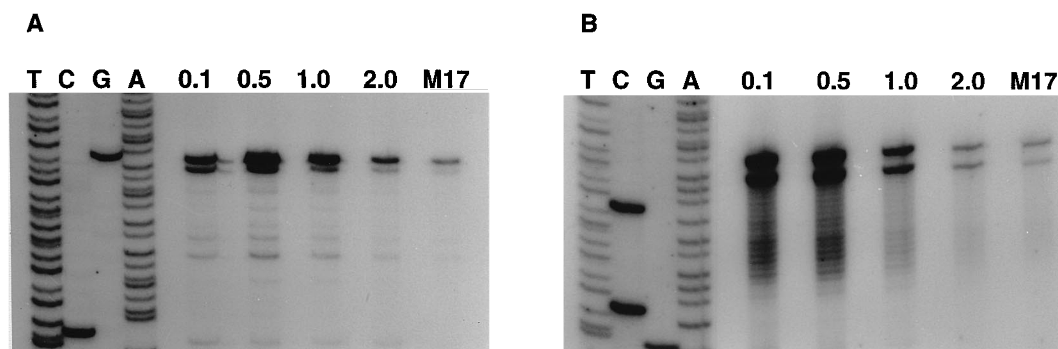

FIG. 2. Primer extension analysis of the *prtP* and *prtM* promoters with MG1363 cells harboring pNZ544 (*prtP*) (A) or pNZ554 (*prtM*) (B). An oligonucleotide complementary to the *gusA* coding strand was used in this assay with equal amounts of total RNA isolated from cells after growth on peptide-rich GM17 (M17) medium or whey-permeate medium with different concentrations (0.1, 0.5, 1.0, and 2.0%) of the peptide source Casitone. Primer-extended products were electrophoresed in parallel with a sequence ladder (lanes T, C, G, and A) generated with the same primer.

and their ratios were comparable to those obtained for pNZ544Δ190 (Fig. 3B; Table 1). From these data, we conclude that all the information needed for full activity and regulation of the *prtP* promoter is contained within a 90-bp region stretching from −40 to +50 relative to the main *prtP* transcription initiation site.

Similarly, the deletion of a 190-bp DNA sequence downstream of the *prtM* promoter (starting at +49 from the first *prtM* transcription initiation site and stretching to the *Eco*RI site just upstream of the *gusA* gene on pNZ554 [Fig. 1]) in plasmid pNZ554Δ190 affected neither the activity nor regulation of the *prtM* promoter. The β-glucuronidase activities of cells harboring pNZ554Δ190 were similar to those of cells harboring wild-type pNZ554 after the growth of cells in 0.1 and 2% Casitone media (Table 1).

To confirm its role in regulation, the 90-bp promoter region was further reduced by deletion of the *prtM* −35 and −10 promoter sequences on the complementary strand. Plasmid pNZ544Δ110 was obtained from pNZ544 by deletion of the 110-bp region downstream of the *prtP* promoter, ranging from position +10 to the *Eco*RI site at position +120 just upstream of *gusA* (Fig. 1). The β-glucuronidase activity obtained after the growth of MG1363 cells harboring this deletion derivative on 0.1% Casitone medium was approximately 2.5 times lower than that of cells harboring wild-type pNZ544 (Table 1). Regulation of the *prtP* promoter also seemed to be reduced in cells containing pNZ544Δ110, as the β-glucuronidase levels obtained after growth in 2% Casitone medium were similar to those of the wild type. The ratio of β-glucuronidase activities on high- and low-concentration Casitone media was only 1.4-fold (Table 1). Primer extension analysis of this deletion derivative (see below) showed similar ratios of cDNA levels on both media (Table 1). These results indicate that the deletion included *cis* control sequences important for *prtP* gene expression.

