Temporal transcription map of the *Lactococcus lactis* bacteriophage sk1

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Bacteriophage sk1 is a small isometric-headed lytic phage that infects *Lactococcus lactis*. The phage has a linear double-stranded DNA genome of 28 kbp, with cohesive ends. RNA was prepared from phage-infected *L. lactis* cells harvested at various intervals after infection, and the RNA molecules were resolved by electrophoresis. Northern blots of these gels were hybridized with sk1 DNA probes and the results obtained from these experiments, together with the results of primer extension analyses, enabled a transcription map of the phage genome to be prepared. Three classes of phage transcripts, designated as early, middle or late based on their time of appearance, were detected. Seven partially overlapping early transcripts were detected; these were transcribed from a 10 kbp region of the phage. The nine middle transcripts were derived from a 2 kbp region, limited by *cos* at one end and the start of the early transcripts at the other. The early and middle transcripts were transcribed divergently from a region mapping at 26 kbp on the sk1 physical map. The four late transcripts were derived from a 16 kbp region of the phage limited at one end by *cos*. The late transcripts were transcribed in the opposite direction to the early transcripts and three of the late transcripts terminated in the same region of the phage genome as three of the early transcripts.

Keywords: small isometric-headed bacteriophage sk1, *Lactococcus lactis*, transcription map

INTRODUCTION

Bacteria belonging to the genus *Lactococcus* are used extensively in the fermentation of dairy products to convert lactose to lactic acid. The fermentation is carried out in a non-sterile environment and the starter bacteria are susceptible to infection by bacteriophages. This phage infection of starter bacteria is still the major cause of slow acid production in cheese manufacture and there is currently considerable interest in using genetic procedures to enhance the phage resistance properties of starter bacteria (Klaenhammer, 1987; Powell *et al.*, 1990; Ward *et al.*, 1992; O'Sullivan *et al.*, 1993). Lactococcal phages have been differentiated into 12 species on the basis of their morphology and DNA homology (Jarvis *et al.*, 1991). The phages isolated from cheese factories are of two major morphological types (Terzaghi, 1976): small isometric-headed phages (Siphoviridae, morphotype B1) and prolate-headed phages (Siphoviridae, morphotype B2). These two types represent 84 % and 13 % respectively of the phage isolated from New Zealand cheese factories (Terzaghi, 1976). Despite the considerable economic significance of phage infection of cheese starter bacteria,

there is little information at the molecular level about the life cycle of these phages or their interaction with their host. Lactococcal phage genes that have been cloned and sequenced include genes encoding some of the structural proteins of the small isometric-headed phage F4-1 (Kim & Batt, 1991; Chung *et al.*, 1991), the lysin genes from ϕ vML3, c2 and ϕ US3 (Shearman *et al.*, 1989; Platteeuw & de Vos, 1992; Ward *et al.*, 1993), and a type II methyltransferase (Hill *et al.*, 1991). In addition, a putative regulator gene *bpi* (Lakshmidēvi *et al.*, 1991) and the *int* gene (Lillehaug & Birkeland, 1993) from temperate lactococcal phages have been cloned and sequenced.

The lactococcal phage sk1 has been shown to have a double-stranded DNA genome, 28 kbp in length (Pillidge & Jarvis, 1988; Powell *et al.*, 1989), the nucleotide sequence of its cohesive ends has been determined (Chandry *et al.*, 1994) and a low-resolution transcript map of the phage has been determined (Beresford *et al.*, 1993). This paper describes the construction of a detailed transcript map for the small isometric-headed lactococcal phage sk1.

