Global transcription pattern of ϕ C31 after induction of a *Streptomyces coelicolor* lysogen at different growth stages

JUAN E. SUÁREZ,¹ TIMOTHY M. CLAYTON,^{2†} ANA RODRÍGUEZ,^{1‡} MERVYN J. BIBB² and KEITH F. CHATER^{2*}

¹Departamento de Microbiología, Facultad de Medicina, Universidad de Oviedo, C/Julián Clavería, s/n 33006, Oviedo, Spain

²John Innes Institute, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK

(Received 14 January 1992; revised 4 June 1992; accepted 26 June 1992)

Using two complementary strategies for low-resolution S1 mapping, the global pattern of ϕ C31 transcription was studied after induction of thermoinducible ϕ C31 lysogens of *Streptomyces coelicolor* A3(2). A complex pattern of early transcripts was seen, with a peak of abundance at about 10 min post-induction. Nearly all of these transcripts were from DNA located to the right of the *c* (repressor) gene and to the left of the *attP* site: a region of about 14 kb. Early transcription was also observed immediately to the left of the *c* gene. The *c* gene itself was also induced, with an earlier expression peak (about 5 min post-induction). Primary late transcripts were generally relatively long, but degraded. They apparently corresponded to most of the 18 kb region to the left of the *c* gene. Some shorter and more persistent late transcripts corresponded to DNA close to or overlapping the *cos* site. Large late transcripts from a region close to the left-hand end of the ϕ C31 genome showed evidence of processing to more stable, smaller RNA species. A failure of older cultures (more than 12 h old) to be induced productively was correlated with a much longer period of early transcription, reduced late transcription, failure to synthesize a major virion protein, and failure to package ϕ C31 DNA. Moreover, heat treatment of the older lysogenic cultures did not result in the ϕ C31-dependent shut-down of host rRNA transcription previously observed for young cultures (Rodríguez *et al.*, *Journal of General Microbiology* (1986) 132, 1695–1701; Clayton & Bibb, *Molecular Microbiology* (1990) 4, 2179–2185).

Introduction

Most detailed studies of temperate bacteriophages have focused on lambdoid coliphages. Studies of temperate phages of bacteria phylogenetically distant from *Escherichia coli*, such as the industrially important streptomycetes, would usefully extend our general understanding of phage biology and lysogeny, as well as providing insights into the molecular biology of the hosts. Temperate phages of streptomycetes have been studied for the last 20 or so years. Probably the most characterized is ϕ C31, a wide-host-range phage morphologically resembling

coliphage λ (Lomovskaya *et al.*, 1980; Suárez *et al.*, 1984; Chater, 1986). ϕ C31 can infect and lysogenize *Streptomyces coelicolor* A3(2), the streptomycete favoured for genetic studies. Like λ for *E. coli*, ϕ C31 has provided useful cloning vectors and tools for molecular genetic studies in streptomycetes (Hopwood *et al.*, 1987; Bruton *et al.*, 1991; Kobler *et al.*, 1991; Kuhstoss *et al.*, 1991).

The genome of ϕ C31 is a 41·2 kb double-stranded DNA molecule (Chater, 1986) with 10 nucleotide complementary single-stranded 3' extensions at its ends (Kobler *et al.*, 1991). A single gene (*c*) located near the centre of the DNA molecule controls lysogeny (Lomovskaya *et al.*, 1980). This gene has been sequenced and its expression studied at the mRNA and protein levels (Sinclair & Bibb, 1988, 1989; Smith & Owen, 1991). Late-expressed temperature-sensitive (*ts*) mutations and host-range mutations all map to the left of *c*, and the single characterized early *ts* mutation (L24) maps to the

* Author for correspondence. Tel. 0603 52571; fax 0603 56844.

† Present address: Police Forensic Science Laboratory, Sandbed Way, Audby Lane, Wetherby, West Yorkshire LS22 4DN, UK.

‡ Present address: Instituto de Productos Lácteos de Asturias, Consejo Superior de Investigaciones Científicas, Crta. Infiesto, s/n 33300 - Villaviciosa, Asturias, Spain.

right of *c* (Lomovskaya *et al.*, 1980). This suggests that the *c* gene may separate the late genes (on the left) from the early genes (on the right), a possibility recently qualified by the discovery that a gene immediately to the left of *c*, encoding a nucleotide kinase-like product, is also transcribed early (Smith *et al.*, 1992; Smith & Owen, 1991). Close to the right-hand end of ϕ C31 DNA is a long region (~8 kb) dispensable for plaque formation, and containing determinants for prophage integration and for interaction of ϕ C31 with restriction-modification systems (Kuhstoss *et al.*, 1991; Chater, 1986).

In this paper, we describe the global transcription of ϕ C31 during the lytic cycle after induction of a heat-inducible prophage. The information is used to clarify the reason(s) why induction is productive only in young cultures.

Methods

Strains, plasmids, phages and culture conditions. Two independently constructed lysogens of *S. coelicolor* A3(2) derivatives were used: *S. coelicolor* 01 (*hisA1 uraA1 strA1* Pgl⁻ SCP1⁻; Rodríguez *et al.*, 1986) and *S. coelicolor* TMC1 (wild-type: Clayton & Bibb, 1990). Both contain the thermoinducible ϕ C31 *cts1* prophage (Novikova *et al.*, 1973). This phage has a 1.72 kb deletion (Δ M) in its *EcoRI*-C fragment (Chater *et al.*, 1981). Conditions for growth and thermal induction of *Streptomyces* cultures were as described by Rodríguez *et al.* (1986) and Clayton & Bibb (1990). Standard recombinant DNA techniques were used to subclone fragments of ϕ C31 *c1* DNA into pBR322, pBR329, and M13mp19 and to prepare plasmid and replicative-form DNA (Sambrook *et al.*, 1989). Conditions for ϕ C31 *c1* propagation were as in Hopwood *et al.* (1985).