Mutagenesis of an inverted repeat within the *prtP* promoter results in a derepressed phenotype. A nearly perfect 18-bp inverted repeat is present within the 90-bp *prt* promoter region, overlapping the transcription initiation sites of both the *prtP* and *prtM* promoters (Fig. 4). As half of this repeat is removed by the deletion in pNZ544Δ110, rendering a deregulated-expression phenotype, we reasoned that these sequences could be involved in the recognition of a dedicated regulator protein. To investigate the role of these sequences in the control of the *prtP* (and *prtM*) promoter in more detail, we studied the effects of small (6- to 10-bp) insertions at different positions in the right (downstream) half of this repeat on the regulation of *prtP* promoter expression. In this way, the symmetry of the repeat would be disturbed without affecting the *prtP* promoter region upstream of the initiation site and hence its activity. To avoid a drastic change in the relatively high AT content (74%) of this promoter region, the insertions consisted of A's and T's only. A 6-bp insertion 9 bp downstream of the major *prtP* transcription start site (position +10) resulted in a 30% increase in β-glucuronidase activity compared with the activity levels of the wild-type construct after the growth of cells in 0.1% Casitone medium (Fig. 4; Table 2). However, in 2% Casitone medium a threefold increase in activity compared with wild-type activity levels was observed; the calculated induction ratio for pNZ5441 was 2.7, instead of the ratio (5.9) found for the wild type (Table 2). A similar 10-bp insertion (pNZ5442) resulted in activity levels comparable to those of pNZ5441 in 0.1% Casitone medium, while its activity in 2% Casitone medium showed a more-than-fivefold increase compared with the β-glucuronidase activity of the wild type in the same medium; the induction ratio was only about 1.5 (Fig. 4; Table 2). Remarkably, the 10-bp insertion at position +13 in pNZ5445 did not alter the β-glucuronidase activity in either medium; the induction ratio was similar to that of the wild type (Fig. 4; Table 2).

TABLE 1. β-Glucuronidase activities in extracts of *L. lactis* MG1363 cells harboring pNZ544 or deletion derivatives[a]

Plasmid	Mean β-glucuronidase activity ± SD (nmol min^{-1} mg of $protein^{-1}$)[b]		Induction ratio[c]
	0.1% Casitone	2.0% Casitone	
pNZ544	37.2 ± 4	6.6 ± 1	5.6 (5.4)
pNZ544Δ190	42.8 ± 6	8.1 ± 2	5.3 (4.2)
pNZ544Δ260	0.7 ± 0.05	0.06 ± 0.02	11.5 (3.9)
pNZ544Δ110	13.7 ± 2	10.1 ± 2	1.4 (1.5)
pNZ554	64.0 ± 4	10.2 ± 3	6.3 (6.2)
pNZ554Δ190	68.0 ± 4	10.5 ± 1	6.5 (ND)

[a] Grown in whey-permeate medium with the indicated concentration of Casitone.

[b] The β-glucuronidase activity in extracts of cells harboring pNZ555, a promoterless derivative of pNZ544 (21), in either medium was lower than 0.03 nmol min^{-1} mg of $protein^{-1}$.

[c] Induction ratios are the quotients of the β-glucuronidase activities obtained on whey-permeate media containing 0.1 and 2.0% Casitone. Data in parentheses are the induction ratios determined from cDNA intensities in primer extension analysis (see text). ND, not determined.

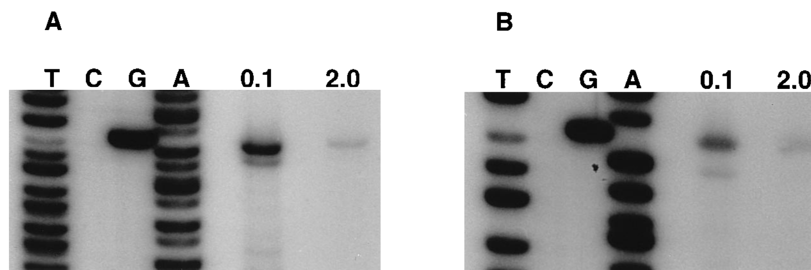

FIG. 3. Primer extension analysis of the *prtP* promoter with MG1363 cells harboring the pNZ544 deletion derivatives pNZ544Δ190 (A) and pNZ544Δ260 (B). An oligonucleotide complementary to the *gusA* coding strand was used in this assay with equal amounts of total RNA isolated from cells after growth on whey-permeate medium with 0.1 or 2.0% Casitone. Primer extended products were electrophoresed in parallel with a sequence ladder (lanes T, C, G, and A) generated with the same primer.

Primer extension analysis was performed with RNA samples derived from cells harboring these pNZ544 insertion derivatives or deletion derivative pNZ544Δ110 to verify the data from the β-glucuronidase assay (Fig. 5). For all constructs, high mRNA levels were obtained after the growth of cells in 0.1% Casitone medium. After growth in 2% Casitone medium, the mRNA levels in cells harboring construct pNZ5445 or wild-type pNZ544 were low, while the mRNA levels in cells harboring construct pNZ5441, pNZ5442, or pNZ544Δ110 were significantly increased (Fig. 5). The intensities of cDNA bands were quantified, and the cDNA product of the constitutive promoter of the chromosomally encoded *usp-45* gene was used as an internal standard (30). The results of this quantification for all four constructs were in good correlation with the data of the β-glucuronidase assay, as concluded from comparisons of the induction ratios calculated in both experiments (Table 2). Moreover, analysis showed that the transcription initiation site was not changed as a result of insertions.

