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Transcriptional Mapping and Nucleotide Sequence of the *Listeria monocytogenes hlyA* Region Reveal Structural Features That May Be Involved in Regulation

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DNA sequence analysis of the regions adjacent to the *hlyA* gene, which encodes listeriolysin O, an essential virulence factor of *Listeria monocytogenes*, revealed the presence of two open reading frames (ORFs): ORF D located 304 base pairs downstream from *hlyA*, and ORF U located 224 base pairs upstream from and in opposite direction to *hlyA*. Promoter mapping performed with RNAs extracted from cells growing exponentially in rich medium showed that the three ORFs are independently transcribed. *hlyA* is transcribed from two promoters separated by 10 base pairs (P1 *hlyA* and P2 *hlyA*). ORF U is transcribed in the opposite direction from an adjacent promoter. These two promoter regions are separated by a palindromic sequence T-T-A-A-C-A-A/T-A/T-T-G-T-T-A-A. This palindrome was also found upstream from the ORF D promoter, suggesting that all three genes are similarly regulated.

Listeria monocytogenes is a gram-positive bacterium responsible for severe infections (meningitis, septicemias) and abortion in humans and several animal species (34). It is one of the best models to study the virulence of facultative intracellular pathogens. *L. monocytogenes* survives and replicates in macrophages (23), and control of the infection requires the emergence of a T-cell-mediated response leading to macrophage activation. For this reason, *Listeria* infection in mice has been widely used to study induction of T-cell-mediated immunity (21).

Taxonomic studies established that the genus *Listeria* includes several species, *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, *L. innocua*, *L. murrayi*, and *L. grayi* (34), of which only the first two are pathogenic, *L. ivanovii* essentially affecting animal species. The low G+C content and 16S RNA sequence analysis (22) indicated that this genus is closely related to the genera *Bacillus*, *Erysipelothrix*, *Lactobacillus*, and *Streptococcus* (34).

Due to the good animal model provided by the experimental murine infection, genetic studies were initiated to identify the virulence factors of this intracellular pathogen (17, 20, 30). These studies took advantage of conjugative transposons initially isolated in streptococci, such as Tn*1545* (10) and Tn*916* (15), which were used to mutagenize genes onto the bacterial chromosome. Recently, we cloned the *hlyA* gene coding for listeriolysin O and showed its essential role in intracellular survival and therefore in virulence of *L. monocytogenes* by gene complementation of a nonhemolytic Tn*917*-induced mutant (9a, 25, 26, 41). The current hypothesis for the role of listeriolysin O is that this secreted thiol-activated protein would help the bacterium to escape from the phagosome compartment to the cytosol, where it can freely replicate. This hypothesis is strongly supported by the fact that, whereas both the wild type and nonhemolytic mutants are able to invade Caco2 cells, the latter are unable to escape from the phagosome (16) and to survive intracellularly.

Two observations indicate that listeriolysin O expression must be regulated and not constitutively expressed: (i) it was reported that there is an inverse correlation between iron concentration in the medium and the hemolytic activity in the culture supernatants (11) and (ii) no increase in hemolytic titer was observed when a multicopy plasmid carrying only the *hlyA* gene was introduced in a wild-type strain (9a). To understand how *hlyA* is transcribed and regulated, we determined the nucleotide sequence of the 4.2-kilobase region containing the 1.5-kilobase *hlyA* gene. This sequence revealed the presence of two open reading frames (ORFs), one (ORF D) located downstream from *hlyA* and in the same orientation and another (ORF U) oriented in the opposite direction and located upstream from *hlyA*. To precisely describe the transcriptional organization of this region, we also mapped the promoters of *hlyA* and those of ORF U and ORF D. The three genes are transcribed independently. *hlyA* appears as a monocistronic unit. ORF U and the *hlyA* promoter regions are separated by a perfect 14-base-pair (bp) palindromic element. The fact that this same palindromic sequence is found upstream from ORF D suggests that expression of the three genes is similarly regulated.

