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Essentiality of the Early Transcript in the Replication Origin of the Lactococcal Prolate Phage c2†

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The genome of the prolate-headed lytic lactococcal bacteriophage c2 is organized into two divergently oriented blocks consisting of the early genes and the late genes. These blocks are separated by the noncoding origin of DNA replication. We examined the functional role of transcription of the origin in a plasmid model system. Deletion of the early promoter P_E1 abolished origin function. Introduction of mutations into P_E1 which did not eliminate promoter activity or replacement of P_E1 with an unrelated but functional promoter did not abolish replication. The A-T-rich region upstream of P_E1, which is conserved in prolate phages, was not required for plasmid replication. Replacement of the P_E1 transcript template sequence with an unrelated sequence with a similar G+C content abolished replication, showing that the sequence encoding the transcript is essential for origin function. Truncated transcript and internal deletion constructs did not support replication except when the deletion was at the very 3′ end of the DNA sequence coding for the transcript. The P_E1 transcript could be detected for all replication-proficient constructs. Recloning in a plasmid vector allowed detection of P_E1 transcripts from some fragments that did not support replication, indicating that stability of the transcript alone was not sufficient for replication. The data suggest that production of a transcript of a specific length and with a specific sequence or structure is essential for the function of the phage c2 origin in this model system.

Bacterial strains of the genus *Lactococcus* are widely used in the manufacture of cultured milk products. Bacteriophage attack of lactococcal starter strains may result in the lysis of susceptible strains and therefore in fermentation failure. Three genetically unrelated groups of lactococcal phages are responsible for most fermentation failures (23). These phages conform to one of two morphotypes: the prolate-headed c2 species and the isometric-headed 936 and P335 species (24). There are no known temperate phages in the c2 and 936 species groups, whereas the P335 species contains both temperate and lytic phages (24). Recently, the genomes of the small isometric 936 group phages sk1 (8) and bIL170 (11), the small isometric P335 group phages r1t (46), TP901-1 (4), Tuc2009 (GenBank accession no. AF109874), bIL285, bIL286, bIL309 (9), and BK5-T (3, 32), and the prolate-headed phages c2 (29) and bIL67 (43) have been sequenced and analyzed. Although there is a wealth of information at the nucleotide sequence level for these lactococcal phages, our current understanding of some aspects of phage biology, such as the replication of the phage genome, is relatively poor.

The 22,163-bp double-stranded linear DNA genome of the prolate-headed phage c2 contains 39 open reading frames (ORFs), which are organized in two divergently oriented blocks consisting of the early genes and the late genes (29). These blocks are separated by a 611-bp noncoding region that contains the origin of replication (*ori*) in *Lactococcus lactis* (47). A 521-bp *ori* fragment, which includes early promoter 1 (P_E1) and late promoter 1 (P_L1), was shown to support plasmid replication (pVA891-*ori*) in *L. lactis* in the absence of phage proteins (47), whereas a 261-bp subfragment (including the conserved sequence upstream of P_E1 but not the sequence downstream of P_E1) did not support plasmid replication. Furthermore, it has recently been demonstrated that phage c2 replicates via theta replication initiated at the *ori* region (5).

Following infection of an *L. lactis* cell by phage c2, three transcripts in the size range from 260 to 360 nucleotides (nt) are made from the P_E1 promoter (28). The level of transcripts made increases during the course of c2 infection (28). Sequence analysis has suggested that these transcripts are not translated, and Lubbers et al. (28) speculated that they might be involved in DNA replication, although no supporting evidence was available. The region upstream of P_E1 is highly A-T rich (78%) and consists of several inverted and direct repeats. It is also highly conserved in the closely related prolate phages bIL67 and ϕ197 (47), in contrast to the region downstream of P_E1, which is not conserved. It has recently been shown that a large panel of lactococcal prolate phage could be divided into three distinct groups based on the sequence of the P_E1 transcript (40). All three *ori* types supported plasmid replication.

Transcription in the origin is essential for replication of numerous phages and plasmids (reviewed in reference 13), but the mechanisms involved vary widely. In coliphage λ, an *ori* transcript is formed, which does not act as a primer for replication (18). The RNA polymerase β subunit also serves as a contact site for DnaA to act as a transcription activator at p_R and thus stimulates transcription-mediated activation of *ori* λ (44). In phage T4, the transcript made from the middle-mode

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origin promoter forms a persistent DNA-RNA hybrid within *ori*(*uvsY*) and is required for initiation of replication from *ori*(*uvsY*) (6). Initiation of replication of plasmid ColE1 requires synthesis of a transcript by RNA polymerase (35). This transcript assumes a particular conformation (33, 34) and then forms a persistent hybrid with the template DNA near the replication origin. The hybridized RNA may be cleaved by RNase H to act as a primer. In the absence of RNase H, the unprocessed transcript can act directly as a primer or indirectly by displacing the nontranscribed DNA strand (12).