With the exception of pNZ544Δ110, in all constructs the *prtM* promoter, positioned on the complementary strand, is still intact (Fig. 1A and 4). All three insertions discussed above disrupt the DNA sequence between the −10 region of the *prtM* promoter and its transcription initiation site. To analyze the effects of these insertions on the regulation of the *prtM* promoter, its activity was quantified by primer extension analysis, as described above. The *prtM* promoter was still active in 0.1 and 2% Casitone media, although the promoter efficiencies in insertion derivatives were slightly lower than that of the wild-type promoter on pNZ544 (data not shown). As expected, transcription initiated 6, 10, and 10 nucleotides upstream of the wild-type initiation site in cells harboring pNZ5441, pNZ5442, and pNZ5445, respectively (data not shown). As shown for the *prtP* promoter, the differences in *prtM* mRNA levels isolated from cells harboring pNZ5441 and pNZ5442 after growth in 0.1 and 2% Casitone media were weaker. The regulation pattern observed for construct pNZ5445 was similar to that of wild-type pNZ544 (data not shown). Altogether, the results clearly demonstrate that specific disruptions of the inverted repeat region, particularly those close to transcription initiation sites, cause derepression of the expression of the *prtP* and *prtM* promoters under peptide-rich conditions.

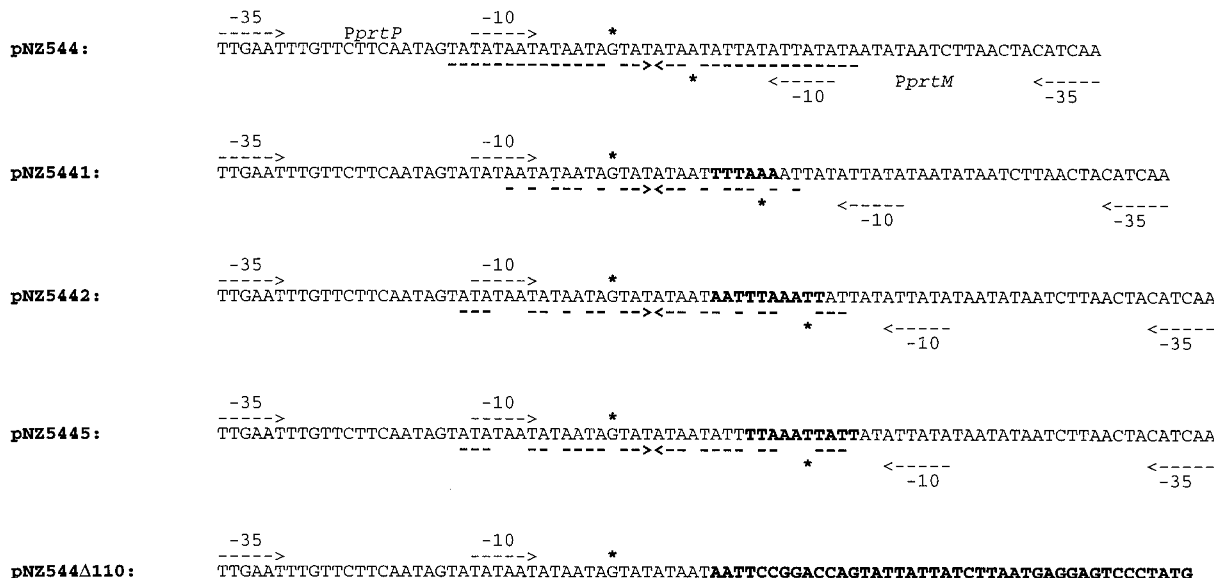

FIG. 4. Nucleotide sequences of the *prt* promoter regions in wild-type pNZ544, deletion derivative pNZ544Δ110, and insertion derivatives pNZ5441, pNZ5442, and pNZ5445. The *prtP* and *prtM* −35 and −10 promoter sequences, represented by small broken arrows over (*prtP*) and under (*prtM*) the sequence, as well as their main transcription initiation sites (asterisks) are shown. The palindromic symmetry around the transcription initiation sites in the wild type and each of the mutated plasmids is indicated by broken, bold arrows. Insertions and the multiple cloning site sequence between the *prtP* promoter and *gusA* in pNZ544Δ110 are in bold.

