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Temporally Regulated Transcriptional Expression of the Genomes of Lactococcal Bacteriophages c2 and sk1

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Transcription maps of the *Lactococcus lactis* subsp. *lactis* prolate phage c2 and small isometric phage sk1 were constructed. Early and late transcripts were demonstrated in phage c2. Early transcription was localized to within a 7.5-kb *Eco*RV fragment, and late transcription included the region which encodes the phage structural proteins and a lysin gene. Early, middle, and late transcripts were demonstrated in phage sk1. Transcription was confined to an 11.3-kb region defined by the three *Eco*RV restriction fragments of 6.2, 4.7, and 0.46 kb during the early part of the sk1 life cycle. Middle gene transcripts extended from the *Eco*RV site (defining the left-hand limit of early gene expression) through the *cos* site and included the 4.3-kb *Pvu*II-*cos* fragment. Late transcription was detected over the remainder of the phage genome. These results indicated that gene expression was temporally regulated at the level of transcription in these two lactococcal phages and that two regions of time-dependent transcription exist in phage c2 and three in phage sk1.

Bacteria belonging to the genus *Lactococcus* have been extensively used as starters in the production of fermented milk products. These bacteria are susceptible to bacteriophage lysis during the manufacturing process. Consequently, resistance to attack by bacteriophage is one of the main criteria applied for selection of lactococcal strains used in the dairy industry (5). This approach has, however, caused many strains with suitable growth characteristics (which would impart desirable properties such as enhanced flavor, aroma, or texture to the final product) to be rejected for industrial application because of their bacteriophage sensitivity.

The development of procedures which allow genetic modification of these bacteria has resulted in much effort being focused on the identification of genetic mechanisms which confer phage resistance on the host strain. Interstrain transfer of suitable genetic information would place less emphasis on the constraint of phage insensitivity as the main criterion in the selection of industrial starters. These studies have tended to concentrate on resistance determinants identified in the bacteria (8, 11, 15). More recently, researchers have turned their attention to the phage genome as a possible source of phage resistance traits. Phage-encoded traits on cloned fragments may interfere with the normal lytic cycle by overproduction or titration of essential regulatory signals necessary for phage propagation (7). Another possible approach is the use of antisense mRNA to inhibit expression of essential phage genes (1, 12).

If exploitation of lactococcal phage genomes as a source of phage-inhibitory mechanisms is to attain maximum potential, a greater understanding of lactococcal phage replication at the molecular level is required. In this study we investigated the transcriptional expression of two lactococcal phages, the prolate phage c2 and the small isometric phage sk1. Data are presented which indicate that this expression is time-dependent and consists of at least early and late genes in c2 and early, middle, and late genes in sk1.

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MATERIALS AND METHODS

Bacterial strains and phage. The lactococcal phages c2 and sk1 (16) and the bacterial strain *Lactococcus lactis* subsp. *lactis* MG1363 (4) were used throughout this study. Cultures were propagated in M17 medium (23) supplemented with 0.5% glucose (M17G). Growth was monitored as the increase in optical density at 600 nm versus time with an LKB Biochrom Ultraspec II spectrophotometer. General phage handling was performed as previously described (9).

DNA manipulations. DNA was isolated from phage prepared by CsCl gradient centrifugation as described previously (10). Digestions of phage DNA with restriction endonucleases were performed according to the manufacturer's instructions (Boehringer Mannheim). Supplementary restriction enzyme sites were mapped relative to sites previously identified (16). DNA fragments separated by agarose gel electrophoresis were transferred to nylon membranes (Hybond N) by the method of Southern (20).