MATERIALS AND METHODS

Strains, plasmids, and culture media. The *L. monocytogenes* strain used in this study was LO28, a wild-type isolate (26). Plasmid pUC18 (43) and phage M13mp18 (27) were used to clone DNA fragments in *Escherichia coli* MC1061 [F$^-$ *araD139* Δ(*ara leu*)*7696* Δ*lacY74 galU galK hsr hsm strA*] (9) or TG1 [K-12, Δ(*lac-pro*) *supE thi hsdD5* F′ *traD36 proAB lacI*^{q} *lacZ* ΔM15] (T. Gibson, Medical Research Council, Cambridge, England), respectively. *E. coli* strains were grown at 37°C in LB medium. For strains containing pUC derivatives, ampicillin was added at a final concentration of 25 μg/ml in liquid and 100 μg/ml in solid medium. Strain TG1 was routinely plated on minimal medium containing 0.2% (wt/vol) glucose and grown at 37°C. Isolated colonies were used to inoculate 2×YT liquid medium (tryptone [16 g/liter; Difco Laboratories, Detroit, Mich.], yeast extract [10 g/liter; Difco], sodium chloride [5 g/liter]) for overnight cultures, which were subsequently diluted for

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infection with M13 derivatives or for transformation. *L. monocytogenes* strains were grown in brain heart infusion broth or agar (Difco) at 37°C.

Chemicals and enzymes. Restriction enzymes and ligase were purchased from Amersham Corp. (Les Ulis, France), Boehringer (Mannheim, Federal Republic of Germany), or Genofit SA (Geneva, Switzerland) and were used as recommended by the manufacturers. [^{35}S]dATP (800 Ci/mmol) and [^{32}P]ATP (3,000 Ci/mmol) were purchased from Amersham.

DNA techniques. Plasmid DNA was purified by ultracentrifugation in cesium chloride gradients (24). Rapid preparation of plasmid DNA was performed by the method of Birnboim and Doly (7). DNA fragments were purified (26) and recombinant DNA techniques were performed (24) as previously described. Hybridizations were performed on Hybond-N nylon membranes (Amersham). DNA probes were labeled by the multiprime labeling system (kit RPN.160 1Y; Amersham). Oligonucleotide primers, synthesized by using the phosphoramidite method (3) with a 380D DNA synthesizer (Applied Biosystems, Foster City, Calif.) were provided by the Unité de Chimie Organique, Institut Pasteur. They were 5′ labeled with [γ-^{32}P]ATP (24) and purified on NACS Prepac Cartridge 1526 NP (Gibco-BRL, Cergy-Pontoise, France). The DNA sequence was determined by the Sanger et al. technique (33) with the Sequenase kit (U.S. Biochemicals, Cleveland, Ohio) and oligonucleotide primers derived from the sequence. Plasmid DNA sequencing was performed as follows. Plasmid DNA (1 to 2 μg) was incubated for 5 min at room temperature in 20 μl of denaturation solution (0.2 M NaOH, 0.2 mM EDTA), ethanol precipitated, and directly sequenced as an M13 derivative.

RNA isolation. Total cellular RNAs were extracted from *Listeria* cultures in exponential growth (A_{600} = 0.7) as described previously (19), except that double-strength Kirby mixture was used. In the case of *L. monocytogenes*, yields were very low (50 μg/500 ml).

Promoter mapping. Both primer extension with reverse transcriptase mapping (6, 18) and S1 nuclease mapping (1) were used. For primer extension, total cellular RNAs (10 μg) were hybridized with 5′-labeled oligonucleotide primer (0.05 pmol) in 5μl of H_2O by boiling the reaction mixture for 5 min, followed by slow cooling to 42°C. Then 1 μl of 0.5 mM dATP, dGTP, dCTP, and dTTP, 0.5 μl of reaction buffer (50 mM Tris hydrochloride [pH 8.3], 500 mM potassium chloride, 100 mM magnesium chloride, 10 mM dithiothreitol), and 9 U of avian myeloblastosis virus reverse transcriptase (Boehringer) were added to the hybridization mixture, which was then incubated for 30 min at 42°C and inactivated at 75°C for 10 min. In some controls, 0.5 μl of RNase solution (10 mg/ml) was added, and the sample was incubated for 1 h at 37°C. It was then twofold diluted in sequence loading buffer (24), denatured for 3 min at 90°C, and loaded on a 6% polyacrylamide–7.6 M urea sequencing gel.