Table 1. DNA probes used to map *sk1* transcripts

DNA probe*	Map position†	Comments‡
EvB	20·5–26·3	Fragment cloned in pJDC9
EvC	15·7–20·4	Fragment cloned in pJDC9
EvF	26·7–28·0	Fragment cloned in pJDC9
EvG	26·3–26·7	Fragment cloned in pJDC9
HiC	22·1–24·5	Fragment cloned in pJDC9
HiC-21	23·6–24·5	HiC deleted by 1·5 kbp from left end by treatment with exonuclease III and cloned in pJDC9
HiC-24	22·6–24·5	HiC deleted by 0·5 kbp from left end by treatment with exonuclease III and cloned in pJDC9
HiC-36	23·9–24·5	HiC deleted by 1·8 kbp from left end by treatment with exonuclease III and cloned in pJDC9
HiD	24·5–26·6	Gel purified from <i>Hind</i> III digest of <i>sk1</i>
MsD	15·0–16·2	Fragment cloned in pJDC9
PvB	9·5–14·6	Fragment cloned in pJDC9
PvD	6·9–9·5	Fragment cloned in pJDC9
PvE	5·1–6·9	Fragment cloned in pJDC9
SaΔA2	19·8–22·3	Spontaneous deletion of SaA2 cloned into pJDC9
SaΔA2-39	21·3–22·3	SaΔA2 deleted by 1·5 kbp from left end by treatment with exonuclease III and cloned into pJDC9
SaE1	14·2–15·7	Fragment cloned in pJDC9
SaG	15·8–16·5	Fragment cloned in pJDC9
SsC	12·1–16·2	Fragment cloned in pJDC9
SsC-6	12·1–15·8	SsC deleted by 400 bp from right end by treatment with exonuclease III and cloned in pJDC9
SsC-44	12·1–16·0	SsC deleted by 200 bp from right end by treatment with exonuclease III and cloned in pJDC9
SsJ	16·2–16·6	Fragment cloned in pJDC9
SsLNPR	24·5–26·0	Product of partial <i>Ssp</i> I digestion of <i>sk1</i> DNA cloned into pJDC9
SsLNPR-23	25·6–26·0	SsLNPR deleted by 1·1 kbp from left end by treatment with exonuclease III and cloned into pJDC9
SsLNPR-28	25·8–26·0	SsLNPR deleted by 1·3 kbp from left end by treatment with exonuclease III and cloned into pJDC9
SsLNPR-33	25·7–26·0	SsLNPR deleted by 1·2 kbp from left end by treatment with exonuclease III and cloned into pJDC9
SsNPR	25·1–26·0	Product of partial <i>Ssp</i> I digestion of <i>sk1</i> DNA cloned into pJDC9
SsNPR-10	25·1–25·3	SsNPR deleted by 0·7 kbp from right end by treatment with exonuclease III and cloned into pJDC9

* *sk1* restriction fragment as described in Fig. 1. Hyphenated numbers indicate restriction fragments that were truncated by treatment with exonuclease III prior to cloning.

† As shown in Fig. 1.

‡ Entire plasmid was used as DNA probe unless otherwise indicated.

METHODS

Bacterial growth conditions. *Lactococcus lactis* subsp. *lactis* strain HID113 (Powell *et al.*, 1990) was grown at 30 °C in M17 medium (Terzaghi & Sandine, 1975) containing 0·5% (w/v) glucose (M17G).

Preparation of phage. Overnight cultures of HID113 were diluted 1:100 into fresh M17G containing 10 mM CaCl₂ and *sk1* phage was added to a final titre of 5×10^4 p.f.u. ml⁻¹. After incubation at 30 °C for 16 h, the lysates, containing 5×10^9 to 1×10^{10} p.f.u. ml⁻¹, were clarified by centrifugation at 13000 *g*

for 15 min, adjusted to pH 7·0 with 2 M Tris/HCl (pH 7·0) and filtered through a 0·45 µm filter. DNA was isolated from the phage particles essentially by the procedure described for the isolation of λ DNA in the Qiagenologist (Qiagen, Chatsworth, CA, USA) except that the volume of lysate was eightfold greater than that recommended for phage λ and the phage were incubated with proteinase K (50 µg ml⁻¹) at 65 °C for 10 min, prior to the addition of SDS.

Isolation of lactococcal RNA. Lactococcal RNA was prepared using a modification of the acid phenol extraction method of

Fig. 1. Restriction map of sk1 DNA. Complete restriction maps for the enzymes *EcoRI*, *EcoRV*, *HindIII*, *PvuII*, *Sau3AI* and *MspI*, and an incomplete map for *SspI*, are shown. The nomenclature for restriction fragments combines the first two letters of the restriction enzyme (except for *EcoRV*, which is called Ev to avoid confusion with *EcoRI*) with the letters A, B, etc., in order of decreasing fragment size. Fragments of the same size are assigned the same letter with a number appended to each (e.g. A1, A2, etc.). Regions where restriction sites have not been determined for *SspI* are hatched. The box marked SaΔA2 shows the location of a cloned *Sau3AI* subfragment which presumably resulted from the spontaneous deletion of the right end of SaA2.