Little is known about mechanisms of lactococcal prolate phage DNA replication, and only slightly more is known about the mechanisms in other lactococcal phage species. A P335 species DNA replication module was identified in the temperate phage Tuc2009, which encoded putative single-stranded DNA binding proteins, a topoisomerase I, a methylase, and a replisome organizer protein (36). The putative replisome organizer protein (Rep_{2009}) has been shown in gel retardation assays to bind to ori_{2009} (36). In another temperate phage, TP901-1, a single-stranded DNA binding protein and a putative replication initiation protein, which are essential for in vivo phage replication, have been identified (37). P335 phages require phage-encoded proteins for DNA replication. This was demonstrated by the ability of cloned phage *ori* fragments to bind and presumably titrate out proteins essential for phage replication, thus conferring the Per phenotype (phage-encoded resistance; φ50, φ31, BK5-T, TP901-1, and Tuc2009) (20, 32, 36, 37, 38). Phage c2 *ori* does not confer a Per phenotype (39), suggesting that DNA replication of this phage relies on a *cis*-acting origin-encoded product and/or host-encoded proteins.

It has been shown that transcription of a divergent region downstream of P_E1 occurs in the three groups of lactococcal prolate phages into which our collection was subdivided (40). Here we demonstrate that production of the P_E1 transcript is required for phage c2 origin function in a plasmid model system. The sequence encoding the transcript produced from P_E1 was essential for the ability to support plasmid replication, and small internal deletions were not tolerated. The data suggest that the transcript probably forms a secondary structure, which is required for its role in replication.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The following bacterial host strains were used in this study. *Escherichia coli* ER2206 [*endA1 thi1 supE44 mcr67* (*mcrA*) (*mcrBC-hsdRMS-mrr*)*114*::IS*10* (*lac*)*U169*/F′ *proAB laqI*^{q} *Z* M15 Tn*10*] was obtained from New England Biolabs, Beverley, Mass. *L. lactis* MG1363 is a plasmid-free, prophage-cured derivative of NCDO712 (17). *L. lactis* NZ9000 is a derivative of strain MG1363 that does not produce nisin and has the *nirR-nisK* genes integrated in the *pepN* gene in the chromosome (27). *L. lactis* NZ9800 is a derivative of strain NZ9700 that does not produce nisin (26). *L. lactis* was grown at 30°C without aeration in M17 medium supplemented with 0.5% (wt/vol) glucose (45). When required, 5 μg of erythromycin per ml or 5 μg of chloramphenicol per ml was added to the medium. *E. coli* was grown at 37°C in Luria-Bertani medium (41). *E. coli* transformants were plated on medium containing 150 μg of erythromycin per ml, 25 μg of chloramphenicol per ml, or 200 μg of ampicillin per ml as appropriate, 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml, and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). For solid media, 15 g of agar per liter was added. Nisin (nisaplin; Aplin and Barrett Ltd., Beaminster, Dorset, England) was added to induce the *nisA* promoter at a concentration of 150 ng/ml for strains NZ9000 and NZ9800.

DNA preparation and manipulation. *L. lactis* was made electrocompetent by growth in the presence of glycine (2.5%) (21). Transformation of *L. lactis* was performed by electroporation as described previously (25). $CaCl_2$-treated *E. coli* cells were transformed as described by Sambrook et al. (41). Plasmid DNA was isolated from *L. lactis* strains by alkaline lysis as described previously (1). Plasmid DNA was isolated from *E. coli* by alkaline lysis as described previously (41) or with a High Pure plasmid purification kit (Roche, Mannheim, Germany). DNA fragments were isolated from gels with a High Pure PCR product purification kit (Roche). DNA manipulations (restriction enzyme cleavage, ligation, phosphorylation, etc.) were carried out by using conditions specified by the manufacturer or by using standard protocols (41). For all PCRs, *Pwo* I polymerase (Roche) was used. DNA sequencing was performed by the dideoxy chain termination method (42) by the Allan Wilson Centre DNA Analysis Services, Massey University, Palmerston North, New Zealand.

Construction of plasmids. The plasmids used or generated in this study are listed in Table S1 in the supplemental material, and the oligonucleotide primers used are listed in Table S2 in the supplemental material. Fragments cloned in recombinant plasmids were verified by DNA sequencing. P_L1 was deleted by PCR by using a pLP201 template with oligonucleotides ori22 and ori7. Plasmid pLP204 was created by ligating a PCR product (template pLP201; primers ori24 and ori23) containing the P_L1 promoter (coordinates 7056 to 7238 in c2) to a PCR product (template pLP201; primers ori25 and ori22) containing the sequence downstream of P_E1 (coordinates 6717 to 7026 in c2). The ligation product was cloned into the EcoRI site of pVA891 (30), resulting in a recombinant *ori* fragment lacking the −10 and −35 hexamers and the intervening sequence of P_E1. Substitutions (T to G) in the consensus −10 region of P_E1 were introduced by amplifying the cloned c2 origin in two separate arms by using a primer that contained the two mutations (template pLP201; primers ori26 and ori22 and primers ori27 and ori23) and then ligating the two PCR products and cloning the fragment into the EcoRI site of pVA891 to generate pLP205. To create plasmid pUC-203, the *ori* fragment of pLP203 was cut out with EcoRI and cloned into the EcoRI site of pUC19. Deletion mutants of the P_E1 transcript were created by progressively shortening the P_E1 transcript-encoding region from the 3′ end by PCR (template pLP201; primers ori7 and ori1 through ori6) and cloning the fragments into the EcoRI site of pVA891 to generate plasmids pLP206 through pLP211.