For S1 mapping, single-stranded ^{32}P-labeled DNA probes were prepared by the modified prime-cut method (5, 13) with the same oligonucleotide primer as in the reverse transcription reaction. Before S1 protection studies, RNA samples (10 μg) were coprecipitated with ^{32}P-labeled probes, suspended in 30 μl of formamide containing 50% (vol/vol) 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Sigma, La Verpilliere, France)–480 mM sodium chloride–2.5 mM EDTA, denatured by heating at 75°C for 5 min, and incubated overnight at 42°C. Samples were divided in three parts, diluted 30-fold with S1 buffer (30 mM sodium acetate, 250 mM sodium chloride, 1 mM zinc sulfate [pH 4.5]). Unprotected probes were digested for 15 min at 37°C with 0, 2, and 100 U of S1 in each third of the sample, respectively. After ethanol precipitation, protected fragments were processed and analyzed on the same calibrated sequencing gel as for reverse transcriptase mapping samples. Sequence reactions obtained with the recombinant M13 single-stranded DNA and the oligonucleotide primer used in the probe preparation were loaded on the same gel and used to identify the transcription starting point. Samples were electrophoresed at 50 W for 3 h to determine the size of the primer extension and S1 digestion reaction products. Bands were examined by autoradiography.

Northern blotting. Formaldehyde agarose gel electrophoresis was performed as described previously (24), except that the final running buffer was 40 mM morpholinepropanesulfonic acid–10 mM sodium acetate–1 mM EDTA (pH 7.0) and that ethidium bromide was added to the gel at a final concentration of 0.6 μg/ml. RNA was blotted onto Hybond-N membranes in 20× SSC buffer (1× SSC is 0.15 M sodium chloride–0.015 M sodium citrate) as described previously (39). Prehybridization (3 h) and hybridization (16 h) with oligonucleotide primers were performed in 6× SSC–5× Denhardt solution–0.1% (wt/vol) sodium dodecyl sulfate buffer containing 100 μg of sonicated carrier DNA per ml in sealed plastic bags at T_d − 5°C, calculated according to the Wallace rule (38). Filters were then washed in 6× SSC–0.1% (wt/vol) sodium dodecyl sulfate–1× Denhardt buffer for 30 min at T_d − 5°C and exposed to film at −80°C with an intensifying screen.

RESULTS AND DISCUSSION

Cloning of overlapping fragments of the *hlyA* gene region in pUC18. A 3,454-bp *Bam*HI chromosomal fragment containing *hlyA* had been cloned from *L. monocytogenes* LO28 in pUC18, giving rise to pLis3. Using as a probe an oligonucleotide primer (21-mer) internal to the *hlyA* gene (positions 1605 to 1625 in Fig. 1), we cloned a 1,886-bp *Eco*RI fragment containing part of *hlyA* and its upstream region, giving rise to plasmid pLis8 (see Fig. 3). Inserts of pLis3 and pLis8 spanned 4,223 bp of the *hlyA* gene region, i.e., 1,488 bp upstream and 1,145 bp downstream from *hlyA*.

Nucleotide sequence analysis, ORF assignments, and protein sequence analysis. The sequence of the 4,223-bp chromosomal region was determined on both strands of the DNA. The sequence of *hlyA* had been reported earlier (26). The presence of putative ORFs upstream and downstream from *hlyA* was screened for by use of a program based on codon usage (37), taking *hlyA* as the reference gene. Two major ORFs, ORF U and ORF D, having good probabilities of being coding sequences, were detected. ORF D, in the same orientation as *hlyA*, started 304 bp downstream from *hlyA* and was not interrupted by a stop codon in the insert of pLis3. ORF U, in an opposite orientation, began 224 bp upstream from the *hlyA* translation initiation codon and was 969 bp long (Fig. 1).

In the case of ORF U, a putative ribosome-binding site (AAAGGAGG) was detected 3 bp downstream from the beginning of the ORF (position 1265 in Fig. 1) and 9 bp upstream from a TTG codon. This codon is rarely used in *E. coli* as a translation initiation codon (14) and may not be used in *L. monocytogenes*. In addition, the region between this start codon and the putative Shine-Dalgarno sequence is rich in Gs and Cs, an unusual feature for translation initiation regions (14). The first ATG present in the ORF has no ribosome-binding site, whereas a second ATG, located 225 bp downstream from the beginning of the ORF, is preceded

