Characteristics of the Developmental Cycle of Actinophage ϕ C31

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Some characteristics of the lytic development of the temperate phage ϕ C31 in *Streptomyces coelicolor* A3(2) were studied using a thermoinducible lysogen. The physiological state of the host and the culture medium influenced the production of progeny virus after induction. The latent period lasted 45 min and the rise period 20–30 min. RNA synthesis in induced cultures was reduced with respect to controls. This reduction was restricted to cellular transcription as evidenced by: (i) no stable RNA being synthesized in induced cultures, and (ii) the proportion of phage specific RNA increasing from 0.5% before induction to more than 30% in induced cultures. Host RNA synthesis proceeded throughout the lytic cycle. Protein synthesis was also reduced in induced cultures, although to a lesser extent than RNA synthesis. Phage DNA synthesis started at around 10 min postinduction, marking the division between the early and late periods of phage development. Host DNA synthesis occurred during the first 20 min after induction, and gradually decreased later.

INTRODUCTION

The complexity of the life cycle of streptomycetes, and their economic interest, have stimulated the construction of cloning systems, using both plasmids and phages as vectors (Suárez & Chater, 1980; Thompson *et al.*, 1980). The phage cloning vehicles are mainly based on the broad host-range, temperate phage ϕ C31, a fact that increases interest in its biology. ϕ C31 has a single molecule of double stranded DNA 41.2 kb long (Chater *et al.*, 1981) and with cohesive ends. It is a naked virus belonging to group B of Bradley's classification (Bradley, 1967) and up to 17 proteins have been detected in the virion (Suárez *et al.*, 1984). The initial study of the developmental cycle of the phage as well as of its genetics was done in Professor N. Lomovskaya's laboratory (for a review see Lomovskaya *et al.*, 1980). In this paper, following Novikova *et al.* (1973), we have obtained a thermoinducible lysogen after induction of which a synchronous development of the phage occurs. By using this lysogen we have studied the general characteristics of phage development, focused mainly on its macromolecular synthesis and the discrimination between phage and cell processes.

METHODS

Bacterial and bacteriophage strains. *Streptomyces lividans* 66 was used as a host to propagate ϕ C31 c1 (a clear plaque mutant) (Lomovskaya *et al.*, 1970) and for enumeration of plaque-forming units (p.f.u.) on solid medium. *Streptomyces coelicolor* A3(2) ϕ C31 sensitive (strain 1915 of the collection of the John Innes Institute, Norwich, UK) was used as a progenitor of the thermoinducible lysogen, *S. coelicolor* O1, obtained after infection with ϕ C31 cts1 (Novikova *et al.*, 1973).

General microbiological methods. *S. lividans* was grown to sporulation in Petri dishes on solid R2YE medium (Chater *et al.*, 1982b) for 5 d at 35 °C. *S. coelicolor* O1 was grown on the same medium for 9 d at 28 °C. In both cases spores were obtained as previously described (Hardisson *et al.*, 1978) and stored in 20% (v/v) glycerol at –20 °C. The media used for germination of *Streptomyces* and propagation of ϕ C31 were GAE (Hardisson *et al.*, 1978); fortified GAE, which had tenfold more yeast extract than GAE; and minimal synthetic medium, which was made

of the GAE salts plus a mixture of 20 amino acids each at 0.1 mM, together with glucose (10 g l⁻¹) and (NH₄)₂SO₄ (2 g l⁻¹).

Propagation and purification of the phage was done on solid or liquid media following essentially the methods described by Chater & Wilde (1976) and Suárez *et al.* (1984). The isolation of thermoinducible lysogens was as previously described (Chater *et al.*, 1982*a*). Germination of lysogenic spores was activated by incubation of 10 ml spore suspensions (10⁸ spores ml⁻¹) in 100 ml flasks at 55 °C for 10 min (Hirsch & Ensign, 1976), followed by cultivation at 28 °C and 200 r.p.m. Germinated cultures were induced by placing them into a water bath at 40 °C for 5 min, and then returning them to 28 °C. The yield of induction was taken as the p.f.u. of the supernatant of induced cultures with respect to controls.