The P_E1 promoter was replaced by the inducible *nisA* promoter (15) by first performing an inverse PCR with the pUC19-ori plasmid, which deleted P_E1 (primers ori24 and ori25). The *nisA* promoter was amplified by PCR (template pNZ8037 [14]; primers ori28 and ori29) to obtain a fragment containing the nisin promoter and a conserved sequence upstream of the position 1 start site (14) but not the *nisA* ribosome binding site. This fragment was then ligated to the inverse PCR product and cloned into the EcoRI site of pVA891 to create pLP212. An inverse PCR with pUC19-ori and primers ori9 and ori10, each containing 14 bp of the P_E1 sequence in an inverted orientation, was performed, and the products were self-ligated. The *ori* fragment containing the inverted P_E1 promoter was cut out with EcoRI and subsequently cloned in pVA891, resulting in pLP213. The P_E1 transcript was replaced with a DNA fragment derived from a lactococcal proteinase gene (domain A of the *prtP* gene) (10) that had a similar G+C content and was a similar length. The proteinase sequence (primers ori11 and ori12; template pHP003) and the P_E1 promoter (primers ori13 and ori14; template pLP203) were amplified by overlap extension PCR by using primers that overlapped in the first PCR. The second PCR (primers ori11 and ori14) was performed by using the external primers and the products of the first PCRs as templates in order to join the two sequences together. The product was cloned into the EcoRI site of pVA891 to generate pLP214. Plasmid pLP216 was created by cloning a PCR product (template pLP207; primers ori15 and ori16) into the NcoI and PstI site of plasmid pVAΩ, which contained a transcriptional terminator (Ω terminator) (16, 40). Plasmids pLP215 and pLP217 through pLP223 were made by inverse PCR by using pUC-203 as the template and primers ori30 and ori31 (pLP215), ori44 and ori45 (pLP217), ori42 and ori43 (pLP218), ori40 and ori41 (pLP219), ori38 and ori39 (pLP220), ori36 and ori37 (pLP221), ori34 and ori35 (pLP222), and ori32 and ori33 (pLP223). The *ori* fragments were recloned into the EcoRI site of pVA891.

Northern blot hybridization. Total RNA from *L. lactis* was extracted from frozen cell pellets (−80°C) by the hot phenol method as previously described (28). Formamide-containing gel loading dye (80% deionized formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) was added to the samples and incubated for 10 min at 75°C before the samples were separated on a 6 M urea–6% polyacrylamide gel by electrophoresis in 1× Tris-borate buffer. RNA was then electrotransferred onto a positively charged nylon membrane (pore size, 0.45 μm; Roche) at 400 mA for 1 h in 1× Tris-borate-EDTA buffer. RNA was fixed to the membrane by UV irradiation. The RNA transfer was visualized

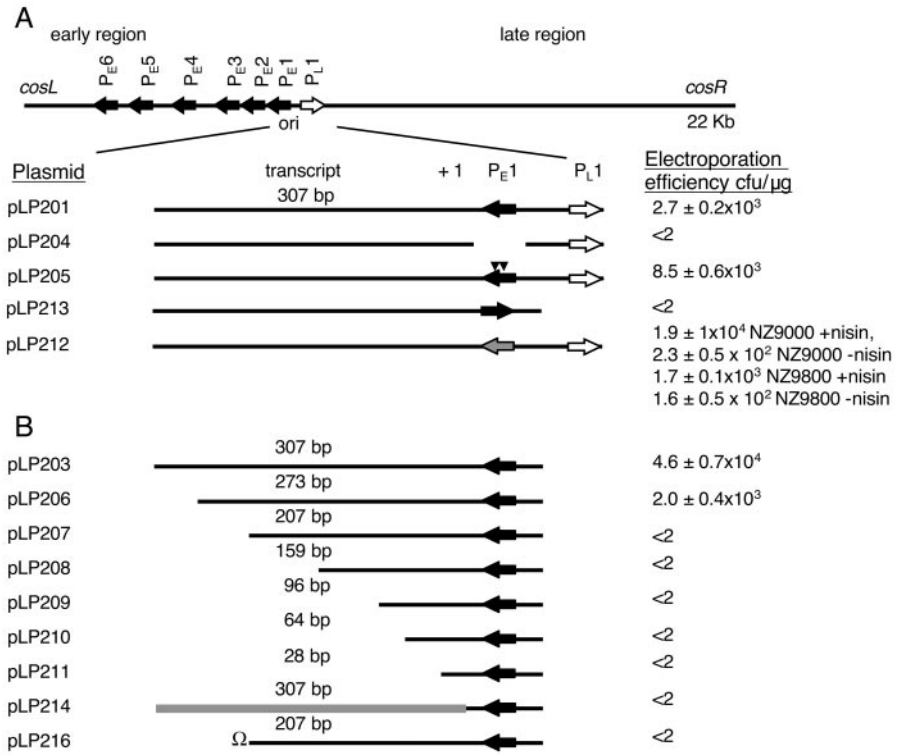

FIG. 1. Schematic representation of the c2 *ori* locus. The positions of the origin fragment in the phage c2 genome relative to the early gene promoter (solid arrows) and the late gene promoter (open arrow) are shown at the top. Below this the c2 *ori* (positions 6717 to 7238 in the c2 sequence) and the derivatives of the origin fragment that were cloned in pVA891 are shown. The abilities of the various constructs (listed on the left) to replicate in *L. lactis* are indicated on the right as mean transformation frequencies from at least three experiments (in the case of pLP212 in the presence and in the absence of nisin); the values varied because of variations in the *L. lactis* transformation frequency between experiments. (A) The solid triangles indicate the position of the base pair changes in the pLP205 insert. The gray arrow represents the *nisA* promoter in the pLP212 insert. In pLP213 P_E1 is inverted. (B) Schematic representation of c2 *ori* fragments cloned in pVA891 carrying shortened DNA sequences coding for the transcript made from P_E1 (pLP201 to pLP211). The length of the remaining sequence of each transcript from the transcription start site is indicated above the transcript. The gray line represents the lactococcal *prtP* sequence replacing the c2 transcript-encoding sequence in the pLP214 insert. Ω in pLP216 represents the transcriptional and translational terminator cloned at the 3′ end of the 207-bp transcript-encoding sequence.

by methylene blue staining of blots (19). Hybridizations were performed by using the ECL system according to the manufacturer's instructions (Amersham Pharmacia) and PCR-generated probes.