van Rooijen & de Vos (1990). *L. lactis* HID113 was grown to an OD_{660} of 0.3 in M17G, then $CaCl_2$ was added to a final concentration of 10 mM. Immediately prior to infection with sk1, a 30 ml sample of the culture was transferred into a 125 ml flask containing 9 ml ice-cold buffer A (20 mM Tris/HCl pH 7.5, 20 mM sodium azide, 400 μ g chloramphenicol ml^{-1}) and quickly chilled by swirling in an ice/ethanol bath. The remaining cells were infected with sk1 at a multiplicity of infection (m.o.i.) of approximately 5. Aliquots (30 ml) of the infected cells, taken immediately after phage addition (time = 0) and at the times indicated in individual experiments, were treated as described for the uninfected sample. The chilled cells suspended in buffer A were harvested by centrifugation, washed once with buffer A and resuspended in 1 ml buffer B (25%, w/v, sucrose, 50 mM Tris/HCl pH 7.5, 1 mM EDTA). After the addition of lysozyme to a final concentration of 1.5 μ g ml^{-1} , and incubation of the mixture on ice for 10 min, the cells were harvested by centrifugation and quickly resuspended in 350 μ l buffer C (20 mM sodium acetate pH 5.2, 3 mM EDTA, 150 mM NaCl) at 0 °C. An equal volume of buffer C containing 2% (w/v) SDS and preheated to 95 °C was added and the mixture was incubated at 95 °C for 1 min. The lysed cells were extracted with an equal volume of a 1:1 mixture of acid phenol (equilibrated with 20 mM sodium acetate, pH 5.2) and chloroform (chloroform:isoamyl alcohol, 24:1, v/v) at 55 °C for 1 min. The aqueous phase was extracted a further four times with acid phenol/chloroform at room temperature, once with chloroform/isoamyl alcohol (24:1) and the RNA was precipitated with 2-propanol. The RNA samples were dissolved in 50 μ l water and incubated with 35 U of RNase-free DNase I to remove any contaminating DNA.

Northern blots and hybridizations. RNA was analysed by electrophoresis through 1% (w/v) agarose gels containing 2.2 M formaldehyde (Maniatis *et al.*, 1982). After electrophoresis, gels were washed several times with water then stained briefly with ethidium bromide to visualize the ribosomal RNA bands and RNA size markers (0.24–9.5 kb, Bethesda Research Labs). Transfer to Hybond N+ membranes (Amersham) was carried out by capillary transfer as recommended by the membrane manufacturer. Hybridizations were carried out at

65 °C for 12 h in a solution containing 5 $\times$ SSPE [1 $\times$ SSPE is 150 mM NaCl, 9.7 mM sodium phosphate, and 1 mM EDTA (pH 7.4) (Maniatis *et al.*, 1982)], 5 $\times$ Denhardt's solution (Maniatis *et al.*, 1982) and 0.5% (w/v) SDS. Final washing of the membrane was with 1 $\times$ SSPE, 0.1% (w/v) SDS at 65 °C for 15 min. ^{32}P -labelled DNA probes were prepared by the random primer procedure (Feinberg & Vogelstein, 1984) using gel-purified phage restriction fragments or cloned phage fragments (Table 1).

Preparation of single-stranded DNA probes. The sk1 DNA fragment SaΔA2 (Table 1, Fig. 1) cloned in pJDC9 (Chen & Morrison, 1988) was used as template for single-stranded probe generation. M13 universal primer or M13 reverse primer (1 μ M) was mixed with 50 ng template DNA. Thermal cycling reactions were performed with 1 U *Taq* DNA polymerase (Boehringer Mannheim), in a reaction mixture containing 50 μ M dCTP, 50 μ M dGTP, 50 μ M dTTP, 1.6 μ M dATP, 2.5 μ Ci [α - ^{32}P]dATP, 10 mM Tris/HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, and 0.1 mg gelatin ml^{-1} . The reactions were cycled 30 times at 94 °C for 1.5 min, 50 °C for 2 min, and 72 °C for 5 min.

Primer extension analysis. Total RNA was isolated from sk1-infected cells as described above. Oligonucleotide primers were synthesized on an Applied Biosystems model 381A DNA synthesizer and approximately 0.25 μ g annealed with 15 μ g RNA in 3 μ l water by incubating at 90 °C for 1 min and then at 42 °C for 5 min. Radiolabelled cDNA was synthesized in 10 μ l total volume reactions containing the RNA/primer mixture, 24 U AMV reverse transcriptase, 15 μ Ci [α - ^{32}P]dATP, 100 μ M dCTP, 100 μ M dGTP, 100 μ M dTTP, 10 μ M dATP, 50 mM Tris/HCl pH 8.5, 50 mM KCl, 20 mM $MgCl_2$, and 10 mM DTT. Reactions were incubated at 42 °C for 30 min then stopped by the addition of 5 μ l 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. The products were analysed, along with DNA sequencing ladders, on 6% (w/v) polyacrylamide gels containing 7 M urea and detected by autoradiography. Cloned sk1 DNA fragments were used as template for sequencing with the Sequenase kit utilizing the same primers used to determine the 5' ends of the RNA.