RNA isolation and estimation. Total RNA was isolated from lactococcal cultures prior to phage infection and at various time points after phage infection according to the following procedure. Batch cultures (1.5 liters) of MG1363 were grown in M17G medium at 30°C from a 2% inoculum to an optical density at 600 nm of 0.1 (approximately 10^8 CFU/ml). $CaCl_2$ was added to a final concentration of 5 mM, and incubation was continued at 30°C for 10 min. The cells were then harvested by centrifugation at 16,000 × *g* for 2 min in a Sorvall RC5C centrifuge. The pellet obtained was resuspended in 30 ml of M17G containing 5 mM $CaCl_2$. The culture was incubated at 30°C for 5 min. A 2.0-ml sample was removed, and the cells were harvested by centrifugation in an Eppendorf centrifuge for 30 s. The supernatant was removed, and the pellet was quickly frozen in an ethanol-dry ice bath. Phage was added to the remaining culture from a high-density phage preparation, to a multiplicity of infection of 10, and the mixture was incubated at 30°C; the start of incubation was recorded as time zero. Samples of 2.0 ml were then removed at the intervals indicated, the cells were harvested by rapid centrifugation, and the pellets obtained were stored in an ethanol-dry ice bath.

To each cell pellet (typically 100 mg) 4.5 weight equivalents of glass beads (<106-μm diameter; Sigma) and 2

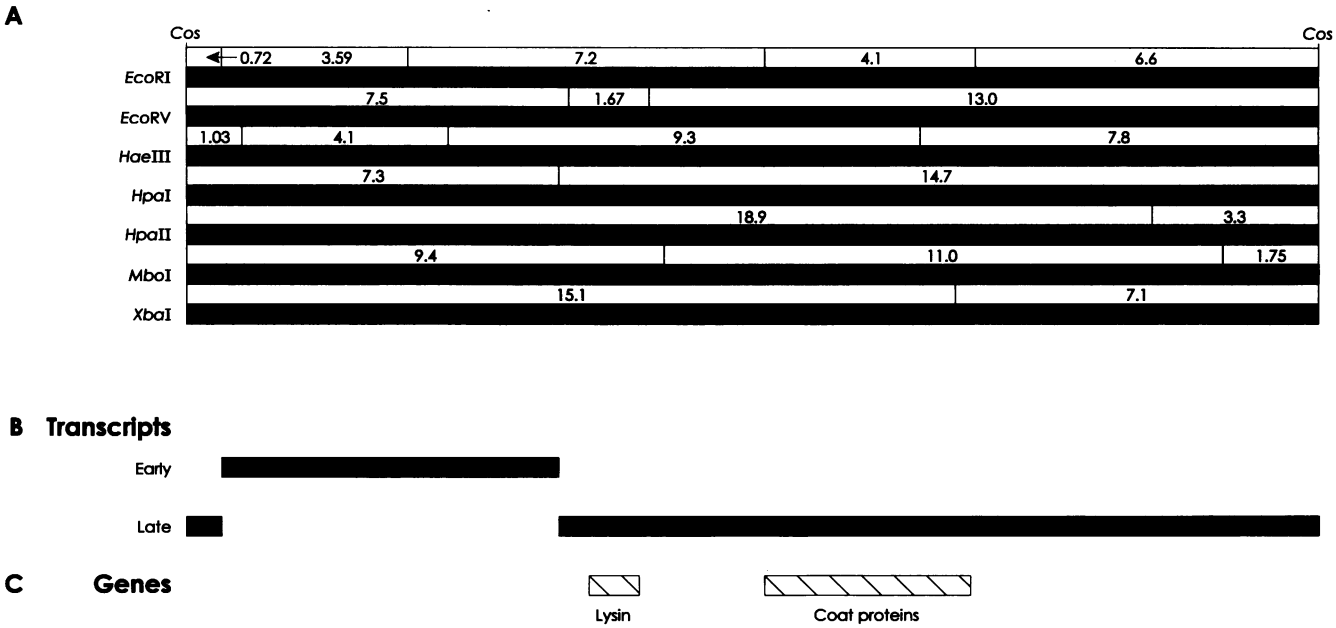

FIG. 1. Restriction and genetic map of phage c2. (A) Restriction endonuclease map of phage c2. Shown are fragments hybridizing to cDNA probes synthesized from RNA isolated 5 min (■) and 15 min (▩) postinfection. Fragment sizes are in kilobases. (B) Summary of the data from transcription mapping experiments, depicting the location of early and late transcripts. (C) Location of lysin (24) and coat protein genes.