TABLE 2. β-Glucuronidase activities in extracts of *L. lactis* MG1363 cells harboring pNZ544 or derivatives[a]

Plasmid	Mean β-glucuronidase activity ± SD (nmol min^{-1} mg of $protein^{-1}$)		Induction ratio[b]
	0.1% Casitone	2% Casitone	
pNZ544	37.2 ± 4	6.1 ± 1	5.9 (4.2)
pNZ5441 (6-bp insertion)	47.2 ± 7	17.3 ± 1	2.7 (2.3)
pNZ5442 (10-bp insertion)	48.8 ± 3	32.7 ± 2	1.5 (2.2)
pNZ5445 (10-bp insertion)	38.8 ± 4	5.5 ± 1	7.1 (5.2)

[a] Grown in whey-permeate medium with the indicated concentration of Casitone.

[b] Induction ratios are the quotients of the β-glucuronidase activities obtained on whey-permeate media containing 0.1 and 2.0% Casitone. Data in parentheses are the induction ratios determined from cDNA intensities in primer extension analysis (see text).

DISCUSSION

We have investigated transcriptional regulation of the oppositely directed genes, *prtP* and *prtM*, required for proteinase production in *L. lactis* by quantitative primer extension studies using transcriptional fusions of the promoterless *gusA* gene from *E. coli* with the *prtP* and *prtM* promoters. mRNA preparations were derived from cells after growth in media with various peptide concentrations. The highest *prtP*- and *prtM*-specific mRNA levels were produced in low-concentration peptide media, while increases in peptide concentrations resulted in decreased mRNA levels. Comparisons of the *prtP*- and *prtM*-specific mRNA levels produced in media with low- and high-level peptide contents showed an approximately eightfold difference. These results are in good agreement with the differences observed previously in β-glucuronidase activity assays performed with both transcriptional fusions (21). The results showed that the production of β-glucuronidase directed by *prtP* and *prtM* promoters was repressed in growth media with high peptide concentrations. Together with the present data, it is convincingly shown that medium-dependent expression of both *prtP* and *prtM* promoters is controlled at the level of transcription initiation. The results of primer extension studies also enabled us to map the transcription start site of the SK11 *prtP* and *prtM* promoters more precisely. The *prtM* start site was found to be positioned on the complementary strand only a few base pairs from the *prtP* transcription start site.

Remarkably, the *prtP* and *prtM* promoters exhibited similar, if not identical, efficiencies under different growth conditions, as concluded from previous β-glucuronidase experiments (21) as well as from the primer extension analysis described above. Identical control of *prtP* and *prtM* expression may reflect the need of cells for a coordinate (1:1) transcription of the *prt* genes to ensure a fixed ratio of their gene products. In this respect, a divergent arrangement of both promoters may be particularly advantageous by providing for an efficient system that can be controlled by the binding of a regulator which affects transcription in both directions.

Deletion analysis of the *prt* promoter region showed that a 90-bp region containing the partially overlapping *prtP* and *prtM* promoters is sufficient for full activity and regulation of both promoters. In this respect, the apparent discrepancy between the (high) *prtP*-specific mRNA levels and (low) β-glucuronidase activity found in cells harboring pNZ544Δ260 (Fig. 3; Table 1) may be the result of blockage at the level of translation initiation caused by steric hindrance or by the formation of partially duplex RNA molecules (23, 24).

All *L. lactis prt* genes analyzed so far contain a region of extensive dyad symmetry which is positioned around the transcription initiation sites of the *prtP* and *prtM* promoters (Fig. 4), though minor differences in the extent of symmetry exist in different strains (13, 31, 36). Our results indicate that specific sequences within this repeat structure may be involved in the regulation of expression of both *prtP* and *prtM* genes. A further deletion of the promoter region starting just downstream of the *prtP* promoter (position +10), which removed half of this dyad repeat and the *prtM* promoter completely, resulted in almost constitutive expression of the *prtP* promoter, as can be concluded from the derepression in β-glucuronidase activity and increased mRNA levels observed in cells harboring pNZ544Δ110 grown at high peptide concentrations (Table 1; Fig. 5). Further evidence to support a regulatory role for sequences within this repeat was obtained by insertion mutagenesis with small DNA linkers that disrupted the palindrome symmetry. In two cases (pNZ5441 and pNZ5442), insertions resulted in strong derepression of β-glucuronidase activity and increased mRNA levels at high peptide concentrations (Table 2; Fig. 5), while the activities of the *prtP* and *prtM* promoters at low peptide concentrations were hardly affected. Taken together, these results strongly indicate that within this region of dyad symmetry, *cis* sequences close to the initiation sites are involved in the recognition or binding of a regulator protein. Similar inverted repeat sequences have been found in or near the promoter regions of many prokaryotic genes and are often involved in the recognition and binding of transcription factors (1, 10, 32). Considering the position of the inverted repeat, i.e., overlapping both transcription initiation sites of the *prtP* and *prtM* promoters, in combination with the observed constitutive expression in cells harboring the *prt* promoter mutants discussed above, we speculate that this regulator protein is a repressor. From our previous work, it was concluded that specific dipeptides (such as prolylleucine) may act, directly or