Primer extension. The avian myeloblastosis virus reverse transcriptase primer extension system (Promega, Madison Wis.) was used according to the manufacturer's instructions. For the analysis, 20 μg of total RNA isolated from *L. lactis* MG1363 and primer 6902prex were used. The sequencing reaction was performed by the dideoxy method by using an AmpliCycle sequencing kit (Perkin-Elmer, Foster City, Calif.) according to the manufacturer's instructions and 20 pmol of primer 6902prex, which had been end labeled with [γ-^{32}P]ATP (Amersham Pharmacia Biotech, Little Chalfont, England).

RESULTS

Transcription of the sequence downstream of P_E1 is required for origin function. The original *ori* plasmid pLP201 (pVA891-*ori*) (30, 47) contained both P_E1 and P_L1 and also 307 bp of the noncoding region downstream of P_E1 (Fig. 1A). This fragment supported replication of the lactococcal origin-screening vector pVA891 (47). To investigate if P_L1 and the highly conserved A-T-rich region between P_E1 and P_L1 played any role in replication, we attempted to introduce plasmid pLP203 into *L. lactis* cells by electroporation. Plasmid pLP203 contains P_E1 and the 307-bp noncoding sequence downstream of P_E1 but not P_L1 or the A-T-rich region (40) (Fig. 1A). The shortened *ori* fragment in pLP203 supported plasmid replication in *L. lactis* (Fig. 1A), with an electroporation frequency that was 1 order of magnitude higher than that for plasmid pLP201. This suggested that P_E1 and the sequence downstream of P_E1 are sufficient for efficient origin function and that P_L1 and the A-T-rich region are not required for replication.

The sequence downstream of P_E1 is very poorly conserved in prolate phages (28) and is transcribed from P_E1 during phage c2 infection. To determine if active transcription was necessary for replication, we constructed several c2 *ori* fragments with modified P_E1 promoters and cloned them into pVA891. The P_E1 promoter itself (-10 and -35 promoter hexamers and the intervening sequence) was deleted by a PCR-based strategy in order to generate plasmid pLP204 (Fig. 1A). This plasmid was unable to replicate in *L. lactis*, as shown by our repeated and reproducible failure to electroporate it into *L. lactis* strain MG1363 compared with parallel controls (Fig. 1A).

The loss of *ori* function by deletion of P_E1 in pLP204 could have been due to lack of a transcript with a necessary mechanistic role or due to DNA conformational changes in the *ori* fragment caused by the internal deletion. To help distinguish

between these possibilities, P_E1 was inverted in plasmid pLP203 to generate pLP213 (Fig. 1A). This construct was a control for conformational changes induced by gross deletion of the promoter, although promoter-inversion-related conformational changes were still possible. In this plasmid, the P_E1 promoter was upstream of, and in the same orientation as, the erythromycin resistance gene of the vector. Plasmid pLP213 could not be electroporated into *L. lactis* cells (Fig. 1A), suggesting that mere binding by RNA polymerase to the *ori* fragment was not sufficient for replication and that directional promoter activity was required.

Unrelated but functional promoter plus the P_E1 transcript-encoding region are sufficient for origin function. To distinguish whether P_E1 promoter activity or the P_E1 sequence was required for c2 *ori* replication, P_E1 was replaced by the inducible *nisA* promoter (14, 15) in order to generate pLP212 (Fig. 1A) and was transformed into *L. lactis* strains NZ9800 and NZ9000. Neither of these strains produces the nisin peptide (26, 27), but both of them allow regulated gene expression under the control of the inducible *nisA* promoter upon addition of the nisin peptide itself to the growth medium. Higher levels of expression of genes under the control of the nisin promoter are achieved in NZ9000 (27). This may be because the effective nisin level is lower in NZ9800 because of nisin binding to NisI or other nisin immunity proteins produced in this strain (27). Plasmid pLP212 was successfully introduced into both strains in the presence of nisin (Fig. 1A). The plasmid could also be introduced even without induction by nisin (Fig. 1A), conditions under which the *nisA* promoter activity was reduced but clearly detectable (see Fig. 4).

To further investigate the requirement for a functional promoter for replication, we created two T-to-G substitutions in the consensus −10 region of P_E1 (TATAAT was mutated to TAGAAG). The substitutions were designed to eliminate or significantly reduce P_E1 promoter activity with minimum impact on the DNA conformation in the origin. The fragment was cloned into pVA891 to produce pLP205 (Fig. 1A), which was assayed for plasmid replication in *L. lactis* MG1363. Despite the sequence changes in the P_E1 promoter, the *ori* fragment in pLP205 supported plasmid replication.