Fig. 2 (continued on facing page). Northern blots of RNA from sk1-infected *L. lactis*, hybridized with: (a), MsD (autoradiograph exposed for 5 d); (b), MsD (autoradiograph exposed for 16 h); (c), EvD; (d), EvF; (e), SsC; (f), EvB; and (g), SaΔA2. One blot was used for panels (a–d), a second was used for panels (e) and (g), and a third was used for panel (f). RNA was prepared from uninfected cells immediately prior to infection (lane U), immediately after infection (lane 0), and at the following times post-infection: 1 min (lane 1), 2 min (lane 2), 3 min (lane 3), 4 min (lane 4), 5 min (lane 5), 6 min (lane 6), 7 min (lane 7), 8 min (lane 8), 9 min (lane 9), 10 min (lane 10), 15 min (lane 15), 20 min (lane 20), 25 min (lane 25), and 30 min (lane 30). Bands marked rRNA designate regions of nonspecific hybridization at the front of the 23S and 16S rRNA species. The sizes (kb) of the transcripts determined by comparison of band mobilities with those of RNA markers are marked to the right of the autoradiograms.

RESULTS

Restriction map of sk1

In order to determine the transcript map for sk1 phage, it was necessary to construct a detailed restriction map of the phage to identify fragments which could be used as

probes against Northern blots of phage mRNA. The sk1 restriction map for the enzymes *EcoRI*, *EcoRV*, *HindIII*, *PvuII*, *Sau3AI* and *MspI*, and an incomplete map for the enzyme *SspI*, is shown in Fig. 1. The previously published *EcoRV* restriction map (Pillidge & Jarvis, 1988; Powell *et al.*, 1989; Chandry *et al.*, 1994) has been modified

to include a new fragment of 50 bp, EvI, which was identified during nucleotide sequence analysis and confirmed by digestion and polyacrylamide gel electrophoresis (data not shown).

Temporal regulation of phage transcripts

RNA was isolated from *L. lactis* at various times after infection with sk1 phage, electrophoresed, and Northern blots of the gels were hybridized with DNA probes from different regions of the sk1 genome (Fig. 2). Tables 2–4 summarize the results of 55 hybridizations of 30 different Northern blots derived from 12 separate RNA preparations. Three temporal classes of phage specific transcripts, designated early, middle and late transcripts, were observed. The seven early phage transcripts (designated E1–E7 in order of decreasing size, Table 2) were first detected 2–5 min after phage infection (Fig. 2a and g, lanes 1–5). The intensity of hybridization to these transcripts decreased during the late stages of the infection (Fig. 2g). The nine middle phage transcripts (designated M1–M9 in order of decreasing size, Table 3) were first detected 7–10 min after infection (Fig. 2d, lanes 7–10) and the intensity of hybridization of DNA probes to these transcripts increased in later samples. Four late phage transcripts (designated L1–L4 in order of decreasing size, Table 4) were first detected approximately 15 min after infection (Fig. 2a, c and e, lanes 15–30) and thereafter the intensity of hybridization to these transcripts increased during the infection.

Early stage transcripts

The region of the phage genome from which the early phage transcripts were derived was determined by hybridizing specific phage fragments to the Northern

blots. All probes that contained DNA from between 15.9 kbp and 25.9 kbp on the map (Fig. 1) hybridized to early transcripts, whereas probes containing DNA from outside this region did not (Table 2, Fig. 3). The early transcripts hybridizing to SsC are not visible in Fig. 2(e), but were visible in a longer exposure of the autoradiograph (data not shown) and are detected with the MsD probe (Fig. 2a), the right end of which is coincident with the right end of SsC (Fig. 1). The left- and right-hand limits to the region were established by SaE1 and EvG, respectively, since neither of these fragments hybridized with any of the early phage transcripts. MsD hybridized to phage transcripts which appeared within 2–3 min of phage infection and also to phage transcripts which first appeared 15 min after infection (Fig. 2a and b). Hence, these fragments overlap the early and late regions of the phage. EvB hybridized to early and middle transcripts (Figs 2f and 3) and consequently overlaps the junction between the early and middle regions of the phage.

The evidence for the mapping of the seven early transcripts is as follows. The early transcripts E3, E4 and E6 hybridized with SsC, SsJ and SaG (Table 2, Fig. 3). However, none of these transcripts hybridized with SsC-6, which is deleted of the 400 bp at the right end of SsC (Table 1, Fig. 3). Therefore, the left ends of E3, E4 and E6 must lie within this 400 bp region. Of these three transcripts, only E6 hybridized with SsC-44 (Table 2), which is deleted of 200 bp from the right end of SsC (Table 1). This indicated that the left end of E6 extended into SsC-44, but not SsC-6, and mapped the left end of this transcript to approximately 15.9 kbp on the sk1 map (Fig. 3). The right end was then mapped on the basis of transcript size to approximately 17.7 kbp (Fig. 3). The right ends of E3 and E4 were also mapped on the basis of transcript size. It is possible that E3 and E4 also extend into SsC-44 and share a common left end with E6, but