volumes of 4 M guanidinium isothiocyanate were added (18). The mixture was vortexed at high speed for 3 min at 4°C and centrifuged in an Eppendorf centrifuge for 5 min, and the supernatant was removed and stored on ice. The cell extraction procedure was repeated twice. The combined supernatants were successively extracted with 1 volume of 25:24:1 phenol-chloroform-isoamyl alcohol until no precipitate formed at the interface and were then extracted twice with 1 volume of 24:1 chloroform-isoamyl alcohol. The RNA was precipitated from the aqueous phase with ethanol, and the pellets obtained were dried under a vacuum. They were then resuspended in diethyl pyrocarbonate-treated water (14) and treated at 37°C for 30 min with 6 U of RNase-free DNase (Promega) in the presence of 40 U of RNase inhibitor (Boehringer Mannheim) in a buffer containing 40 mM Tris-HCl (pH 7.6), 10 mM NaCl, 6 mM $MgCl_2$, and 2.5 mM $CaCl_2$.

Total RNA yield was estimated by measuring A_{260}, which typically indicated a yield of 60 to 90 μg of RNA per time point. RNA was stored at −20°C until required.

Nucleic acid labelling. RNA from phage-infected cells was used as a template to synthesize cDNA, with a modification of the Superscript Preamplification System (BRL) and [α-^{32}P]dCTP, 10 mCi/ml (Amersham). Five micrograms of RNA in a total volume of 10 μl was heated to 70°C for 10 min and collected by centrifugation for 5 s in an Eppendorf centrifuge, and then 1 μl of random hexamer (50 ng/μl) was added and incubation was continued at 70°C for a further 10 min. The mixture was quickly chilled on ice before collecting by centrifugation for 5 s. To the reaction mixture was added 2 μl of 10× synthesis buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl, 25 mM $MgCl_2$, 1 mg of bovine serum albumin per ml), 1 μl of a 10 mM deoxynucleoside triphosphate mix (10 mM [each] dATP, dTTP, and dGTP), 2 μl of 0.1 M dithiothreitol, 3 μl of [α-^{32}P]dCTP, and 1 μl of Superscript reverse transcriptase (200 U/μl). This mixture was incubated at room temperature for 10 min and then for 50 min at 42°C. The reaction was terminated by incubating at 90°C for 5 min.

Hybridizations were performed at 65°C overnight as described previously (14). The filters were washed in 2× SSC (1× SSC is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]–0.1% sodium dodecyl sulfate (SDS), and 1× SSC–0.1% SDS for 10 min at room temperature; this was followed by a high-stringency wash in 1× SSC–0.1% SDS at 65°C for 15 min. Washed filters were exposed to X-ray film (Agfa Curix RP1) in the presence of intensifying screens at −100°C.

RESULTS

RNA isolation. Total RNA was isolated from phage-infected cultures as described in Materials and Methods. Synchronous infection was achieved by a high multiplicity of infection (10:1) and pretreatment of the host cells with $CaCl_2$. Samples were removed at 5-min intervals after phage infection. Lysis of the host culture occurred 45 min postinfection by phage c2 and 60 min postinfection by sk1, indicating that one life cycle had been completed at this time.