Extraction of nucleic acids from mycelial cultures. For cultures of *S. coelicolor* 01, the extraction procedures were as in Rodríguez *et al.* (1986); for *S. coelicolor* TMC1, procedures were as in Clayton & Bibb (1990).

S1 mapping. For low-resolution global mapping of transcripts, the procedures were essentially those of Smith & Chater (1988). Unless stated otherwise, each reaction used 2 μ g RNA and 100 ng DNA. In brief, the mixture was incubated in formamide-containing buffer at 85 °C for 10 min, then at 64 °C for 3.5 h, before adding S1 nuclease (871 units, 37 °C, 1 h). After precipitation, alkaline denaturation and electrophoresis in alkaline 1% (w/v) agarose gels, the protected DNA was visualized by probing Southern blots with suitable ³²P-nick translated DNA probes (Sambrook *et al.*, 1989). S1 mapping analysis of *rrnD* transcription was done with a ³²P-end-labelled 570 bp DNA probe, as described by Clayton & Bibb (1990).

Dot-blot analysis of RNA. RNA samples (1 μ g) were spotted on nitrocellulose filters (prewetted in 20 $\times$ SSC and then dried). The filters were dried and processed for hybridization as for Southern blots (Sambrook *et al.*, 1989).

Analysis of proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Mycelial suspensions were washed in ice-cold 50 mM-Tris/HCl, 10 mM-EDTA, pH 8.0, and lysed by sonication. After centrifugation (15 min, 4 °C, Eppendorf microcentrifuge) the supernatants were analysed by SDS-PAGE as in Laemmli (1970) (see Suárez *et al.*, 1984, for precise details).

Results

Strategy for mapping of ϕ C31 transcripts at different times after prophage induction

SphI fragments of ϕ C31 *c1* DNA were cloned into pBR322 (to give the pKC series of plasmids), and *EcoRI* fragments were cloned into pBR329 (the pUO series) (Fig. 1). The resulting plasmids were used in low-resolution S1 mapping of RNA obtained at different time intervals (10, 20, 30, 40 and 50 min) after initiating induction of a young exponential-phase culture of a heat-inducible ϕ C31 lysogen (*S. coelicolor* 01, 7 h after inoculating the culture with spores).

Two S1 mapping strategies were adopted (Fig. 2). In type A experiments, full-length ϕ C31 virion DNA was protected by mRNA. Southern blots of the protected fragments were then probed with radiolabelled preparations of each of the plasmid subclones. This showed the sizes of transcripts present in the induced culture, their approximate origins, and the time course of their accumulation. In the type B experiments, the DNA to be protected was from individual plasmid subclones, and Southern blots of protected fragments were probed with radiolabelled total ϕ C31 *c1* DNA. This showed the lengths of the larger overlaps between the subcloned ϕ C31 DNA fragments and the phage transcripts (in general, protected DNA of less than 0.5 kb might not have been seen in these experiments). In relating type A and B experiments, comparable size was generally taken to indicate transcript identity. It is important to note that these experiments do not distinguish between rightward and leftward transcription. No type B experiments were done for the fragments *EcoRI*-E or *EcoRI*-G. In three autoradiographs, all derived from a single type A S1 nuclease digestion (Fig. 3), an intense smear was obtained with a 30 min sample, yet no equivalent signal was obtained in the type B experiment; this was attributed to a presumptive failure to expose some of the ϕ C31 DNA to the nuclease in this one digestion mixture, through inadequate solubilization or mixing.

Overall summary of S1 mapping results

The data are shown in Fig. 3 and are summarized in Fig. 1. No transcription (even of the *c* gene – see next section) was detected before induction in this first series of experiments. At least 13 different transcripts, ranging from 0.5 kb to 4.5 kb, were detected from the approximately 18 kb region between *SphI* site 8.3 and *EcoRI* site 19. These were all early transcripts, since they were most abundant in the earliest sample (10 min post-induction). In most cases, their levels at later time-points declined dramatically. There was no evidence of comparably

Fig. 1. Physical map of ϕ C31 Δ M *cts1* (Chater, 1986) with locations of transcripts made during the lytic cycle of the phage and of probes used in RNA studies. The upper map shows the *Sph*I (S) and *Eco*RI (R) site numbering used in the paper. The fragments cloned into pBR322 (pKC series) or pBR329 (pUO series) are indicated, as are the locations of the repressor (*c*) gene, the phage attachment site (*attP*), the two major dispensable regions (hatched boxes) and the cohesive ends (*cos*). The regions used as dot-blot probes (DBP C, D, E, A and B) are shaded on the block map in the centre of the figure. The lower part of the figure shows transcripts, the sizes and positions of which are deduced from the data in Fig. 3 (together with information from Smith & Owen, 1991). Continuous lines indicate the observed sizes of the transcripts, and the dotted lines indicate the possible limits of the DNA segments which encode the transcripts. Sizes are in kb. The RNA species k, l, m and n may be underestimated in size, and other species from this region not detected because there is a deletion (Δ M) of 1.72 kb from the *cts1* prophage, whereas DNA used in S1 mapping was undeleted.

abundant very long early transcripts from this region. In contrast, RNA accumulating late in infection (from 20 min onwards) mainly protected DNA to the left of the *c* region. Thus, the leftmost approximately 18 kb of ϕ C31 DNA, extending a short distance into the right-hand end (these ends are presumably joined during the replicative cycle), is the late region. In contrast to the discrete and generally fairly short bands protected with early mRNA, the DNA protected by late mRNA ran as smears on the autoradiographs, consistent with the occurrence of a small number of relatively long, and significantly unstable, late mRNA species.