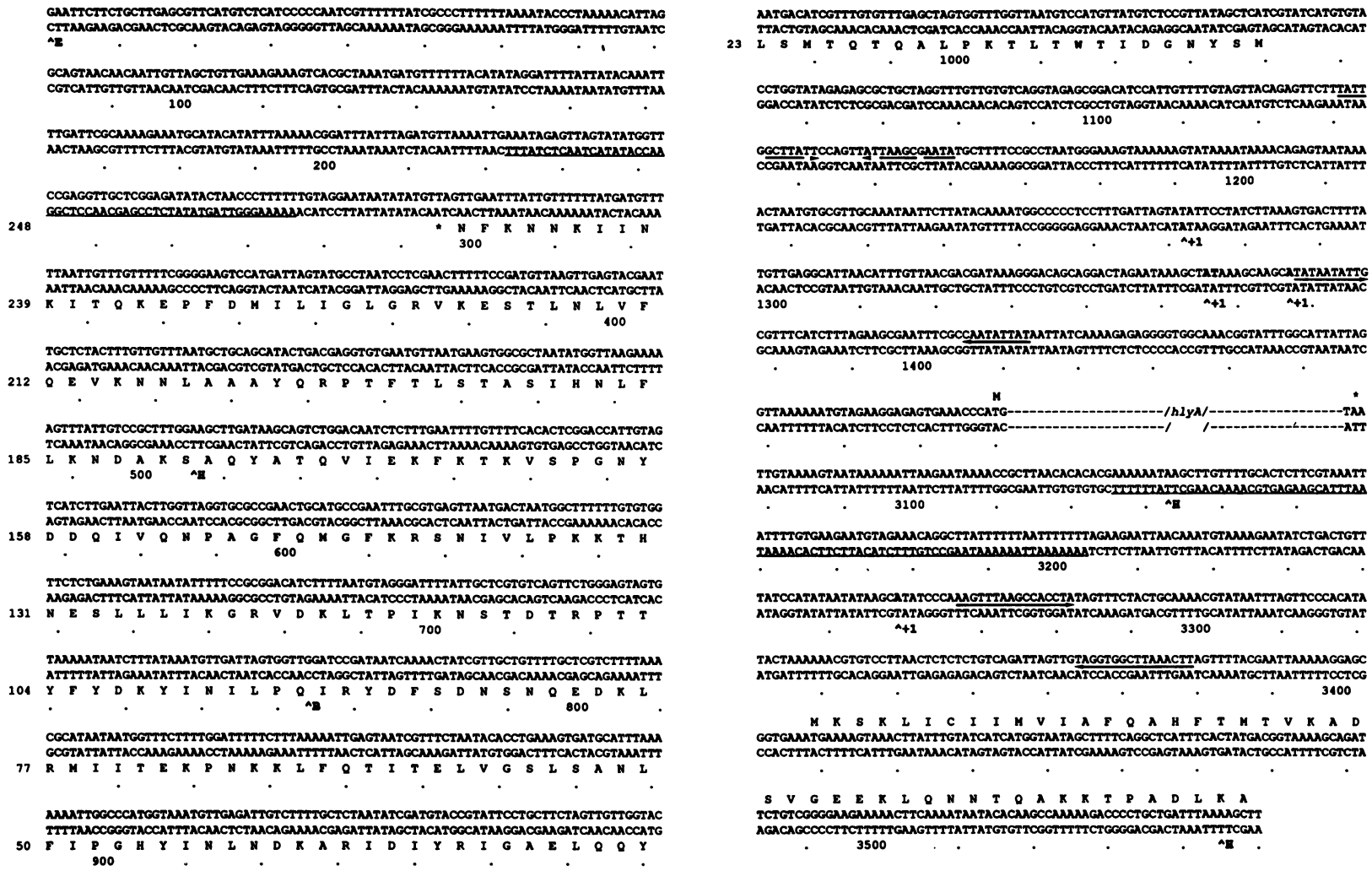

FIG. 1. Nucleotide sequence of the *hlyA* region. The sequence presented here corresponds to the region spanning ORF U, *hlyA*, and the 5′ end of ORF D (the complete sequence of ORF D will be published elsewhere). Nucleotide coordinates are indicated below the sequence. The deduced amino acid sequences of ORF U, *hlyA*, and ORF D are presented. Their coordinates are given in the margin. Possible cleavage sites for signal peptidase in the ORF D amino acid sequence are after Ala-24 or Gly-28 (42). Restriction sites are indicated (E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI). The transcriptional start points (+1) are indicated. Palindromic structures in untranslated leader sequences are indicated by arrows, and putative transcriptional terminators are underlined (see the text).