Functional importance of the P_E1 transcript length and sequence. Waterfield et al. (47) previously showed that a 261-bp subfragment that included P_E1, P_L1, the conserved A-T-rich region between the two promoters, and a 48-bp P_E1 transcript (starting from the position 1 nucleotide) cloned in pVA891 (previously not designated, now designated pLP202) did not support replication in *L. lactis*. This result suggested that the transcript had to be a particular length to support replication. To identify the minimal DNA fragment that exhibited *ori* activity, we generated six deletion constructs of the transcript made from P_E1 by progressively shortening the DNA sequence coding for the transcript from the 3′ end. The *ori* fragments containing P_E1 and the downstream sequence but not the late promoter (P_L1) (pLP206 to pLP211) (Fig. 1B) were cloned into pVA891. The shortened 273-nt transcript (from the position 1 nucleotide) in pLP206 still supported replication, but the next-shortest truncated-transcript construct (207 nt; pLP207) did not replicate. None of the transcripts shorter than 207 nt supported replication (Fig. 1B). To eliminate the possibility of deleterious run-on transcription into the vector in the deletion mutants, we inserted a transcriptional terminator (omega terminator [20]) at the 3′ end of the transcript of pLP207 (to generate pLP216 [Fig. 1B]). The presence of the terminator did not restore replication, indicating that run-on transcription did not make the plasmid genetically unstable.

The region downstream of P_E1 is not conserved among prolate phages (47). This prompted us to investigate if a particular transcript sequence, and not just a minimum transcript length, was required for replication. Thus, we replaced the P_E1 transcript-encoding region with a DNA fragment that had a similar G+C content and was a similar length, which was derived from a lactococcal proteinase gene (domain A of the *prtP* gene) (10), and cloned it into pVA891 to generate pLP214 (Fig. 1 B). This DNA fragment was designed to encode a transcript that could not be translated, and it was cloned in the antisense direction with respect to the *prtP* gene upon which it was based. The resulting plasmid, pLP214, did not replicate in *L. lactis*, suggesting that the specific sequence or conformation of the native *ori* transcript was critical for its stability or function. To investigate this, scanning mutagenesis was performed with the DNA sequence coding for the transcripts made from P_E1. The 10-, 13-, and 48-bp deletions used were designed to disrupt the predicted secondary structures (40) in the transcripts made from P_E1 (pLP217 to pLP223). Of the seven deletion plasmids constructed, only pLP217 replicated in *L. lactis* (Fig. 2).

The ColE1 plasmid replication origin contains a G tract consisting of six G residues, which is essential for replication (22). A similar G tract has also been found in the P_E1 transcript of the prolate phages c2, biL67, c6A, and 923 (40). The phage c2 G tract consists of seven G residues, whereas the 923 *ori* has eight G residues and bIL67 *ori* has seven G residues interrupted by the nucleotides CTA. In order to examine if the G tract played a role in c2 replication, the seven G residues were deleted in the c2 *ori* fragment by using a PCR-based strategy. The fragment was cloned into pVA891, generating pLP215, which was assayed for its ability to replicate in *L. lactis*. This plasmid did not replicate in *L. lactis* (Fig. 2).

Recombinant plasmids from the deletion and modification experiments described above (Fig. 1 and 2) were verified by DNA sequencing of plasmid DNA isolated from *L. lactis* cells. Thus, we can rule out the possibility that plasmid integration into the chromosome, rather than autonomous replication, was being measured in plasmids harboring recombinant c2 *ori* fragments.

P_E1 promoter analysis in recombinant *ori* plasmids. The transcription start site of the early transcripts synthesized from P_E1 has been determined for phage c2 (28). To investigate if transcription from P_E1 in the plasmid system started from the same position, primer extension analysis was performed. A single cDNA fragment was produced in the primer extension experiments by using the 6902prex primer (Fig. 3). The transcription start site was thus mapped to a nucleotide corresponding to the same A nucleotide identified as the transcription start site in phage c2 during infection (28).

To examine if *ori* transcripts were produced from replicating constructs, we performed Northern blotting using total cellular RNA from the corresponding *L. lactis* strains harboring replicating plasmids. The probe was generated by amplification of

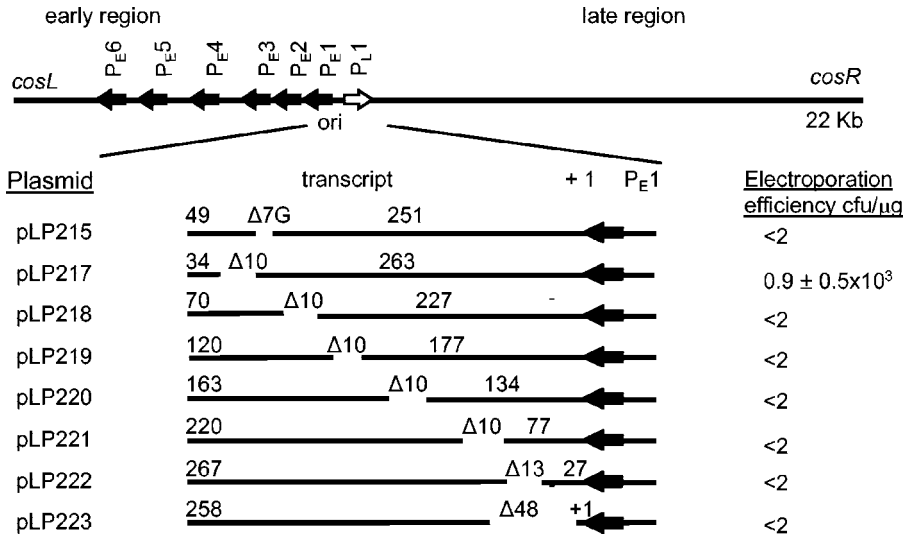

FIG. 2. Schematic representation of plasmids used in scanning mutagenesis. The c2 *ori* fragments carrying deletions in the transcript-encoding sequence were cloned into pVA891 and assayed for the ability to replicate in *L. lactis*. The numbers above the lines indicate the sizes (in base pairs) of the fragments 5′ and 3′ of the deletion. The spaces between the solid lines indicate the positions of deletions, and the numbers preceded by Δ indicate the lengths of the deletions (in base pairs).