Early transcription of the repressor gene

Transcription of the *c* region in uninduced cultures was analysed by using radiolabelled *Sph*I-G fragment to

probe the fragments resulting from protection of ϕ C31 DNA. In spite of the high amounts of RNA used (up to 100 μ g per reaction) no hybridization was found, though other experiments did detect transcription (see below, and Sinclair & Bibb, 1989; Smith & Owen, 1991) (Fig. 3). In contrast, 10 min after the start of induction three DNA fragments of approximately 1.35, 1.5 and 2.1 kb were protected, identifying mRNA species a, b and c, respectively. All three were apparently internal to the *Sph*I-G segment, because they were not detectably changed when that fragment was used in the protection. Smith & Owen (1991) reported RNAs of 2070, 1560 and 1270 nucleotides in similar experiments on a ϕ C31 *cts1* lysogen, and located them precisely in relation to the nucleotide sequence. The latter information is incorporated into Fig. 1. These RNA molecules were almost absent by 20 min, and undetectable in later time samples.

Fig. 2. Strategies used in global S1 mapping. In the type A example, mRNA is used to protect full-length ϕ C31 DNA from S1 nuclease, and protected segments are identified in Southern blots by probing with particular 32 P-labelled restriction fragments of ϕ C31 DNA. This reveals the length and approximate location of transcripts. In the type B example, an exactly reciprocal strategy reveals the extent of overlap of transcripts with particular fragments.

Transcription of early genes to the right of the repressor gene

For ease of presentation, each apparent mRNA species is dealt with separately, working rightwards from the *c* region.

A relatively abundant and persistent early RNA (d; 1.4 kb) was detected in a type A experiment with the *SphI*-[C+I] probe, but not with probes for the flanking

SphI-G or *EcoRI*-E fragments. A similarly sized band was detected in the type B experiment, though it was of comparatively low abundance and persistence.

An approximately 2.6 kb mRNA (e) is transcribed from sequences entirely located between *SphI* sites 8.6 and 9.1, and overlapping *EcoRI* site 9. It was detected in type A experiments with the *SphI*-C and *EcoRI*-E probes, and was unchanged in the type B experiment done with *SphI*-C. This mRNA was abundant at 10 min post-induction, but it was undetectable in the later samples.

An approximately 2.2 kb mRNA (f) traverses *SphI* site 9.1, and could have its leftmost end close to *EcoRI* site 9. In type A experiments it gave a weak signal with *SphI*-C as probe, a strong signal with *EcoRI*-E, and no signal with *EcoRI*-G. A 1.4 kb fragment (f') detected in the type B experiment with *SphI*-C probably resulted from overlap of mRNA f with the right-hand end of *SphI*-C. Very little mRNA f was detectable at 20 min post-induction, and none in later samples.

An approximately 4.5 kb mRNA (g) has one end to the left of *SphI* site 9.1 and extends to the right of *EcoRI* site 11, being detected in type A experiments by the *SphI*-C+I, *EcoRI*-E and *EcoRI*-G probes. It may be co-terminal at its left end with mRNA f. It is of low abundance, perhaps because of a short half-life: a smear extended from the front of the 4.5 kb band, and no signal was detected at 20 min or later. In type A experiments, the band was strongest with *EcoRI*-E as probe, even weaker signals being obtained with *SphI*-[C+I] and *EcoRI*-G probes.

Fairly abundant mRNAs of approximately 1.6 kb (h) and approximately 1.0 kb (i) have most if not all of their lengths between *EcoRI* sites 11 and 13. They were detected in type A experiments only with *EcoRI*-G, and were virtually undetectable by 20 min post-induction.

An approximately 2.5 kb mRNA (j), also fairly abundant but short-lived, has one end close to *EcoRI* site 11, and the other end about 1 kb to the right of *EcoRI* site 13. The 2.5 kb band was detected in type A experiments with *EcoRI*-G and *EcoRI*-C, giving a stronger signal with *EcoRI*-G. Probably, this mRNA gave rise to a 1.0 kb band (j') observed with type B experiments using *EcoRI*-C.

Several mRNA species appeared to be transcribed entirely from sequences between *EcoRI* sites 13 and 19. In type A experiments, bands k (0.8 kb, strong), l (2.0 kb, weak), m (1.6 kb, strong), and n (0.5 kb, weak) were relatively persistent: they were still detectable with RNA isolated 20 min post-induction, using *EcoRI*-C as probe, though they were undetectable by 40 min post-induction. A type B experiment with *EcoRI*-C as the protected DNA fragment gave relatively weak hybridization, but bands apparently corresponding to mRNA species l and

Fig. 3. RNA-protected fragments of ϕ C31 DNA obtained with RNA made during the lytic development of the phage. The larger panels are the results of 'type A' experiments (Fig. 2). In each of these panels, RNA extracted from lysogens at the indicated times after heat induction was used to protect ϕ C31 DNA against S1 nuclease digestion. The protected DNA was detected by alkaline agarose gel electrophoresis and Southern blotting, followed by hybridization with the indicated 32 P-labelled probes (see also Fig. 1), and autoradiography. For the EcoRI-E and -G panels, longer exposures are also shown. For all the other regions, a small second panel shows the result of a 'type B' experiment (Fig. 2), in which the RNA was used to protect the appropriate fragment of ϕ C31 DNA, and the eventual Southern blot was probed with 32 P-labelled ϕ C31 DNA. Each 'type B' panel represents that RNA time sample giving the strongest signal. In some cases, a series of pBR327-derived size markers (provided by D. J. Lydiate: Lydiate *et al.*, 1986) was probed with pBR327. In others, a similar (but not identical) series was provided by F. Malpartida (John Innes Institute). The markers MM are readily seen only with the early time samples, in which a longer exposure was used to detect the protected DNA fragments. Tracks marked with black crosses are blank, and in tracks marked with white crosses a presumptive fault in the S1 digestion has given a very strong false signal (the latter three marked tracks were from a single S1 nuclease digest).