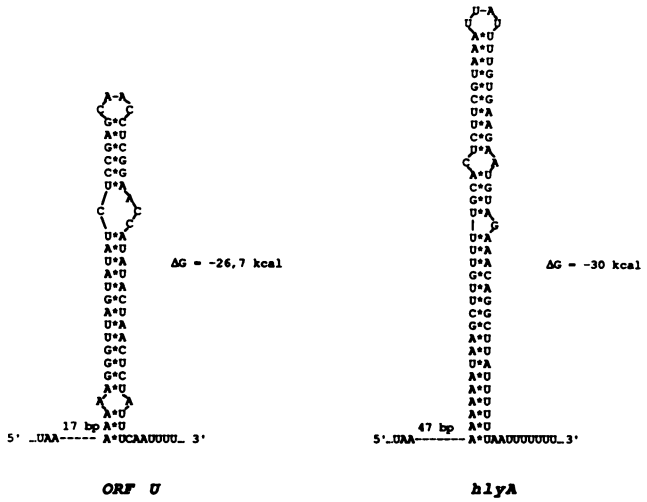

FIG. 2. Secondary structures of putative terminators for ORF U and ORF D. ΔGs were calculated as described previously (8, 40).

by a putative ribosome-binding site, AGG. If this second ATG is considered to be the translation initiation codon, the protein encoded by ORF U would be 248 amino acids long. The amino acid composition deduced from the sequence of ORF U is not unusual, and the amino acid sequence does not present any characteristics of a secreted protein. Its hydropathicity pattern does not reveal either long hydrophobic or hydrophilic regions or alternance of such regions as in transmembrane proteins. However, interestingly, the most hydrophobic region contains a stretch of 18 amino acids, from amino acid position 49 to amino acid position 68, which has homology with the helix-turn-helix motif of one class of DNA-binding protein (28): Ile-Phe-Leu-Asn-Ala-Ser-Leu-Ser-Gly-Val-Leu-Glu-Thr-Ile-Thr-Gln-Phe-Leu-Lys-Lys (Fig. 1). This indicates that the protein could be a DNA-binding protein.

In the case of ORF D, three ATG codons are located 27, 54 and 84 bp downstream from the beginning of the ORF (position 3382 in Fig. 1). Only the first is preceded, 8 bp upstream, by an heptanucleotide (AAAGGAG) that is complementary to the 3′ end of 16S rRNA (22) and is considered to be a ribosome-binding site. This ATG is probably the initiation codon of ORF D. In addition, the beginning of the protein sequence deduced from the ORF D sequence has all the characteristics of signal sequences of gram-positive bacteria (44), indicating that ORF D probably encodes a secreted protein. This may be relevant for virulence, since ORF D is unique to *L. monocytogenes*: an *L. monocytogenes* ORF D probe did not hybridize with *L. ivanovii* and *L. seeligeri*. This was not the case for *hlyA*, which can be detected in *L. ivanovii* and *L. seeligeri*, or for ORF U, which