the sequence for the P_E1-derived *ori* transcript in pLP206, including the P_E1 promoter. The P_E1 transcript was detected in all the replicating plasmids (Fig. 4). The sizes of the major transcripts corresponded to the sizes determined in a previous study for c2-infected cells when RNase protection was used (40), and the major species contained 260, 265, 280, and 295 nt. An additional species consisting of around 150 nt was also present in samples with the largest amounts of transcript (pLP201, pLP203, and pLP206). The substitutions in the −10 region of the P_E1 promoter in pLP205 significantly reduced the transcript level compared with the level in plasmid pLP201 but did not abolish promoter activity. The lower level of transcription was obviously sufficient for replication. Plasmid pLP212 (with the *nisA* promoter) also produced the *ori* transcript in the

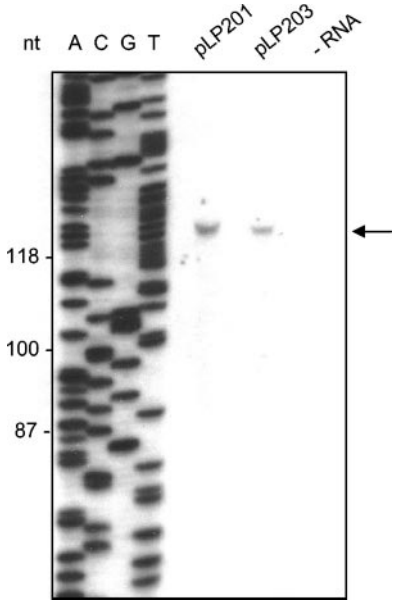

FIG. 3. Primer extension analysis to identify the transcription start site in the pVA891-*ori* plasmids. The primer extension reaction was performed with total RNA isolated from *L. lactis* containing plasmid pLP201 or pLP203 by using primer 6902prex. The primer extension products in both lanes are indicated by the arrow. The same primer was used for the sequencing reaction with pLP201 (lanes A, C, G, and T). Sizes (in nucleotides) are indicated on the left.

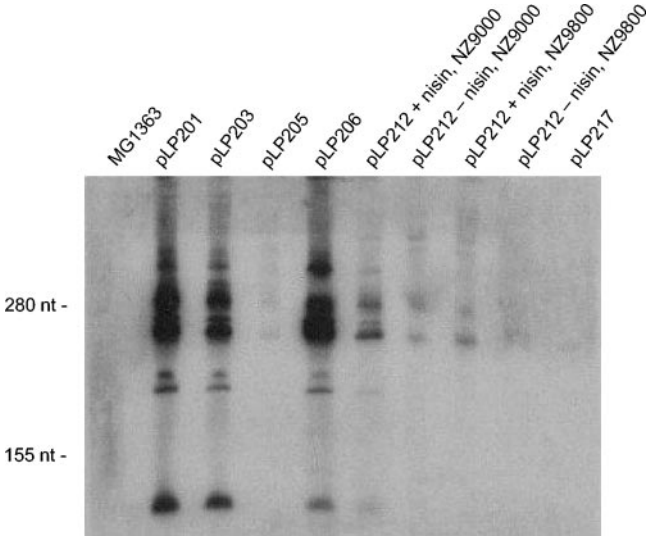

FIG. 4. Northern hybridization analysis of the c2 *ori* fragments that support plasmid replication. Twenty micrograms of total RNA from *L. lactis* cells harboring a plasmid was electrophoresed on an acrylamide gel, blot transferred, and then probed with a PCR product (template pLP206; primers ori1 and ori7) and detected by the ECL system. MG1363, no plasmid; pLP constructs, MG1363 transformed with the plasmids indicated; NZ9000 and NZ9800, two lactococcal host strains for pLP212, in which the *ori* transcript is placed under control of the *nisA* promoter; + nisin, growth in the presence of nisaplin; − nisin, growth in the absence of nisaplin.