m were detected, as well as the 1.0 kb putative j' band. The 0.8 kb band (k) was not detected in the type B experiment. Any of these RNA species that traverses the ΔM deletion present in the $\phi C31$ *cts1* prophage would give rise to an S1-sensitive D-loop when $\phi C31c1$ DNA or pUO25 (both containing the undeleted *EcoRI*-C fragment) was used in S1 mapping, so their true lengths may be longer than estimated in this work.

Late mRNA from the region to the left of the *c* gene

A diffuse smear was seen in type A experiments with *SphI*-D, -B or -[F + I] (Fig. 3, right). The smear was first visible at 20 min and increased in intensity in later samples. When the *SphI*-D fragment (3.32 kb) was used in a type A experiment, bands of about 7, 5, 3.2, 2.7, 2.0, 1.4 and 1 kb became increasingly obvious from 20 to 50 min, against a comparably intensifying background smear (9 kb–1 kb). A type B experiment with *SphI*-D gave relatively weak bands, one of about 4.5 kb (perhaps indicating full-length protection) and others of about 3.8, 3.2, 2.7 and 2 kb in a faint background smear. In contrast, the smear was featureless when *SphI*-B or -[F + I] were the probes in type A or type B experiments, with virtually no full-length protection in the type B experiment. Interestingly, the *SphI*-[F + I] probe also gave two faint but definite discrete bands of about 1.6 kb and 0.8 kb, with RNA from 10 min post-induction. This indicates that part of the region between *SphI* sites 8.01 and 8.3 is transcribed early, in agreement with the finding by Smith & Owen (1991) of early transcription from the region immediately to the left of the *c* gene. The simplest interpretation of these results is that from about 20 min post-induction one or a small number of relatively long mRNA species are made, between them traversing most of the approximately 17 kb between *SphI* sites 2.1 and 8.2. This RNA is rapidly degraded, with some of the degradation leading to the accumulation of more stable forms with endpoints in preferred positions.

Small late transcripts that increase in amount throughout the late stages of the lytic cycle

With samples from 20 min onwards, bands of 0.8 kb (o'), 1.3 kb (p) and 2.2 kb (q) steadily increased in amount in type A experiments with *SphI*-E + H (a probe that encompasses the *cos* site). A faint background smear including higher molecular mass material also increased coordinately with these bands. In a type B experiment a 1.3 kb band (p?) was again seen, but a 1.4 kb band (o) replaced the 0.8 kb o' band and the 2.2 kb band q was apparently shortened to 2.1 kb. Possibly, the 1.4 kb band (o) includes the *cos* site, which is continuous in the *SphI*-

Fig. 4. Dot-blot of RNA isolated after heat induction of 7 h cultures of *S. coelicolor* TMC1, using probes A–E (Fig. 1).

E + H probe but discontinuous in $\phi C31c1$ DNA. The bands p and q do not include the *cos* site, since they were not reduced in size in the type A experiments. If the apparent size differences between bands q and q' were correct, then q would overlap *SphI* site 16.2 (q and q' must fall mainly within the *SphI*-E fragment, since they are larger than the *SphI*-H fragment). However, differences in molecular mass standards and electrophoresis conditions mean that we are not confident that q and q' differ in size. It is not excluded that these RNA species (o, p, q) are relatively stable products from processing of the major late mRNA.

RNA dot-blot analysis of the accumulation of mRNA corresponding to selected regions of $\phi C31$

To extend some aspects of the transcription analysis described above, comparable RNA samples were made from an induced 7 h culture of an independently prepared $\phi C31$ *cts1* lysogen, TMC1. These were analysed by RNA dot-blot experiments (Fig. 4). Selected regions of $\phi C31$ *c1* DNA were radiolabelled for use as probes, as follows (see Fig. 1): the *cos* region (probe C); a region just to the right of *cos* (probe D); the *SphI*-I fragment immediately to the left of the *c* gene (probe E); a fragment internal to the *c* gene itself (probe A); and a fragment located about 7 kb to the right of the *c* gene (probe B). RNA samples were isolated 5, 10, 15 and 20 min after the start of the 5 min heat induction period.

Fig. 5. Dot-blots of RNA isolated from cultures of *S. coelicolor* TMC1 grown for different lengths of time, and subjected to heat induction. RNA was isolated before, and 20 min after, initiating heat treatment. Probes A–E are shown in Fig. 1. The probe A 7 h control dot was misplaced on the original filter, so the relevant section of the photographed autoradiograph has been moved for presentation purposes without modifying the appearance of the spot.

These experiments showed that *c* gene transcription was high at 5 min, and was already declining by 10 min (Fig. 4, probe A). In contrast, the other probe for early transcription (probe B) revealed little transcription at 5 min, but a maximum at 10 min. This demonstrated the occurrence of two classes – ‘immediate’ and ‘delayed’ – of early transcript. Late transcripts, detected with probes C and D, were increasing by 10 min and continued to accumulate markedly through the later time points. Probe E, the *Sph*I–I fragment, revealed marked increases in transcription at 10 min and 15 min, and little further increase. The pattern of transcription in this region was not clear in the S1 mapping experiments, in which the *Sph*I–I fragment was used only in combination with other larger fragments, but it seems possible that the 1.6 kb and/or 0.8 kb transcripts detected at 10 min with the *Sph*I–[F + I] probe in Fig. 3 may correspond to the signal detected with probe E and to the early transcript traversing part or all of *Sph*I–I that was reported by Smith & Owen (1991).