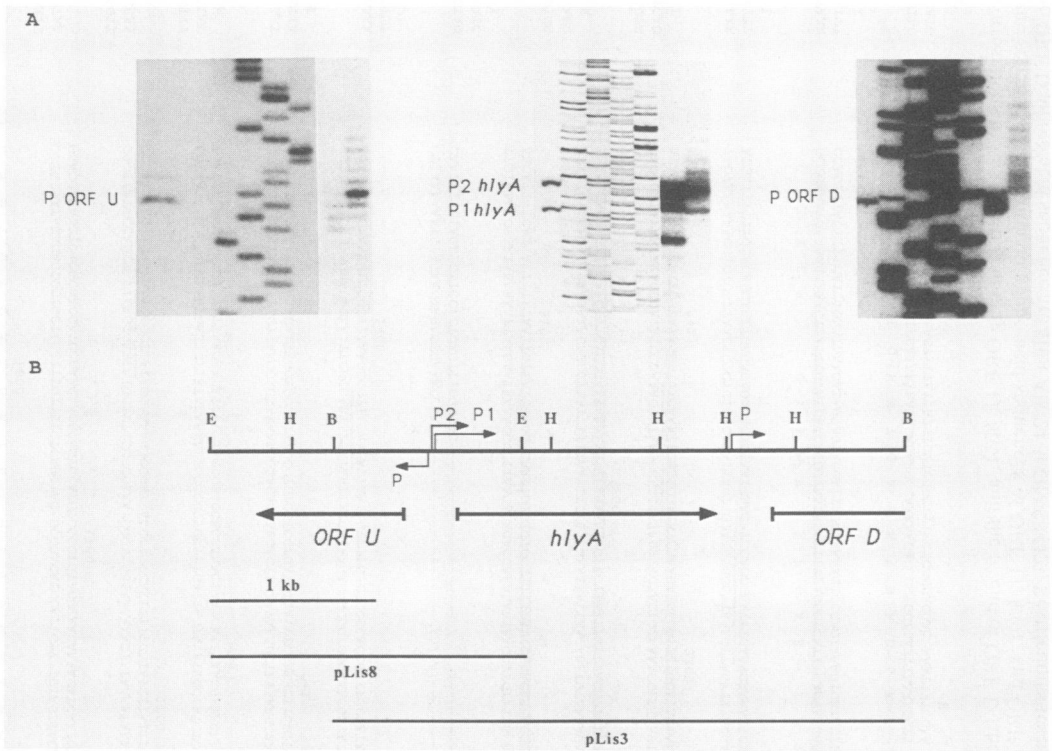

FIG. 3. Mapping of *hlyA*, ORF U, and ORF D transcripts by primer extension and S1 mapping analysis. (A) ^{32}P end-labeled oligonucleotide primers corresponding to positions 1226 to 1245, 1605 to 1625, and 3435 to 3454 in Fig. 1, respectively, were used for reverse transcription and S1 mapping analysis of ORF U, *hlyA*, and ORF D transcripts as described in Materials and Methods. The DNA products were separated by electrophoresis on an 6% polyacrylamide gel simultaneously with the Sanger dideoxy-chain termination ladder produced with the identical primer and the sense or antisense strand of the pLis3 insert cloned into M13mp19 as the template. Lanes (from left to right): reverse transcription reactions, G, A, T, and C tracks of sequencing reactions, S1 mapping reactions obtained with 100 and 2 U of S1 nuclease. (B) Schematic representation of the transcriptional organization of the *hlyA* region. Under the line representing the region, the three ORFs identified by the sequence have been indicated as well as the inserts of pLis8 and pLis3. Restriction sites are indicated (E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI).

is also detected in *L. ivanovii* at low stringency (Gormley et al., manuscript in preparation).

Palindromic sequences of 69 and 54 bp were found downstream from *hlyA* and ORF U stop codons, respectively (Fig. 2). These structures have calculated ΔGs of −30 and −26.7 kcal (ca. 125.5 and 111.7 kJ), respectively. They are indicative of putative transcription termination signals (29).

Promoter mapping and transcriptional organization of the region. Promoters of *hlyA*, ORF U, and ORF D were mapped by both S1 mapping and primer extension analysis with RNAs extracted from cells growing exponentially in rich medium. Both techniques gave identical results; the reverse transcription allowed a more precise determination of transcriptional start sites (Fig. 3). Two transcriptional start sites for *hlyA* were reproducibly identified at positions 1367 (P1 transcript) and 1356 or 1357 (P2 transcript) (Fig. 1). These transcriptional start sites are separated by 10 bp and located 122 and 133 bp, respectively, from the translation initiation codon of *hlyA*. The P1 transcript starts with an A, and the P2 transcript starts with an A or a T. Both transcripts detected were of similar intensity; the longer one (originating at P2) was slightly more abundant than the shorter one (Fig. 3).

Transcriptional start sites for the two putative ORFs were identified, implying that not only *hlyA* but also ORF U and ORF D were transcribed independently in *L. monocytogenes*. The transcriptional start site of ORF U was localized at A or T in position 1272 or 1273 (Fig. 1). This position is 232 bp from the presumed translation initiation codon of ORF U. Thus the two divergent ORFs, *hlyA* and ORF U, have their transcriptional start sites separated by 84 bp when considering P2 of *hlyA* or 95 bp in the case of P1 of *hlyA*. The −35 position of the ORF U promoter is thus 14 bp from the −35 position of P2 *hlyA*. The characteristic features of this intergenic region will be described below. The transcriptional start site of ORF D was localized at an A in position 3259, 181 bp from the translation termination codon of *hlyA* and 65 bp downstream from the putative terminator of *hlyA*. The beginning of this transcript is located 150 bp upstream from the putative translation initiation codon of ORF D. No longer transcript was detected for ORF D, which might have started at either one of the two promoters of *hlyA* or elsewhere.