Macromolecular synthesis in induced and non-induced cultures. The biosynthesis of DNA, RNA and proteins in induced and non-induced cultures was measured by continuous incorporation and pulse labelling (3 min) of [*methyl*-³H]thymidine (10 µCi ml⁻¹, 30 Ci mmol⁻¹) (1 Ci = 37 GBq), [³H]uridine (2 µCi ml⁻¹, 27 Ci mmol⁻¹) and L-[4,5-³H]leucine (2 µCi ml⁻¹, 50 Ci mmol⁻¹) into TCA-insoluble material. The processing of radioactive samples has been previously described (Hardisson *et al.*, 1980), although in this case the samples taken to measure protein biosynthesis were heated to 90 °C in TCA solution, for 15 min, in order to hydrolyse the [³H]Leu-tRNA.

Nucleic acid isolation. ϕC31 DNA was extracted from virions purified on a CsCl gradient with phenol, essentially as described by Chater & Wilde (1976). Host DNA was extracted as described by Chater *et al.* (1982*b*). Pulse-labelled DNA and RNA were extracted from cultures germinated for 7 h. At fixed times [*methyl*-³H]thymidine (25 µCi ml⁻¹) or [³H]uridine (10 µCi ml⁻¹) was added to shaken induced and non-induced 10 ml cultures of *S. coelicolor* O1. The incorporation of the radioactive precursor was stopped, after 3 min at 28 °C, by pouring the culture over crushed-frozen 50 mM-Tris/HCl buffer (pH 8.0 at 4 °C) containing 25 mM-sodium azide. The cells were immediately centrifuged at 15000 r.p.m. for 1 min at 4 °C. The sediment was then washed with 2 ml ice-cold TE buffer (pH 8.0) containing 20% (w/v) sucrose and then resuspended in 1 ml 50 mM-Tris/HCl (pH 8.0) containing 1 mM-EDTA and 20% sucrose in the case of DNA extraction, or 1 ml 50 mM-sodium acetate buffer (pH 5.0) containing 1 mM-MgCl₂, heparin (1 mg ml⁻¹) and 20% sucrose for RNA extraction; 1 ml of the same buffer without sucrose but with 2% (w/v) SDS was added to the suspension and the tube was heated to 60 °C for 5 min in the case of DNA extraction or boiled in a water bath for 1 min for RNA extraction; 4 ml of a phenol/chloroform (1:1, v/v) mixture saturated with the appropriate buffer (0.1 M) was added to the hot extract and after mixing, the phases were separated by centrifugation. The phenol/chloroform extraction was repeated twice, followed by extraction of the aqueous phase with chloroform/isoamylalcohol (24:1, v/v). The nucleic acid present in the aqueous phase was precipitated with ethanol and resuspended in a small volume of TE buffer (10 mM-Tris/HCl, 1 mM-EDTA, pH 8.0) and in the case of DNA extraction incubated with pancreatic RNAase (40 µg ml⁻¹) for 1 h at 37 °C followed by further phenol extraction and ethanol precipitation. The pellet was resuspended in TE buffer.

Nucleic acid hybridization. Hybridization of ³H-labelled RNA (5 µg, specific activity 8–10 × 10³ c.p.m. µg⁻¹) or ³H-labelled DNA (10 µg, 3.5 × 10³ c.p.m. µg⁻¹), synthesized *in vivo*, to host DNA (100 µg) or ϕC31 DNA (25 µg) immobilized on nitrocellulose filters was done essentially as described by Gillespie & Spiegelman (1965). Hybridizations were done at 65–67 °C for 20 h. The radioactivity of the filters was determined as previously described (Hardisson *et al.*, 1980).

Kinetics of RNA decay after addition of rifampicin. Cultures incubated for 7 h were induced and 10 min later pulse-labelled with [³H]uridine (2 µCi ml⁻¹) for 3 min. Uninduced control cultures were processed in parallel. After the labelling period, rifampicin (200 µg ml⁻¹) was added to the cultures to stop RNA synthesis, samples were taken at timed intervals and the radioactivity remaining as TCA-insoluble material after 30 min on ice was considered as the stable RNA fraction.

RESULTS

Properties of the lysogenic S. coelicolor O1 strain

Although we attempted to induce lysogenic *Streptomyces* with mitomycin C or UV light, no phage production was observed (data not shown). An alternative approach to obtain synchronous development of ϕC31 was to construct a thermoinducible lysogen, *S. coelicolor* O1, which showed essentially the same biological properties as others previously described (Novikova *et al.*, 1973). The highest yield of phages was obtained with induced cultures, previously germinated for 7 h, when they showed short germ tubes. No phages were obtained from heat-treated cultures germinated for less than 3 h or for more than 10 h.