The effect of culture age on heat induction of a ϕ C31 lysogen

For biochemical studies of the lytic cycle of ϕ C31, it would be convenient to induce cultures relatively late in

growth to increase the quantities of material readily available for analysis. However, previous results (Rodríguez *et al.*, 1986; Sinclair, 1987) showed that productive induction was obtained only for the few hours after germ tube emergence. By 10–12 h, yields of ϕ C31 after induction were negligible. This corresponded approximately with the cessation of rapid growth. It was important to establish whether this failure in induction was due to a block early in the lytic cycle, preventing the normal course of ϕ C31 transcription, or to a late block, perhaps affecting lysis of the host and release of progeny virions. To examine this question, suitable sets of RNA samples were analysed by dot-blotting using the probes described in the preceding section. The RNA samples were made 20 min after the start of induction, from TMC1 cultures aged 7, 10, 15, 20 and 40 h. Rapid growth occurred until about 14 h, after which little further increase in optical density was observed. Control RNA samples were made from uninduced mycelium of the same age from the same culture vessel. In interpreting the data, it is necessary to compare results from the induced and control cultures, because the controls themselves showed an interesting progression with time; it should also be remembered that by 20 min after induction in the earlier experiments, the early transcripts had declined significantly, while the late transcripts were

Fig. 6. Type A S1 mapping experiments on RNA isolated from cultures of *S. coelicolor* 01 grown for 19 h and subjected to heat induction. RNA was isolated before, and 35, 65 and 95 min after, initiating heat treatment. The probes are shown in Fig. 1.

accumulating rapidly (the 7 h samples of Fig. 4 illustrate this).

After 10 h and 15 h of culture, there seemed to be a slowing in the progression of induced lytic transcription, in that 'delayed early' mRNA was more abundant 20 min after induction than in 7 h samples at the same interval after induction (Fig. 5, probe B). By 15 h of culture, two of the late transcripts were greatly reduced in abundance 20 min after induction, as if late transcription were either delayed, or absent altogether (Fig. 5, probes D and E). By 20 h, there was no evidence at all of heat induction of ϕ C31 early or late transcription over background spontaneous events.

By 10 h, the level of *c* gene transcripts had increased markedly in the uninduced control culture, and no

significant further increment was observed in response to induction (Fig. 5, probe A). In a very old culture (40 h), the *c* gene transcripts were virtually absent with or without induction (Fig. 5, probe A). Surprisingly, transcripts corresponding to the *cos* region (Fig. 5, probe C) were also more abundant in older uninduced cultures, in this case being present even at 40 h. This may indicate a specialized stationary-phase mode of transcription, or an increased stability of this part of the late mRNA. The possibility of fortuitous hybridization of the *cos* probe to host RNA was ruled out by the absence of any signal with RNA from an equivalent non-lysogenic culture.

In these experiments, RNA was extracted only up to 20 min after the start of induction. To investigate further the apparent delay in transcription in older cultures,

Fig. 7. A major ϕ C31-specific protein accumulates after induction of young, but not of old, cultures of a ϕ C31 lysogen. Cell-free extracts of 7 h and 24 h cultures of *S. coelicolor* 01 were made before (U), and at the indicated times (min) after, heat induction. They, and controls of molecular mass markers (MM) and purified ϕ C31 virions, were subjected to SDS-PAGE and visualized by staining with Coomassie Blue. The ~ 29.5 kDa ϕ C31-specific protein is arrowed. *The 30 and 40 min (7 h induction) samples were inadvertently switched.

RNA was extracted from a 19 h culture of strain 01 both before induction and at 35, 65 and 95 min after the start of induction, and analysed by type A S1 mapping as described earlier (Fig. 6). Transcription in the *c* gene region (*Sph*I-G probe) was detectable in the uninduced 19 h culture, and in substantially increased amounts at all times after induction, giving the same S1 mapping pattern as at 7 h. No transcription of the remainder of the early region (probes *Sph*I-[C+I] and *Eco*RI-E, -G and -C) nor any late transcription (probes *Sph*I-[E+H], -D, -B and -[F+I]) was seen in the uninduced 19 h culture. Early mRNA was abundant at 35 min post-induction in the 19 h culture; but little was detectable after 65 min. Late mRNA was also detected at 35 min post-induction in the 19 h culture, though it was not very abundant (except the RNA corresponding to *Sph*I-D). It was much less abundant by 65 min. In contrast to the dot-blot experiments with independent RNA preparations, no significant transcription in the *cos* region (probe *Sph*I-[E+H]) was detected in this 19 h culture before

induction. This disparity might be due to the lower sensitivity of the S1 mapping experiments and their inability to detect very short transcripts in the experimental conditions used.

In summary, these results all suggest that, in older cultures, transcription of the early genes to the right of the *c* region is greatly delayed, and there is a consequent reduction in the extent of late transcription. This reduction was less marked for the *Sph*I-D region, perhaps indicating that RNA for this region is relatively stable.