Table 2. Hybridization of DNA probes to sk1 early transcripts

DNA probe*	Transcript (kb):						
	E1 (7.1)	E2 (5.7)	E3 (3.9)	E4† (3.5)	E5† (3.5)	E6 (1.8)	E7 (0.7)
EvB	+	+	—	—	+	—	+
EvC	+	+	+	+	+	+	—
HiC	+	+	—	—	+	—	—
HiC-21	+	+	—	—	—	—	—
HiC-24	+	+	—	—	—	—	—
HiC-36	+	—	—	—	—	—	—
HiD	+	—	—	—	—	—	+
MsD	—	—	+	+	—	+	—
SaΔA2	+	+	—	—	+	—	—
SaΔA2-39	+	+	—	—	+	—	—
SaG	—	—	+	+	—	+	—
SsC	—	—	+	+	—	+	—
SsC-6	—	—	—	—	—	—	—
SsC-44	—	—	—	—	—	+	—
SsJ	—	—	+	+	—	+	—
SsLNPR	—	—	—	—	—	—	+
SsLNPR-23	—	—	—	—	—	—	+
SsLNPR-28	—	—	—	—	—	—	+
SsLNPR-33	—	—	—	—	—	—	+
SsNPR	—	—	—	—	—	—	+
SsNPR-10	—	—	—	—	—	—	+

+, DNA probe hybridized with sk1 transcript; —, no hybridization of DNA probe with sk1 transcript was detected.

* sk1 restriction fragment as described in Table 1 and Fig. 1.

† The assignment of probes hybridizing to E4 or E5 was inferred from the transcript map (Fig. 3).

were not detected by hybridization with SsC-44 because of their comparatively low abundance (Fig. 2a) and small extent of overlap with the probe. The failure to observe hybridization of the 3.9 kb E3 transcript with either SsC-44 or SaΔA2, although these fragments are separated by only 3.5 kbp (Fig. 3) is consistent with this suggestion. The left ends of the E3, E4 and E6 transcripts are therefore very similar (or identical) and these transcripts overlap each other (Fig. 3).

The early transcripts E1, E2 and E5 hybridized with HiC (Table 2), suggesting that these transcripts also overlap (Fig. 3). While E4 and E5 are the same size (3.5 kb), E5 must be a different transcript to E4, which hybridized to SaG, because 5.5 kbp of DNA separates HiC from SaG (Fig. 1). The failure of HiC-24 to hybridize with E5 (Table 2) enabled the right end of this transcript to be placed between map positions 22.0 and 22.5 kbp (Fig. 3). Similarly, because E2 hybridized to HiC-21, but not to HiC-36 (Table 2), its right end was located between map positions 23.7 to 23.9 kbp (Fig. 3). Similarly, E1 hybridized to SsLNPR and HiD but not to SsNPR (Table 2) and therefore its right end was placed between 24.6 and

25 kbp (Fig. 3). The left ends of E1, E2 and E5 were then deduced from the size of these transcripts.

The early transcript E7 hybridized with SsNPR and SsLNPR and their subfragments SsNPR-10 and SsLNPR-28 (Table 2). These data indicated that most of this transcript was from within SsNPR, with a right end close to the 25.9 kbp position on the map (Fig. 3).

Orientation of the early phage transcripts

The direction of transcription of the early phage genes encoding E1, E2 and E5 was determined by using probes consisting of single-stranded DNA covering the phage SaΔA2 fragment (Fig. 1). These probes from either end of the cloned fragment were prepared as described in Methods and hybridized to Northern blots of RNA isolated from sk1-infected cells. Only the probe with the 5' end oriented to the left of the maps shown in Figs 1 and 3 hybridized to the phage RNA (data not shown), indicating that E1, E2 and E5 are oriented in the right to left direction of the maps shown in Figs 1 and 3. Nucleotide sequence data subsequently showed that the open reading frames encoded by the early phage genes were all oriented in the right to left direction, thus confirming the hybridization data (data not shown).

Determination of the 5' end of early transcripts by primer extension analysis

Primer extension analyses (Fig. 4a and data not shown) were used to map precisely the 5' ends of E1 and E5 to map positions 25 kbp and 22.7 kbp respectively (Fig. 3). In both cases two 5' ends of the transcripts were detected (Fig. 4a, b and d). Putative promoter sequences which showed homology with other lactococcal promoters (van de Guchte *et al.*, 1992) were present in the DNA upstream of the 5' end of both transcripts (Fig. 4b and d). Primer extension experiments also detected the 5' end of a transcript within SaΔA2 approximately 200 bp from its left end at 19.9 kbp on the sk1 map (Fig. 3). This 5' end was located within an open reading frame and was not preceded by a recognizable promoter sequence (Fig. 4c). It may be the 5' end of E3, although as discussed above, no hybridization of this transcript with the SaΔA2 probe was detected (Fig. 2g, Table 2). Identification of the 5' ends of other early phage transcripts was prevented by the instability of molecular clones containing appropriate regions of the phage DNA and a lack of nucleotide sequence data.