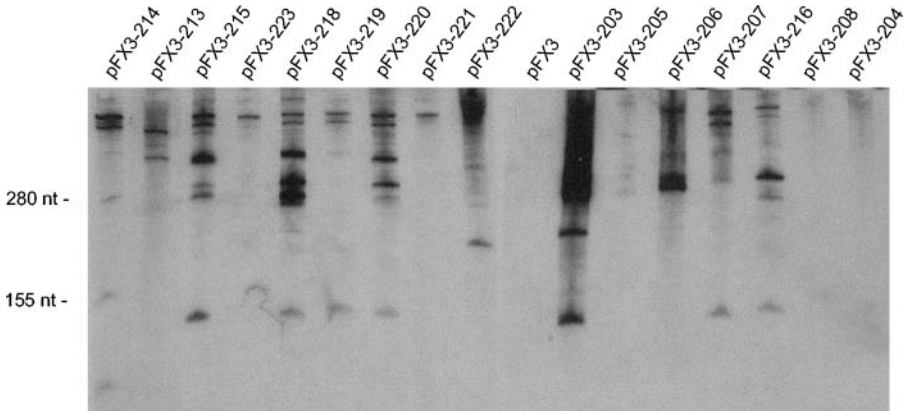

FIG. 5. Northern hybridization analysis of the c2 *ori* fragments that did not support plasmid replication. The fragments were recloned into a plasmid (pFX3) that contains an origin of replication for gram-positive bacteria. Twenty-microgram portions of total RNA from *L. lactis* strain MG1363 harboring the plasmids were electrophoresed on acrylamide gels, blot transferred, and then probed with different PCR products and detected by the ECL system. The migration positions of two marker bands (280 and 155 nt) are indicated on the left. Each lane is labeled with the designation of the plasmid construct (see Fig. 1 and 2 for schematic representations of the constructs). The following PCR-generated probes were used: lane pFX3-214, template pLP214 and primers ori11 and ori12; lane pFX3-213, template pFX3-213 and primers ori4 and M13 Rev; lanes pFX3-215, pFX3-223, pFX3-218, pFX3-219, pFX3-220, pFX3-221, pFX3-222, pFX3, pFX3-203, pFX3-205, pFX3-206, pFX3-207, pFX3-216, pFX3-208, and pFX3-204, template pLP206 and primers ori1 and ori7.

absence of nisin in both host genotypes. Plasmid pLP217 produced extremely low levels of the P_E1 transcript (Fig. 4). Therefore, the *ori* transcript was present in all replicating plasmids.

Transcript stability in nonreplicating *ori* derivatives. To investigate the P_E1 transcript stability in modified *ori* fragments that did not support replication, we recloned the c2 fragments from several of the nonreplicating pLP series of plasmids into the vector pFX3, which has a functional replication origin for gram-positive bacteria (48). The three smallest *ori* fragments could not be recloned in pFX3. The inserts of the replication-competent plasmids pLP203, pLP205, and pLP206 were also recloned into pFX3 as controls. Total RNA was analyzed by Northern blot hybridization with a specific probe. For pFX3-214, this analysis was based upon the cloned *prtP* fragment in the plasmid. For pFX3-213, the probe was based on a fragment that included the *ori* transcript, the inverted P_E1 promoter, and the pFX3 vector sequence (102, 28, and 126 bp, respectively). For all other plasmids, the probe was based on the P_E1-derived *ori* transcript in pLP206 that included P_E1.

The transcript produced from P_E1 was readily detectable in pFX3-203 and pFX3-206 (Fig. 5). The *ori* fragment of pLP205 cloned into pFX3 produced very low levels of the P_E1 transcript, similar to the levels observed with pLP205 (Fig. 4). No transcript was detected in pFX3-204 (complete P_E1 promoter deletion). In the deletion mutant pFX3-207, the P_E1 transcript was clearly detectable, although this fragment did not support replication. For pFX3-208, no transcript was detected. Addition of the omega terminator to the 3′ end of the pLP207 *ori* insert significantly increased the stability of the transcript (corresponding to pLP216 and pFX3-216). The presence of the omega terminator did not eliminate the larger hybridizing species produced by this and other plasmids, which probably represented transcript read-through into the vector. Comparison of the transcripts in pFX3-207 and pFX3-216 (Fig. 5) did show that there was an increase in the abundance of the *ori* transcript of the appropriate size and that there was a significant reduction in the amount of larger transcripts.

The Northern blot analysis of pFX3-213 showed that the inverted P_E1 promoter was still active and initiating transcription in the opposite direction. A very low level of transcript could be detected in pFX3-214. Among the internal deletion mutants, a transcript of the expected size could not be detected in pFX3-219, pFX3-221, and pFX3-223, but the transcripts were present in abundance in pFX3-215, pFX3-218, and pFX3-220.

DISCUSSION

In this study we examined the relationship between the structure and organization of the phage c2 origin and its ability to mediate DNA replication. To our knowledge, this is the most detailed investigation to date of lactococcal prolate phage DNA replication, which is characterized in this phage by a lack of a requirement for phage-encoded proteins. We elected to use a model system in which the phage *ori* supported replication of a plasmid in *L. lactis*. The *ori* region was originally inferred from sequence analysis and plasmid cloning (29, 47) and was also recently examined by using the two-dimensional gel technique (5) to analyze replicating c2 phage. Furthermore, the function of the corresponding *ori* region in prolate phages bIL67 and 923 was recently confirmed (40). In contrast to several isometric-headed phages (φ50, φ31, TP901-1, BK5-T, and Tuc2009) (31, 32, 36, 37, 38), the c2 *ori* does not confer a Per phenotype (39). The cloned c2 *ori* is also obviously functional in the absence of phage proteins, in contrast to the *ori* regions of some isometric-headed phages (BK5-T and φ31) (22, 32). The cloned sk1 origin of replication could support plasmid replication if the N-terminal 179 codons of ORF 47 and the intergenic region between ORFs 47 and 48 were present on a plasmid (8). In contrast, the data for phage c2 suggest that phage-encoded proteins are not required for DNA

replication, which is therefore entirely dependent on host replication proteins.