Transcription mapping of phage c2. The restriction map of phage c2 previously determined in this laboratory (16) was extended to define the location of *Hpa*I and *Hpa*II restriction enzyme sites (Fig. 1A). Phage c2 DNA was digested with enzymes *Eco*RI, *Eco*RV, *Hae*III, *Hpa*I, *Hpa*II, *Mbo*I, and *Xba*I, and the DNA fragments were separated by gel electrophoresis (Fig. 2A). DNA was transferred to nylon filters and probed with ^{32}P-labelled cDNA synthesized from total RNA isolated from phage c2-infected cultures at 5 (Fig. 2B) and 15 (Fig. 2C) min postinfection. Only cDNA derived from phage transcripts would be expected to hybridize to the phage DNA, as it has been demonstrated previously that c2 DNA does not hybridize with the host genome (unpublished data).

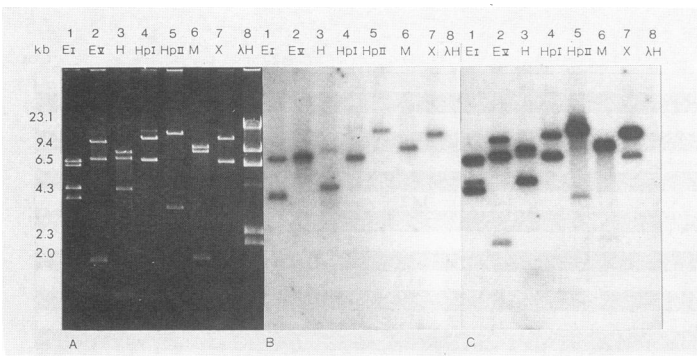

FIG. 2. Hybridization of cDNA with phage c2 DNA. (A) Gel electrophoresis of phage c2 DNA digested with the following enzymes (lanes 1 through 7, respectively): *Eco*RI (EI), *Eco*RV (EV), *Hae*III (H), *Hpa*I (HpI), *Hpa*II (HpII), *Mbo*I (M), and *Xba*I (X). Lane 8, λ *Hin*dIII molecular weight markers. (B and C) Corresponding autoradiograph probed with cDNA synthesized from RNA isolated 5 min (B) and 15 min (C) postinfection.

Analysis of the hybrid fragments indicated that at 5 min postinfection transcription was concentrated on a region located near the left-hand *cos* site (Fig. 1B). Three *Hae*III fragments hybridized with the probe (Fig. 2B, lane 3). These fragments were 1.03, 4.1, and 9.3 kb, respectively, and were located immediately adjacent to each other on the restriction map. The data from the *Eco*RV and *Hpa*I digests (Fig. 2B, lanes 2 and 4) indicated that only one fragment from each digest hybridized with the probe. These fragments were 7.5 and 7.3 kb, respectively, and each was located immediately adjacent to the left-hand *cos* end (Fig. 1A). In neither digest was there any evidence to suggest that the region transcribed extended to the adjacent 1.67- or 14.7-kb fragments. These data indicated that the right-hand limit of transcription 5 min after phage c2 infection was located in the 9.3-kb *Hae*III fragment, before the region of overlap with the 1.67-kb *Eco*RV or 14.7-kb *Hpa*I fragments. The 0.72-kb *Eco*RI fragment located immediately adjacent to the left-hand *cos* site did not show any homology to the cDNA synthesized from the RNA isolated 5 min after phage infection; hence, the left-hand limit of early transcriptional expression was located within the 1.03-kb *Hae*III fragment before the region of overlap with the 0.72-kb *Eco*RI fragment. These data define the region of early transcription and are depicted in Fig. 1B. At 10 min postinfection the same transcription pattern was observed (data not shown).

Transcriptional activity was much more extensive at 15 min after infection with phage c2 (Fig. 2B). In each of the digests examined, all the DNA fragments hybridized with the cDNA probe. In experiments in which the autoradiographs were overexposed, evidence was obtained that the 0.72-kb *Eco*RI fragment hybridized to the 15-min probe (data not shown). This indicated that at late times during the life cycle of c2 its transcription occurred over the remainder of the genome, where expression was not detected 5 min postinfection. Transcriptional activity was still detected within the early gene region. The region of exclusive late gene transcription is indicated in Fig. 1B. This pattern of transcriptional expression was maintained until lysis of the host culture occurred at 45 min.