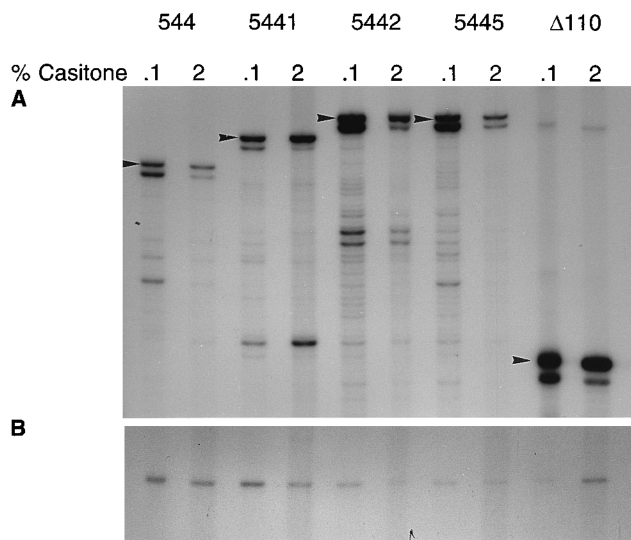

FIG. 5. Primer extension analysis of the *prtP* promoter with MG1363 cells harboring pNZ544, deletion derivative pNZ544Δ110, and insertion derivatives pNZ5441, pNZ5442, and pNZ5445. (A) An oligonucleotide complementary to the *gusA* coding strand was used in this assay with equal amounts of total RNA isolated from cells after growth on whey-permeate medium with 0.1 or 2.0% of the peptide source Casitone. The significant primer extended products (with lengths of 188 [pNZ544], 194 [pNZ5441], 198 [pNZ5442], 198 [pNZ5445], and 78 [pNZ544Δ110] bases) have been indicated by arrowheads. (B) As an internal control, equal amounts of each RNA sample were also analyzed with an oligonucleotide specific for the *usp-45* gene (30).

indirectly, as effectors of this regulator (21). The present data suggest that upon the interaction of effector molecules, the affinity of the putative repressor to the *prt* operator region is increased, resulting in repression of transcription.

A similar organization of the *prtP* and *prtM* genes was found in *L. lactis* WG2; both transcription initiation sites were mapped and found to be in close proximity to each other and positioned on opposite strands (31). In the same study, the strengths of these promoters were analyzed with promoter-probe vectors; both were found to be weaker than other previously isolated lactococcal promoters (e.g., promoters P32 and P59). However, they did not take into consideration the (potential) medium dependency of the activities of the promoters, as the analysis was carried out with cells grown only under peptide-rich conditions (GM17). Therefore, it is possible that the efficiencies of both WG2 *prt* promoters were underestimated as a result of repression of transcription under these conditions.

Our further research is aimed at the identification of the putative repressor and isolation of its gene. The latter will allow us to overproduce the repressor protein for use in DNA-binding studies with the *prt* promoter region in order to identify the exact position(s) of the DNA stretch(es) involved in regulation. The construction of regulator mutants will enable us to determine whether this repressor is specific for the *prt* genes. Recent work in our laboratory has shown that expression of genes coding for the aminopeptidase PepN and the X-prolyl-dipeptidyl-peptidase PepXP is regulated by the peptide content of the medium, as described for the *prt* genes (22).

ACKNOWLEDGMENTS

We thank Oscar Kuipers, Elaine Vaughan, and Roland Siezen for their continuous interest in our investigations and for critically reading the manuscript.

This research was partially financed by European Union contract BIO2-CT94-3055.

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