Middle phage transcripts

The three middle transcripts M1, M2 and M3 hybridized with EvF and EvG (Fig. 2d, Table 3) and were first detected 7–10 min after infection. In addition, a number of smaller transcripts (M4–M9) ranging in size from 0.2 kb to 0.9 kb were observed to hybridize with EvF at later time points. As these transcripts did not hybridize to SsLNPR or EvD, they must have been transcribed from

Fig. 3. Transcript map of phage sk1. The position and length of phage transcripts (a) and DNA probes (b) used to construct the map are shown relative to a scale showing the size of the sk1 physical map. Arrows indicate the directions of transcription of the phage genome based on hybridization and sequence data. Grouping of the transcripts into early (E), middle (M), and late (L) is based on the time of appearance of transcripts as described in the text. Transcripts are represented by solid lines and labelled E1, E2, etc., in order of decreasing size. The numbers in parentheses indicate the predicted length of the transcripts in kb. Uncertainties in the location of the ends of transcripts are indicated by broken lines. Transcripts marked with (†) migrated more slowly than the biggest molecular size marker and their sizes were obtained by extrapolation of the standard curve. The location and length of the hybridization probes are shown as thick lines beneath the name of the DNA probe. DNA probes are named according to the nomenclature described in the legend to Fig. 1 and are defined in Table 1.

DNA mapping between 25.8 kbp and 28.0 kbp on the sk1 map (Fig. 3). In view of the size and number of the middle transcripts and the small region of the genome from which they are derived, at least some of these transcripts must overlap. Nucleotide sequence data showed that the open reading frames encoded by the middle genes of the phage were all oriented in the left to right direction on the physical map shown in Fig. 1 (data not shown). Thus it was concluded that the early and middle phage genes are transcribed in opposite directions from a region located at approximately 26 kbp on the sk1 map. The middle transcripts did not appear to span the sk1 *cos* region, as EvD did not hybridize to any transcripts until 15 min post-infection (Fig. 2c). A weakly hybridizing smear of large late transcripts was detected with the EvF probe (Table 4), indicating that a small amount of transcription initiating from a promoter within EvF may proceed

across *cos* into the late region of the genome in the final stages of phage infection.

Late phage transcripts

Various DNA probes located between 0 kbp and 16 kbp on the physical map shown in Fig. 1 hybridized to phage transcripts which were not detected until approximately 15 min after infection (Figs 2a, b, c and e, Table 4). Because the discrete bands of these transcripts migrated more slowly than the biggest available size marker (9.5 kb), their sizes were determined by extrapolation of the standard curve and are therefore only indicative values. Some probes hybridized to discrete transcripts of 14, 13, 11 and 10 kb (Table 4, Fig. 2b and e). However, in contrast to the Northern blots of the early and middle transcripts, where the phage transcripts yielded sharp

Fig. 4. Primer extension analysis of the 5' end of sk1 early transcripts. (a) Autoradiograph showing electrophoretic separation of a sequencing ladder and the products of primer extension from a 17-mer oligonucleotide complementary to a portion of E5 annealed to RNA isolated from *L. lactis* 10 min after infection with sk1 phage. Lanes 1 and 2 contain 3 and 8 µl respectively of primer extension reaction mixture. Lanes A, C, G and T contain the relevant nucleotide sequencing reactions which utilized cloned sk1 HiC fragment as template and the oligonucleotide used for primer extension as primer. The nucleotide sequence on the right margin is that of the sense strand of the DNA and reads from right to left of the transcript map shown in Fig. 3. (b), (c) and (d) Nucleotide sequences surrounding the 5' ends of transcripts E1, E3 and E5 respectively. The 5' ends of the transcripts as determined by primer extension are indicated with arrowheads. Nucleotide sequences which show homology with -10 and -35 regions of promoters from Gram-positive and Gram-negative organisms are underlined. The 5' ends of E1 (b) and E5 (d) are located within intergenic regions of the phage DNA, while the 5' end of E3 (c) is located within an open reading frame, the deduced amino acid sequence of which is shown beneath the nucleotide sequence.