A major ϕ C31 virion protein is readily detectable in a young culture late in the lytic cycle, but is not made in an older culture after induction

The relative deficiency of late mRNA synthesis observed after induction of old cultures of ϕ C31 *cts*1 lysogens suggested that there should be a deficiency of virion

Fig. 8. Amplification and processing of ϕ C31 DNA in young (7 h) and old (15 h) cultures of *S. coelicolor* 01 before (U) and after heat induction. Total mycelial DNA preparations were digested with *Eco*RI, then heat-treated to separate ϕ C31 cohesive ends, and subjected to agarose gel electrophoresis. *Eco*RI-digested DNA from ϕ C31 c1 virions was included, and shows a larger *Eco*RI-C fragment than that from the lysogens, because the *ctsI* prophage contains a deletion inside this fragment (Fig. 1).

structural proteins, the probable major products of the late genes. SDS-PAGE analysis of cell-free extracts of 7 h and 24 h cultures, taken at intervals after induction, revealed at least one apparently phage-specific protein that accumulated late in lytic development in the young culture (Fig. 7). This protein, of about 29.5 kDa, had the same mobility as the major protein detectable in purified ϕ C31 virion preparations (Suárez *et al.*, 1984). There was no detectable induction of this protein in the 24 h cultures, even 185 min after induction (Fig. 7). It is unlikely that the 29.5 kDa protein is a host heat-shock protein, since no protein of this size was reported in a survey of the heat-shock response in several *Streptomyces* spp. (Guglielma *et al.*, 1991).

ϕ C31 DNA that accumulates late in the induction cycle is processed to linear monomers in young, but not old, cultures

DNA was extracted from a 7 h culture of the 01 lysogen at different times after induction, then digested with *Eco*RI and subjected to agarose gel electrophoresis (Fig. 8). Faint traces of ϕ C31-specific bands were detectable in the uninduced and 15 min post-induction samples,

Fig. 9. Effect of culture age on the ability of prophage induction to inhibit host rRNA transcription. RNA was extracted from cultures of *S. coelicolor* TMC1 of different ages, both before (U) and 20 min after (I) initiating heat induction. Samples were subjected to S1 mapping (see Methods and Clayton & Bibb, 1990). The six RNA-protected bands correspond to the four *rrnD* promoters (P_1 – P_4), an unassigned S1 nuclease site (S) and a processing site (p16S). Size markers (M) were end-labelled fragments of pBR322 digested with *Hpa*II. Sizes are given in nucleotides (nt).

possibly reflecting a low proportion of spontaneously induced cells in the population. Samples taken at 30 min or later showed conspicuous ϕ C31-specific bands. The autonomous state of this DNA is revealed by the equimolar quantity of the *Eco*RI-CAM fragment in relation to other fragments. This fragment is interrupted in the prophage state because it contains the *attP* site involved in crossing-over with the host chromosome. During replication, the phage DNA is expected to be circular and/or concatameric, whereas packaging into virion particles is expected to involve cleavage at the *cos* site into linear monomers. This cleavage results in the conversion of a 12.5 kb *Eco*RI fragment into the 9.6 kb (B) and 2.96 kb (F) fragments that make up the left and right ends of DNA extracted from virions. The latter fragments were fairly abundant in the DNA samples taken late in the induction cycle (55 min onwards), though barely detectable at 30 min, when the phage DNA was less abundant.

In samples taken from an older culture (15 h), ϕ C31 DNA was amplified even in the uninduced control, and little, if any, further amplification could be seen at various times after induction. The *Eco*RI-C fragment was fully represented, indicating that the DNA was extrachromosomal, but the *Eco*RI-B or -F fragments were not detectable (even after heating and rapid cooling of the DNA to separate any hydrogen-bonded cohesive ends). Thus, none of the DNA was packaged. The low level (if any) of replication after induction, and the absence of packaging, are compatible with the relatively low levels of late transcription and the absence of a major virion protein, as discussed in the previous section.

The ability of ϕ C31 to inhibit transcription of host rRNA genes is lost with increasing culture age

Induction of a ϕ C31 prophage leads to inhibition of host rRNA transcription (Rodríguez *et al.*, 1986; Clayton & Bibb, 1990). The effect of culture age on this inhibition was analysed by S1 mapping of transcripts from the *rrnD* rRNA gene cluster using the TMC1 RNA samples, isolated at different stages of culture, with and without prophage induction. A dramatic reduction in *rrnD* transcription occurred 20 min after induction of a 7 h culture (Fig. 9); however, inhibition declined with increasing culture age, and by 20 h the S1 mapping profiles with or without prophage induction were extremely similar. The ability to inhibit transcription from host rRNA promoters appears to be an early phage function, beginning about 5 min after induction (Clayton & Bibb, 1990); in cultures older than 10 h, either this phage early function is delayed beyond 20 min or it is unable to take place at all.

Discussion

Four temporal classes of ϕ C31 mRNA have now been described. Other data (Sinclair & Bibb, 1989; Smith & Owen, 1991) showed that the *c* gene is transcribed during uninduced growth of lysogens ('prophage transcription'). Increased transcript accumulation for the *c* region immediately after repressor inactivation, reported by Sinclair & Bibb (1989) and Smith & Owen (1991), was also seen in the present work, and defines an 'immediate early' phase of lytic cycle transcription. Probably, this is a simple consequence of negative autoregulation of *c* transcription. Higher abundance of *c* gene transcripts in older uninduced cultures of a *ctsI* lysogen may indicate a more rapid turnover of the repressor in cultures entering stationary phase, reducing the effective repressor concentration, and allowing increased *c* transcription to occur. It would be interesting to know whether *c* gene transcription in a wild-type (*c*⁺) lysogen showed the same dependence on culture age.