Northern blot analysis allowed sizing of the *hlyA* transcripts. When total RNAs extracted in the same conditions as those used for the promoter mapping experiments were separated by electrophoresis, transferred onto nylon membranes, and hybridized with the oligonucleotide primer (positions 1605 to 1625 in Fig. 1) used for the primer extension analysis of the *hlyA* transcripts, one single band was detected that corresponded to transcripts of about 1,800 nucleotides (Fig. 4). This is exactly the size calculated for RNAs originating at the identified *hlyA* promoters and ending at the putative terminators. Thus, *hlyA* appears to be a monocistronic unit, in agreement with our previous complementation studies: when a plasmid carrying only *hlyA* was introduced in a transposon-induced nonhemolytic avirulent mutant, both the hemolytic phenotype and the virulence were restored, demonstrating that the transposon insertion had no polar effect on adjacent genes necessary for virulence (9a). As a corollary, it appears that terminators in *L. monocytogenes* would have the same structural features as *E. coli* rho-independent terminators (29). A schematic drawing of the transcriptional organization of the region is presented in Fig. 3B and 5B.

In regions of divergent transcription with back-to-back

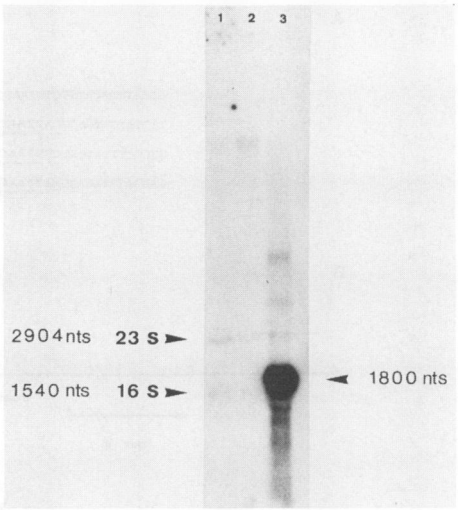

FIG. 4. Northern blot analysis of *hlyA* transcripts. Lanes: 1 and 2, *E. coli* total RNAs; 3, *L. monocytogenes* LO28 total RNAs. nts, Nucleotides.

promoters in procaryotes and their viruses (reviewed in reference 4), the distances between the transcription initiation sites range from 75 bp (*X-rpsU* of *E. coli*) to 513 bp (*malT-malP* of *E. coli*), and the distances between the translational start sites range from 103 bp (*cI-cro* of bacteriophage lambda) to 611 bp (*malT-malP* of *E. coli*). In the case described here, these two distances are 84 and 449 bp, respectively, and therefore are not exceptional. In contrast, the untranslated leader regions were rather long, 122 and 133 bp in the case of *hlyA* and 232 bp in the case of ORF U. This was also the case for the ORF D leader, which is 150 bp long. The potential secondary structures detected in these leaders (Fig. 1) are an indication of putative regulations in those regions. Since divergent transcription units often code for a regulatory protein (R) and a nonregulatory polypeptide (S) (4), the homology of the protein encoded by ORF U with some DNA-binding proteins suggests that the ORF U-*hlyA* region could be of the type R-S. ORF U would play a regulatory role, controlling either the expression of *hlyA* and of ORF U itself or that of other loci.