(b)

CAATATTATTATTCTTTACAAGTCTAAATAAAAGTATATAATTAATTTATCATCAAGAAAGGAGGTAAAA
 -35 -10

(c)

GTAAACTATAACTTTGTTTTCCCTACGGTTGAAAAAGTATTTATCACTAATGATAATAGTAGTCAATCACA
 V N Y N F V F P T V E K V F I T N D N S S Q S

(d)

AAAATATCATCTAATATTTGACAACGATAATTAATTTTGGTATAATAGTATATATAGAAATAAAGGAGAAC
 -35 -10

bands, probes such as EvD hybridized to a smear of phage transcripts ranging in size from about 300 bases to greater than 10 kb (Fig. 2c). This smearing may have resulted from non-specific processing of phage transcripts, transcript degradation, the presence of transcripts of increasing size with a common 5' end located within EvD, or a combination of these factors.

The right ends of the L1, L2 and L3 were placed at (approximately) 15.7 kbp on the sk1 map because the

transcripts hybridized with MsD but not EvC (Fig. 3, Table 4). Their left ends were located by transcript size and, for L1 and L2, confirmed by hybridization with EvD. Unlike the other late transcripts, L4 did not hybridize with MsD although it did with SsC (Fig. 2b and e). It is therefore likely that the 3' end of this transcript is located to the left of the 3' ends of the other three late phage transcripts. Its 5' end may therefore coincide with that of the 11 kb transcript (Fig. 3). Nucleotide sequence data showed that the open reading frames in the sk1 late

Table 3. Hybridization of DNA probes to sk1 middle transcripts

DNA probe	Transcript (kb):								
	M1 (1.3)	M2 (1.2)	M3 (1.1)	M4 (0.9)	M5 (0.8)	M6 (0.7)	M7 (0.6)	M8 (0.3)	M9 (0.2)
EvB	+	+	+	—	—	—	—	—	—
EvF	+	+	+	+	+	+	+	+	+
EvG	+	+	+	+	+	—	—	—	—
HiD	+	+	+	+	+	—	—	—	—

+, DNA probe hybridized with sk1 transcript; —, no hybridization of DNA probe with sk1 transcript was detected.

Table 4. Hybridization of DNA probes to sk1 late transcripts

DNA probe	Transcript (kb):			
	L1 (14)	L2 (13)	L3 (11)	L4 (10)
EvD	+	+	sm	sm
EvF	±	±	—	—
MsD	+	+	+	—
PvB	+	sm	sm	sm
PvD	+	sm	sm	sm
PvE	+	sm	sm	sm
SaE1	+	sm	sm	sm
SsC	+	+	+	+
SsC-6	+	+	+	+
Ssc-44	+	+	+	+

+, DNA probe hybridized with sk1 transcript; sm, the hybridization pattern was smeared, preventing the detection of individual bands; ±, the hybridization to these transcripts was very weak; —, no hybridization of DNA probe with sk1 transcript was detected.

region were all oriented in the left to right direction of the map shown in Fig. 1 (data not shown).

In addition to the late transcripts derived from the 0–16 kbp region of the phage genome, a late 2.2 kb RNA species, migrating between the 16S and 23S rRNA bands, was detected in some RNA preparations (Fig. 2d and g). When present, this RNA species hybridized to *EcoRV* fragments B, C, F and G and therefore is not a single RNA species and may be breakdown products of early and middle transcripts.

DISCUSSION

The propagation of bacteriophages containing a double-stranded DNA genome generally involves two or three time-dependent phases of phage gene expression. These phases of phage gene expression are usually controlled at the level of transcription and result in a complex, often

overlapping, time-dependent pattern of phage transcripts. For example, transcription of the *Escherichia coli* phage T7 is essentially unidirectional along the linear genome, but a complex pattern of transcripts is observed due to the initiation of transcription at several points, the processing of long transcripts and partial readthrough of transcription termination sites (Dunn & Studier, 1983). T7 transcription is also regulated temporally by the synthesis of a T7-encoded RNA polymerase which recognizes T7 middle and late promoters. The work described in this paper has shown that the small isometric-headed lactococcal phage sk1 exhibits a complex pattern of transcription which is temporally regulated into three distinct phases corresponding to early, middle and late stages of infection.

By analogy with other bacteriophages, it is likely that the sk1 early transcripts are initiated from promoters recognized by the (unmodified) host RNA polymerase. Seven early sk1 transcripts were detected 2–5 min after phage infection and were derived from a 10 kbp region of the phage genome located between 15.9 and 25.9 kbp on the sk1 map (Fig. 3). These transcripts were oriented in the right to left direction on the sk1 map and ranged in size from 0.7 to 7.1 kb. Three of the transcripts (E1, E6 and E7) do not overlap and cover the entire 10 kbp of the early region of the genome, while the other four transcripts (E2–E5) overlap regions of the genome covered by E1 and E6.