'Delayed early' transcription, reaching a peak of RNA accumulation 10 min after induction, was detected by dot-blot analysis (Fig. 4) in a region about 6 kb to the right of the *c* gene. The distinction between immediate early and delayed early transcription was not made for the remainder of the early region. Smith & Owen (1991) also reported an early transcript reading leftwards through *Sph*I site 8.3, which may be related to one or both of the faint early transcripts that we detected with the *Sph*I-[F+I] probe (Fig. 3). Overall, the early region extends between a position close to *Sph*I site 8.2 and an undefined position in the *Eco*RI-C fragment – a length of 15–17 kb. Genetic evidence, from studies with temperature-sensitive mutants, had already indicated that an early gene was to the right of *c*, but most *ts* mutants (among those that were sufficiently stable to allow analysis) defined late genes which mapped to the left of the *c* region (Chinenova & Lomovskaya, 1975; Lomovskaya *et al.*, 1980; Sladkova & Rebentish, 1980). Detailed deletion analyses have revealed only two extensive regions of ϕ C31 that are dispensable for plaque formation: the *c* region, and a region of about 8 kb including restriction sites 13–19. Thus, about 10 kb of DNA between these regions fulfils essential early functions. It is not obvious why stable *ts* mutants in this early region should be relatively rare.

The low-resolution S1 mapping of this region does not suggest that a single major early mRNA is sequentially transcribed and translated across the whole region, though it is not excluded that such a primary transcript may be formed and very rapidly processed into the series of relatively short RNA species detected both in our work and in a more detailed transcription analysis of part of the early region (*Sph*I site 8.6 to *Eco*RI site 11) carried

out in parallel by Ingham & Smith (1992). (We note that the latter experiments detected additional transcripts to those that we observed.) The apparent pattern of early transcription in ϕ C31 contrasts with observations on coliphage λ , in which only two major promoters (P_L and P_R) are used for early transcription. Multiple RNA species are generated from these λ promoters mainly by control of termination, though processing close to the 5' end of the λ leftward transcript also occurs (Friedman & Gottesman, 1983). The essential early region of ϕ C31 is currently being subjected to further detailed transcription analysis and DNA sequencing (C. Ingham & M. C. M. Smith, personal communication; C. J. Bruton, personal communication; G. J. P. Murphy, N. Hartley & K. F. Chater, unpublished).

The right-hand dispensable region has also proved to contain early genes. This is consistent with results indicating that at least two different functions that interact with host-controlled restriction-modification systems are in this region (Chater, 1986). Our S1 mapping analysis in this region is incomplete, because the *ctsI* prophage carries a deletion (Δ M) of 1.72 kb from the *EcoRI*-C fragment (Chater *et al.*, 1981).

Late transcription extends over an approximately 18 kb segment including most of the *SphI*-F, B, D, H and E fragments. Possibly, there is a single major mRNA covering all this region even including *cos*, though it is equally possible that several fairly large transcription units are involved. A single RNA species of this length would probably take as much as 5–10 min to transcribe (Bremer & Yuan, 1968). Not surprisingly, considering that ϕ C31 mRNA has a half-life of only 1–1.5 min (Rodríguez *et al.*, 1986), only degraded molecules were detectable. Some apparently more stable species are generated during degradation, giving perceptible striations in some of the tracks in S1 mapping. Transcripts of DNA close to or including *cos* appeared to be particularly stable. A small segment of ϕ C31 DNA that includes *cos* has been sequenced (Kobler *et al.*, 1991), but the sequence does not give any clues about possible gene products or other sequence features.

The construction of integrating vectors carrying the ϕ C31 *att* region (Kuhstoss *et al.*, 1991), and unpublished nucleotide sequencing studies by S. Kuhstoss (personal communication) and H. Rausch (personal communication), suggest that *attP*, together with integration and possibly excision functions, is in the *SphI*-E fragment. Possibly, the transcripts q and/or p specify components of the site-specific recombination system that would be needed for prophage excision. If so, it is surprising that these transcripts are abundant late in infection.

Cultures of a heat-inducible lysogen could be induced to release ϕ C31 only during the early stages of rapid growth (Rodríguez *et al.*, 1986). This effect is also seen at

the transcription level. As culture age increases, early transcription seems to be delayed, and early transcripts are abundant for much longer (as if early transcription were not properly shut off). Both late transcription and DNA synthesis are greatly reduced, and at least one – perhaps all – of the major late proteins is not made. There is little or no production of mature ϕ C31 particles inside the cells, as judged by the absence of cleavage at the *cos* site of amplified ϕ C31 DNA. The deficiencies in the induction cycle in old cultures are mirrored by observations on the shut-off of host rRNA gene transcription: in young cultures, induction is rapidly followed by *rrnD* shut-off, but in older cultures, heat treatment has no effect. These results suggest changes in host factor abundance at different stages of growth, such that either required host factors decrease in amount, or inhibitory host factors increase, as culture age increases. In any case, it is clear that biochemical studies of events during the lytic cycle can at present be done only with young, rapidly growing cultures.

J.E.S. was the recipient of a fellowship from the Fundacion Juan March (Spain). T.M.C. was funded by the John Innes Foundation. A.R. was funded by the Fondo de Investigaciones Sanitarias. Construction of pKC plasmids was carried out by K.F.C. during tenure of a Fulbright Scholarship in the laboratory of R. Losick (Harvard University). The work was also supported by a block grant to the John Innes Institute from the Agricultural and Food Research Council, and by the John Innes Foundation. We thank D. A. Hopwood for comments on the manuscript.