Comparison of the promoter sequences for *hlyA*, ORF U, and ORF D. Four promoter sequences were identified: two for *hlyA*, one for ORF U, and one for ORF D. The four promoters have −10 regions related to the *E. coli* consensus sequence, but only one of them (P1 *hlyA*) has a −35 region similar to the *E. coli* consensus sequence (32). The three others lack a −35 region, a feature characteristic of positively regulated promoters (31). This would suggest that *hlyA* has one constitutive promoter and a second promoter that requires an activator. By comparing the sequences of P1 *hlyA*, P ORF U, and P ORF D, we identified a palindromic sequence of 14 bp that is conserved in the three promoters and located just upstream from the −35 region (Fig. 5). Moreover, in the intergenic sequence ORF U-*hlyA*, the palindrome T-T-A-A-C-A-A/T-A/T-T-G-T-T-A-A is shared by the two back-to-back promoters. This palindromic structure might be recognized by regulatory DNA-binding proteins. Since P2 *hlyA* and P ORF U lack an *E. coli* consensus −35 region, an activator is possibly involved (31). Moreover, the homologies between the −40 regions of P2 *hlyA* and P ORF U promoters and that of ORF D suggest that *hlyA*, ORF U, and ORF D could have the same activator. How-

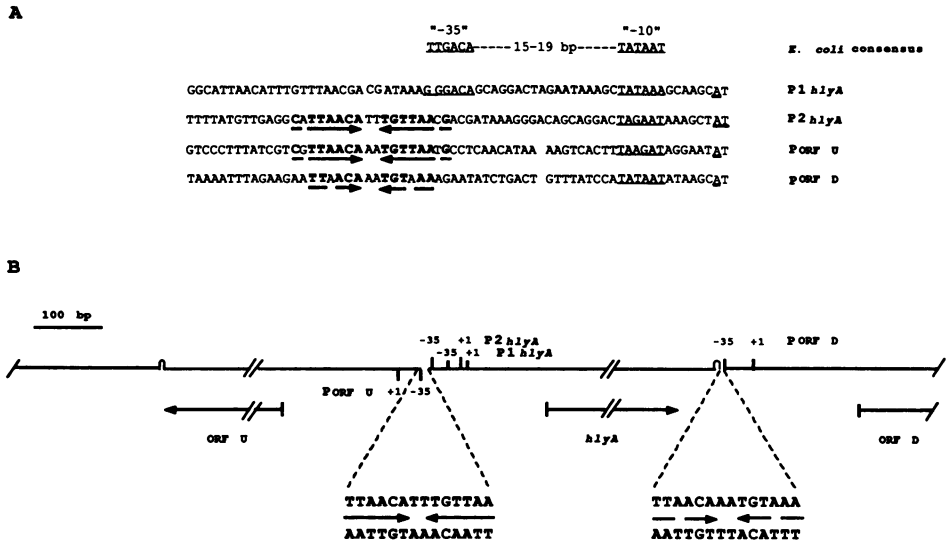

FIG. 5. (A) Promoter comparison. (B) Schematic drawing of the *hlyA* region.

ever, since no other promoter has been mapped and sequenced in *L. monocytogenes*, the possibility cannot be excluded that the palindrome is a characteristic of all *Listeria* promoter regions.

The hypothesis of a requirement for a positive regulator for *hlyA* expression would explain the absence of an increase in the hemolytic titer when a multicopy plasmid carrying *hlyA* was introduced in a wild-type *L. monocytogenes*. The simplest explanation for such an observation is that the activator concentration would be a limiting factor. Nevertheless, the regulation of *hlyA* is probably more complex than a simple activation. It has been reported that iron down-regulates the expression of listeriolysin O (11). Such a regulation could be mediated by a repressor as in *E. coli*, where iron-regulated promoters are repressed by the *fur* gene product in the presence of iron (2). Fur has also been shown to regulate expression of the diphtheria toxin gene *tox* cloned in *E. coli* and a Fur-binding site was identified in the *tox* promoter region (35). In the case presented here, no site homologous to the Fur consensus binding site was detected in the promoter regions.

It has been recently reported that listeriolysin O expression is under heat shock control (36). The promoters identified here do not share homologies with *E. coli* heat shock promoters (12), and there is no evidence that the 14-bp palindromic sequence identified in this study has any relationship to a heat shock response.

In conclusion, to understand the regulation of the expression of an essential virulence factor in *L. monocytogenes*, we have described the structural organization of the region encoding listeriolysin O. *hlyA* appears as a monocistronic unit that can be transcribed from two promoters spaced by 10 bp. *hlyA* is adjacent to another gene, ORF U, transcribed in opposite direction. These two transcription units are separated by the perfect palindrome T-T-A-A-C-A-A/T-A/T-T-G-T-T-A-A. Since this palindrome is also found in the promoter region of ORF D, a species specific gene located downstream from *hlyA*, it can be suggested that the three genes are under a similar regulation.

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