The P_E1 promoter gives rise to high levels of early transcript in c2-infected cells (28), and the sequence of the P_E1 promoter was strictly conserved in eight diverse prolate phages (40), although the transcript sequence was not conserved. This suggests that there was some selection for the P_E1 promoter sequence. However, although *ori* transcription was essential for supporting replication in the present study, the sequence of the promoter was not critical, as long as the promoter was functional. Introduction of two base pair changes in the −10 region of P_E1 (TATAAT to TAGAAG) was tolerated, and the resulting plasmid (pLP205) replicated. Analysis of the *ori* transcription in pLP205 confirmed that the ability to drive transcription was retained by the mutated promoter, although it was dramatically reduced. This observation is consistent with a previous report that some mutations in the −10 region of *L. lactis* promoters weaken the promoter strength but do not abolish it completely (25). The P_E1 promoter could also be replaced with the inducible *nisA* promoter (pLP212) without affecting replication. The *nisA* promoter showed some activity even in the absence of nisin, and the amount of transcript made was clearly sufficient to allow replication in the plasmid system. Leakiness of the *nisA* promoter in *L. lactis* has been reported only when cells were grown in lactose or galactose (7), which is not a potential explanation for our findings. However, our construct lacked the *nisA* gene ribosome binding site and conserved sequence around it, which were present in previous studies on this promoter regulation (14). These sequences were omitted to eliminate a translation signal (which is absent from the P_E1 transcript) and to avoid changing the essential 5′ end of the transcript and its position 1 start site. Overall, the 152-bp nisin promoter fragment in our construct lacked 15 nucleotides at the 5′ end and 153 bp at the 3′ end compared with the *nisA* promoter fragment characterized in pNZ8008 (14). The absence of these regions may have contributed to the *nisR*-independent transcription which we observed.

The *ori* deletion studies reported here and the ability of the P_E1 transcript template sequences of other prolate phage to support replication in the absence of the upstream A-T-rich region and P_L1 (40) show that these two conserved regions are not required for replication in the plasmid system. However, during lactococcal infection, the phage c2 genome replicates via theta forms emanating from the *ori* region (5) and another replication mechanism that could not be clearly resolved by the two-dimensional gel analysis but that gave rise to replication forks throughout the circularized genome. A role for the A-T-rich region in this mechanism cannot be excluded. Notwithstanding this, the plasmid model system facilitated a range of manipulations that would be difficult or prohibitively laborious in whole phage. However, it does have certain limitations. We used the ability of constructs to be electroporated into *L. lactis* as a qualitative assay for replication, but we could not distinguish between replication efficiencies once the plasmid had been established. The growth rate under erythromycin resistance selection could not be used as an indication of the copy number of the constructs, because one copy of the gene is sufficient to protect the cells. Future experiments with reporter genes or quantitative PCR may be used to explore the plasmid copy number in order to establish the replication efficiency of derivative *ori* constructs.

The essential role of transcription in the ability of the phage c2 *ori* region to support plasmid replication is consistent with observations for other phages, plasmids, and bacteria (2, 6, 18, 35). In phage c2, this promoter activity might be essential for localized strand melting to allow binding of replication proteins or might be required for producing a transcript with a mechanistic role. The former option now seems unlikely for two reasons. First, the promoter inversion abolished replication; hence, RNA polymerase-mediated melting of the transcript template region was not sufficient for replication. Second, the integrity of the nucleotide sequence of the P_E1 transcript was essential, suggesting a role for the RNA product. This template sequence could not be replaced by an unrelated fragment with a similar G+C content, and progressive deletion from the 3′ end abolished replication. Deletion of a G tract abolished replication, and the resulting ΔG tract transcript carried the 3′-most internal deletion in a series of internal deletions that did not support replication. Disruption of the transcript, beginning somewhere between positions 263 and 251, and anywhere further upstream abolished replication. These internal deletions were designed to coincide with regions that were predicted to form the stem-loop structure elements of the P_E1 transcript. The transcripts produced from three of these scanning deletion constructs were detectable, ruling out the possibility that RNA instability or enhanced degradation interfered with the function of transcripts capable of supporting replication. It was reported recently that chimeric fragments from the P_E1 transcript template sequences of phages c2 and 923 did not support replication (40) and that the modeled RNA structures of the P_E1 transcripts of these two phages, as well as the third type identified, were very different. Based on these findings and the data obtained with the progressive and internal deletions in this study, the most likely function of the P_E1 transcript is that it assumes a specific secondary structure and anneals to the origin region to initiate or facilitate replication. This annealing event could expose the noncoding strand or provide a primer. For example, in ColE1 plasmid replication, DNA polymerase I catalyzes leading strand formation after cleavage of an RNA-DNA hybrid by RNase H (22), and DNA polymerase I is therefore required for replication of ColE1 in the presence of RNase H. It is therefore noteworthy that phage c2 plates with 100% efficiency on a lactococcal strain deficient for DNA polymerase I (40), ruling out the possibility that the function of the P_E1 transcript is as a primer for initiation of replication by this polymerase. The primary transcript might be processed by other enzymes, and such a processing event might even depend on a specific transcript conformation. With or without processing, the c2 *ori* transcript might have a mechanism of action similar to that involved in R-loop formation during initiation of chromosomal replication at *oriC* of *E. coli*. This transcript does not form a primer (2) and functions even when it lacks a 3′-hydroxyl group. The continuing production of the phage c2 P_E1 transcript while progeny phage genomes accumulate within the cell suggests that there is an ongoing requirement for substantial amounts of the transcript. Further work is required to determine the mechanistic role of the c2 *ori* transcript in the replication of phage DNA.

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