References

- BREMER, H. & YUAN, D. (1968). RNA chain growth rates in *Escherichia coli*. *Journal of Molecular Biology* **38**, 163–180.
- BRUTON, C. J., GUTHRIE, E. P. & CHATER, K. F. (1991). Phage vector that allow monitoring of secondary metabolism genes in *Streptomyces*. *Bio/Technology* **9**, 652–656.
- CHATER, K. F. (1986). *Streptomyces* phages and their application to *Streptomyces* genetics. In *The Bacteria: a Treatise on Structure and Function*, vol. 9, *Antibiotic-producing Streptomyces*, pp. 119–158. Edited by S. W. Queener & L. E. Day. Orlando, Florida: Academic Press.
- CHATER, K. F., BRUTON, C. J. & SUÁREZ, J. E. (1981). Restriction mapping of the DNA of the *Streptomyces* temperate phage ϕ C31 and its derivatives. *Gene* **14**, 183–194.
- CHINENOVA, T. A. & LOMOVSKAYA, N. D. (1975). Temperature-sensitive mutants of actinophage ϕ C31 of *Streptomyces coelicolor* A3(2). *Genetika* **11**, 132–141.
- CLAYTON, T. M. & BIBB, M. J. (1990). Induction of a ϕ C31 prophage inhibits rRNA transcription in *Streptomyces coelicolor* A3(2). *Molecular Microbiology* **4**, 2179–2186.
- FRIEDMAN, D. I. & GOTTESMAN, M. (1983). Lytic mode of lambda development. In *Lambda II*, pp. 21–51. Edited by R. W. Hendrix, J. W. Roberts, F. W. Stahl & R. A. Weisberg. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- GUGLIELMA, G., MAZODIER, P., THOMPSON, C. J. & DAVIES, J. (1991). A survey of the heat shock response in four *Streptomyces* species reveals two *groEL*-like genes and three GroEL-like proteins in *Streptomyces albus*. *Journal of Bacteriology* **173**, 7374–7381.

- HOPWOOD, D. A., BIBB, M. J., CHATER, K. F., KIESER, T., BRUTON, C. J., KIESER, H. M., LYDIATE, D. J., SMITH, C. P., WARD, J. M. & SCHREMPF, H. (1985). *Genetic Manipulation of Streptomyces. A Laboratory Manual*. Norwich: The John Innes Foundation.
- HOPWOOD, D. A., BIBB, M. J., CHATER, K. F. & KIESER, T. (1987). Plasmid and phage vectors for gene cloning and analysis in *Streptomyces*. *Methods in Enzymology* **153**, 116–166.
- INGHAM, C. J. & SMITH, M. C. M. (1992). A transcript map of the early region of the *Streptomyces* bacteriophage ϕ C31. *Gene* (in the press).
- KOBLE, L., SCHWERTFIRM, G., SCHMIEGER, H., BOLOTIN, A. & SLADKOVA, I. (1991). Construction and transduction of a shuttle vector bearing the *cos* site of *Streptomyces* phage ϕ C31 and determination of its cohesive ends. *FEMS Microbiology Letters* **78**, 347–354.
- KUHSTOSS, S., RICHARDSON, M. A. & RAO, R. N. (1991). Plasmid vectors that integrate site-specifically in *Streptomyces* spp. *Gene* **97**, 143–146.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LOMOVSKAYA, N. D., CHATER, K. F. & MKRTUMIAN, N. M. (1980). Genetics and molecular biology of *Streptomyces* bacteriophages. *Microbiological Reviews* **44**, 206–229.
- LYDIATE, D. J., IKEDA, H. & HOPWOOD, D. A. (1986). A 2.6 kb DNA sequence of *Streptomyces coelicolor* A3(2) which functions as a transposable element. *Molecular and General Genetics* **203**, 79–88.
- NOVIKOVA, N. L., LOMOVSKAYA, N. D. & KAPITONOVA, O. N. (1973). Adsorption and growth of actinophage ϕ C31 in germinating spores of *Streptomyces coelicolor* A3(2). *Mikrobiologia* **42**, 513–518.
- RODRÍGUEZ, A., CASO, J. L., HARDISSON, C. & SUÁREZ, J. E. (1986). Characteristics of the development cycle of actinophage ϕ C31. *Journal of General Microbiology* **132**, 1695–1701.
- SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- SINCLAIR, R. B. (1987). *The repressor (c) gene of Streptomyces phage ϕ C31*. PhD thesis, University of East Anglia, Norwich.
- SINCLAIR, R. B. & BIBB, M. J. (1988). The repressor gene (*c*) of the *Streptomyces* temperate phage ϕ C31: nucleotide sequence, analysis and functional cloning. *Molecular and General Genetics* **213**, 269–277.
- SINCLAIR, R. B. & BIBB, M. J. (1989). Transcriptional analysis of the repressor gene of the temperate *Streptomyces* phage ϕ C31. *Gene* **85**, 275–282.
- SLADKOVA, I. A. & REBENTISH, B. A. (1980). Physical mapping of *Streptomyces coelicolor* A3(2) actinophages. III. A restriction endonuclease cleavage map. *Molekularnaya Biologiya USSR* **14**, 1131–1136.
- SMITH, C. P. & CHATER, K. F. (1988). Cloning and transcription analysis of the entire glycerol utilization (*gylABX*) operon of *Streptomyces coelicolor* A3(2) and identification of a closely associated transcription unit. *Molecular and General Genetics* **211**, 129–137.
- SMITH, M. C. M. & OWEN, C. W. (1991). Three in-frame N-terminally different proteins are produced from the repressor locus of the *Streptomyces* bacteriophage ϕ C31. *Molecular Microbiology* **5**, 2833–2844.
- SMITH, M. C. M., INGHAM, C. J., OWEN, C. E. & WOOD, N. T. (1992). Gene expression in the *Streptomyces* temperate phage ϕ C31. *Gene* **115**, 43–48.
- SUÁREZ, J. E., CASO, J. L., RODRÍGUEZ, A. & HARDISSON, C. (1984). Structural characteristics of the *Streptomyces* bacteriophage ϕ C31. *FEMS Microbiology Letters* **22**, 113–117.