Transcription mapping of phage sk1. A restriction map of the chromosome of phage sk1 was previously determined (16). Filters prepared from phage sk1 DNA, digested with combinations of restriction enzymes (Fig. 3A), were probed with cDNA synthesized from RNA isolated 5, 10, and 30 min after infection with phage sk1. Fragments of less than 0.8 kb were not on the filters as they had been run off the gel. Analysis of the data (Fig. 4) indicated that transcriptional expression of the phage genome was occurring in a temporally regulated manner.

Within 5 min of phage sk1 infection, phage-specific transcripts were being synthesized within the host cell. Hybridization of the cDNA probe from RNA synthesized 5 min postinfection indicated that transcriptional activity was focused within a portion of the phage genome (Fig. 4B). The *Cla*I-*Xho*I-*Sal*I digestion of sk1 DNA resulted in five fragments (Fig. 4A, lane 2), only one of which, the 14.7-kb fragment (Fig. 4B, lane 2), hybridized with the 5-min probe. A similar situation was observed in the *Pvu*II digest, where hybridization was limited to a 13.6-kb fragment (Fig. 4B, lane 5). The 6.2- and 4.7-kb *Eco*RV fragments hybridized with the 5-min probe (Fig. 4B, lane 4). In the *Cla*I-*Hin*dIII digest, three fragments of 8.37, 2.4, and 2.15 kb hybridized

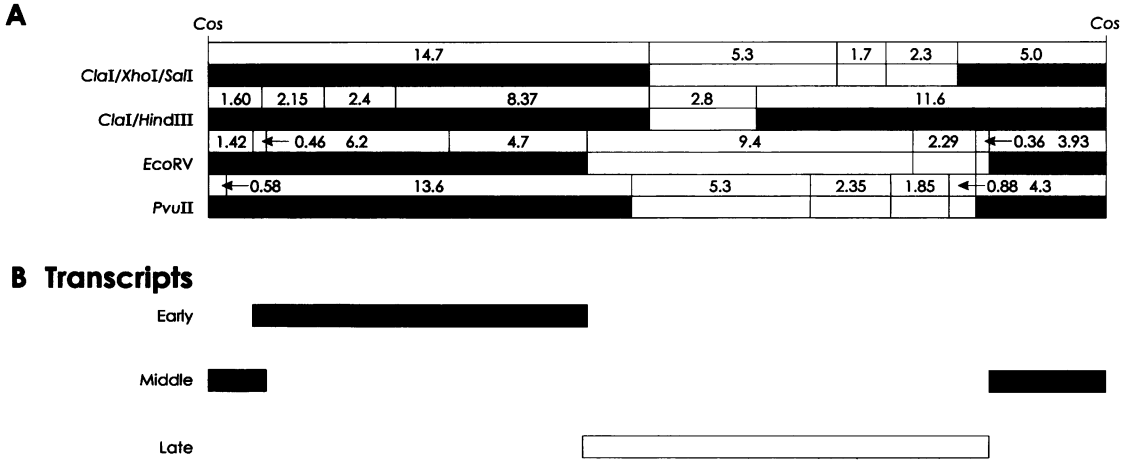

FIG. 3. Restriction and genetic map of phage sk1. (A) Restriction endonuclease map of phage sk1. Shown are restriction fragments hybridizing to cDNA probes synthesized from RNA isolated 5 min (■), 10 min (▭), and 30 min (▭) postinfection. Fragment sizes are in kilobases. (B) Summary of the data from transcription mapping experiments depicting the location of early, middle, and late transcripts.