The 5' ends of E1 and E5 were determined by primer extension studies to be located in intergenic regions between open reading frames and each was preceded by nucleotide sequences similar to consensus lactococcal and *E. coli* promoters (Harley & Reynolds, 1987; van de Guchte *et al.*, 1992). While promoter cloning experiments are required to prove the functionality of these putative promoters and eliminate the possibility that E1 and/or E5 arose by processing of a larger transcript, these data suggest that sk1 phage contains at least two early promoters. At least one additional early promoter would be necessary to initiate transcription of E7. The 5' end of another transcript, which mapped to approximately 200 bp from the left end of SaΔA2, may be that of E3. Because this end lies within an open reading frame and was not preceded by a recognizable lactococcal promoter sequence, it is likely that this transcript was derived by processing of a larger transcript. Interestingly, early transcripts larger than 7.1 kb can be weakly detected in hybridizations with SaΔA2 (Fig. 2g) and other probes from the early region (data not shown) and may represent unprocessed transcripts. These data suggest that the early sk1 transcripts identified in this study include primary transcripts, initiated from at least three early promoters, and processed products of (some of) these primary transcripts. The presence of processed and overlapping transcripts has been observed in lytic phage that infect *E. coli* and *Bacillus subtilis* (Christensen & Young, 1983; Panganiban & Whiteley, 1983a, b; Dunn & Studier, 1983). Their synthesis may be a mechanism to achieve differential levels of gene expression from a limited number of promoters.

The precise locations of the 3' ends of the early transcripts have not been determined. However, E3, E4 and E6 terminated within a 400 bp region within SsC and may share a common termination point. In agreement with the results obtained in this investigation, Beresford *et al.* (1993), using phage cDNA probes, also determined that the sk1 early transcripts were derived from a region of the phage genome corresponding to EvB, EvC and EvG.

By analogy with other lytic bacteriophages, it is likely that middle and late transcripts are initiated from promoters that are not recognized by the host RNA polymerase and that transcription from these promoters is dependent on phage-encoded proteins produced in previous phases of the phage replication cycle. The nine middle sk1 phage transcripts were first detected 7–10 min after phage infection and were derived from a 2.1 kbp region of the phage genome between 26 kbp and the phage *cos* site at 28 kbp on the map of sk1 (Fig. 3). These transcripts ranged in size from 0.2 to 1.3 kb and were oriented in the opposite direction to the early transcripts. While the precise ends of the middle transcripts were not determined, it is obvious that the transcription pattern in this region is quite complex and that many of the transcripts overlap. Either there are multiple transcription initiation and termination sites within this region, or most of the middle transcripts are likely to be processed products of larger middle transcripts. In contrast to the results of Beresford *et al.* (1993), no evidence that the middle transcripts traversed *cos* and were derived from the EvD region of the phage was obtained in this investigation (Figs 2c and 3). However, weak hybridization of EvF with large late transcripts was observed 15 min after phage infection, indicating that some late transcripts may initiate from the same region as the middle transcripts and traverse *cos*. These transcripts could be initiated from a late promoter within the middle region of the phage genome or by antitermination of transcripts initiated from a middle promoter.

The four late phage transcripts were not detected until 15 min after phage infection and were derived from a 16 kbp region of the phage genome between 0 and 16 kbp on the sk1 map (Fig. 3). Like the middle transcripts, they were oriented in the opposite direction to the early transcripts. Three of the late transcripts (L1, L2 and L3) and three of the early transcripts (E3, E4 and E6) terminated within the 1.2 kbp of DNA spanned by MsD, suggesting that there may be a common region of transcription termination for these convergent early and late transcripts. A region containing back-to-back terminators capable of terminating convergent transcription has recently been identified in the *L. lactis* chromosome (Llanos *et al.*, 1992). The mapping of the end-points of the late transcripts was less precise than that of the early and middle transcripts because of the large transcript sizes and the smeared appearance of Northern blots hybridized with probes from the late region. The smeared hybridization pattern could result from the presence of a series of nascent late transcripts of increasing size initiating from a common initiation site, or from the rapid processing or degradation of full-length transcripts. It is also possible that there are

a number of transcription initiation and termination sites within the late region and that these contribute to the range of transcript sizes observed.

As observed with the early and middle transcripts, the late phage transcripts also overlap each other. Given the uncertainty in the size of the large late transcripts, it is possible that the 5' end of L1 is close to *cos* and that this transcript therefore spans the entire late region. If the lactococcal (or phage encoded) RNA polymerase synthesizes RNA at a similar rate to the *E. coli* enzyme (Bremer & Yuan, 1968), it would take approximately 5 min to synthesize a 16 kb transcript. This may provide an additional level of temporal regulation of phage gene expression. However, as the 3' end of L1 is common to L2 and L3, which are approximately 13 and 11 kb in length, the delay in translation of proteins from the distal ends of these transcripts would be less than 5 min. Further work will study the regulation of gene expression and mechanisms of mRNA processing in this phage.

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