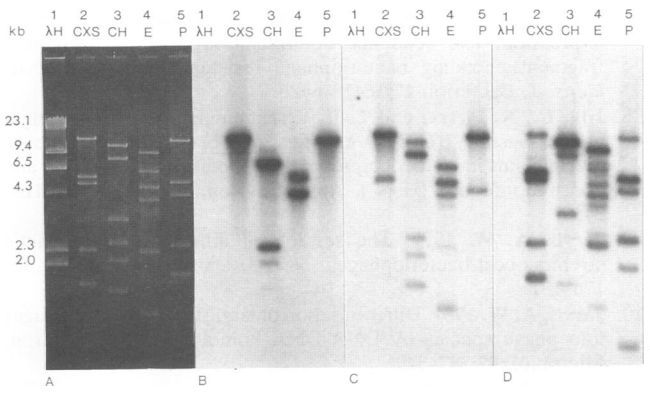

FIG. 4. Hybridization of cDNA with phage sk1 DNA. (A) Gel electrophoresis of phage sk1 DNA digests. Lane 1, λ *Hin*dIII molecular weight markers; lanes 2 through 5, respectively, sk1 DNA digested with *Cla*I-*Xho*I-*Sal*I (CXS), *Cla*I-*Hin*dIII (CH), *Eco*RV (E), and *Pvu*II (P), respectively. (B through D) Corresponding autoradiograph probed with cDNA synthesized from RNA isolated 5, 10, and 30 min postinfection, respectively.

strongly with the probe. The 1.6-kb *Cla*I-*Hin*dIII fragment hybridized faintly to this probe, whereas the 1.42-kb *Eco*RV fragment showed no hybridization (Fig. 4B, lanes 3 and 4). All these fragments were located within the same region of the phage sk1 genome. These data indicated that transcription 5 min postinfection was located within the three adjacent 6.2-, 4.7-, and 0.46-kb *Eco*RV fragments (Fig. 3B).

At 10 min after phage infection, transcripts were being synthesized from a greater portion of the phage genome (Fig. 4C). In addition to the fragments which hybridized at 5 min, a number of other fragments in all four digests examined hybridized with the 10-min probe. In the *Cla*I-*Xho*I-*Sal*I digest a 5-kb fragment hybridized and in the *Cla*I-*Hin*dIII digest two further fragments of 1.6 and 11.6 kb hybridized with the probe (Fig. 4C, lanes 2 and 3). The 3.93- and 1.42-kb fragments in the *Eco*RV digest hybridized, as did a 4.3-kb fragment in the *Pvu*II digest (Fig. 4C, lanes 4 and 5). These combined data suggest that transcription was occurring across the *cos* site at 10 min postinfection and that a further approximately 6 kb of the sk1 genome was being transcribed at this time (Fig. 3). Evidence was obtained from overexposed autoradiographs (data not shown) that low levels of transcription occurred immediately to the left of the region strongly transcribed at 10 min, since hybridization was observed with the 2.3- and 1.7-kb *Cla*I-*Xho*I-*Sal*I fragments, the 2.35-, 1.85-, and 0.88-kb *Pvu*II fragments, and the 2.29-kb *Eco*RV fragment.

By 30 min postinfection, transcriptional activity extended to the remainder of the phage genome (Fig. 3 and 4D). The area which was hybridized weakly to the 10-min probe was expressed strongly at this time. The 10-min specific transcripts were still present in the infected cells. The strength of the hybridization signal from many of the fragments which hybridized at 5 and 10 min postinfection was reduced; this was particularly evident with the fragments from the left-hand side of the phage genome.

DISCUSSION

The generation of new phage within an infected cell requires the coordinated control of a number of interacting pathways. Data from a number of intensively studied phages indicate that much of this control is exerted at the level of transcription (13). During most phage infections, transcription can be conveniently divided into at least early and late stages, with late stages usually requiring prior transcription and translation of the early region in order to proceed. The current study was undertaken to determine the transcriptional organization of the genomes of phages c2 and sk1, which are well-studied representatives of the two most commonly occurring phage species in the dairy industry.

A method for the isolation of total RNA from phage-infected cultures was developed. Concentrated cell cultures were used so that significant yields of RNA could be achieved with minimal manipulation. Synchronous infection was important to ensure that RNA isolated at any time point was not contaminated by transcripts from a different stage in the phage cycle.

The data obtained indicated that gene expression was temporally regulated at the level of transcription in both lactococcal phages examined. In the case of c2, two regions of transcriptional activity were detected during the phage replication cycle. These were referred to as early transcription, which occurred between 0 and 10 min postinfection, and late transcription, which began between 10 and 15 min postinfection and continued for the duration of the replication cycle (Fig. 1). Attempts to clone DNA fragments from the early gene region of c2 have proven to be largely unsuccessful (24). This may be due to the presence within this region of lethal genes which interfere with host cell metabolism. Gene mapping experiments within the late gene region have indicated that this portion of the genome encodes a lysin (24) and phage coat protein genes (unpublished data). During the replication cycle of phage sk1, three phages of transcription, which are referred to as early, middle, and late transcription phases, were observed. Early transcription appeared within 5 min of infection, by 10 min postinfection middle transcripts appeared, and by 30 min transcriptional activity had extended into the late region (Fig. 3). The apparently more complex transcriptional pathway observed for phage sk1 may be a consequence of its larger genome size (29 kb) relative to c2 (22 kb) (16).

Most phages examined to date with genomes greater than 10 kb exhibit temporal regulation of transcriptional expression (13). Three or four sets of transcripts can usually be defined in this manner. T7, which is a well-studied example of the T-odd coliphages, has a genome of 40 kb which is transcribed in three blocks. The first set of genes to be transcribed uses host RNA polymerase (21), while the two late sets use a phage-encoded RNA polymerase (22). T4, an example of the T-even phages, has a genome size of 166 kb and has four sets of genes. Expression of the early genes is mediated by host RNA polymerase. One of the products of early gene expression is a protein which modifies host RNA polymerase, altering its specificity so that it has a preference for delayed early phage promoters. Middle gene expression is modulated by a promoter-binding protein, and late gene expression is controlled by a phage-encoded sigma factor, α^{gp55} (13). Phage-encoded sigma factors also play a role in control of gene expression in the *Bacillus subtilis* phage SPO1 (6). Temporal gene expression has also been observed in coliphage λ (19) and is mediated by an antitermination system brought about by phage-encoded proteins (17). No temporal control of transcription has been observed in either the small filamentous (e.g., f1 [3]) or small polyhedral (e.g., φX174 [2]) phage. These phages have genome sizes of 6.4 and 5.3 kb, respectively. All promoters seem to be active throughout the replication cycle. The data presented here

demonstrate temporal regulation of transcriptional expression in lactococcal phages and therefore imply the presence of sigma factors or other activator systems. This would also be expected on the basis of the genome sizes of the two phages studied.

The hybridization signal obtained from late transcripts of phage c2 was much less intense than that observed from the early gene region (Fig. 2). This is surprising, as it would be expected that the late transcripts, which include the genes for the phage coat proteins, would need to be translated to high levels, a situation which could be helped by increased transcription of this region. A possible explanation for this anomaly is that at early times postinfection the phage transcripts have to compete with host transcripts for available translational factors and, therefore, a high concentration of phage mRNA is needed. At later times during the replication cycle this situation may be reversed as cell growth is inhibited. This would imply that host transcription-translation is shut off, thus making available a greater portion of the cells' translation machinery for phage gene expression.

No appreciable decrease in the amount of early transcripts occurred during the phage c2 life cycle (Fig. 2). Whether this was due to continued synthesis of both sets of transcripts or to stability of the early transcripts is not yet known. During sk1 infection a progressive replacement of transcripts was observed (Fig. 4). The early transcripts were degraded late in the phage life cycle, suggesting specific demarcation between the three phases of gene expression in this phage.

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