The one-step growth curve of ϕC31 in 7 h germinated spores of *S. coelicolor* O1 showed a latent period of about 45 min, followed by a rise period of 20–30 min. The burst size was 100–200

Fig. 1. One-step growth curve of ϕ C31 *cts* development in *S. coelicolor* O1. The cultures were germinated until most of the spores showed short germ tubes and were then thermally induced. ○, Non-induced cultures or cultures germinated in minimal medium; ●, induced cultures in GAE medium; □, induced cultures in fortified GAE medium. Results are means of three determinations.

in GAE and over 500 in fortified GAE. No phage release was observed from spores germinated in minimal synthetic medium (Fig. 1).

Macromolecular synthesis by induced and non-induced cultures

All the subsequent experiments, which included radioactive precursor incorporation, were done with spores germinated for 7 h at 28 °C in GAE medium in order to obtain an acceptable 'burst size' along with a significant incorporation of label into macromolecules.

RNA synthesis was severely reduced in induced cultures with respect to controls, as measured by continuous incorporation of [5-³H]uridine (Fig. 2*a*) or by pulse labelling (Fig. 2*b*). This effect was not due to the heat treatment, as no differences were detected between 'induced' and 'non-induced' non-lysogenic cultures (data not shown). Protein synthesis was also reduced by the induction treatment, although to a lesser extent than RNA synthesis (Fig. 2*c, d*). In contrast, after 10 min of induction (Fig. 2*e*), DNA synthesis showed an increase that was particularly evident in pulse experiments (Fig. 2*f*), probably indicating the start of phage DNA replication.

Hybridization analysis of the RNA synthesized after induction

In order to determine the proportion of ϕ C31 RNA synthesized after induction, pulse-labelled RNA extracted from *S. coelicolor* O1 was hybridized with ϕ C31 DNA immobilized on nitrocellulose filters (Fig. 3). Before induction, the amount of radioactivity which bound to the filters was very low, around 0.5% of the c.p.m. applied, probably due to spontaneous induction of some cells of the population and to the repressor coding system of the phage. After induction, a continuous increase in the fraction of phage-specific RNA was seen, with an inflexion point after 10 min of induction, reaching a maximum of over 30% of the c.p.m. applied at 30 min. The degree of hybridization between labelled RNA and chromosomal *S. coelicolor* 1915 DNA did not change either with RNA from non-induced or from induced cultures (Fig. 3), indicating that cellular RNA synthesis proceeded throughout the latent period. (Note that the hybridizations to host DNA were done under conditions in which the more abundant RNA species would have been in excess; these results are therefore not quantitative.)

Effect of rifampicin on RNA metabolism

Rifampicin was used as a tool to discriminate between unstable RNA (mainly mRNA) and stable RNA (rRNA and tRNA), synthesized by non-induced and induced cultures.

The addition of rifampicin to induced and non-induced germinating cultures resulted in a halt of [5-³H]uridine incorporation into RNA. In non-induced cultures (Fig. 4*a*) there was a short delay in the inhibition effect, probably due to continued production of the RNAs whose

Fig. 2. Macromolecular synthesis in induced and non-induced cultures of *S. coelicolor* O1 as measured by incorporation of exogenous, labelled precursors into TCA-insoluble material. (a, b) RNA; (c, d) Protein; (e, f) DNA. (a), (c) and (e) refer to continuous incorporation of label by induced (○, △, □) and non-induced (●, ▲, ■) cultures. (b), (d) and (f) refer to pulse labelling (3 min) with induced (□) and non-induced (▨) cultures. Results are means of three determinations.

Fig. 3. Hybridization of pulse-labelled RNA synthesized by non-induced and induced cultures of *S. coelicolor* O1 to $\phi C31$ (■) or *S. coelicolor* 1915 chromosomal DNA (□) immobilized on filters. DNA was used at 25 and 100 μg per filter, respectively, and RNA at 5 μg ($8-10 \times 10^3$ c.p.m. μg^{-1}) per filter. Hybridization was done at 67 °C for 20 h in $2 \times$ SSC and the unspecifically bound RNA was eliminated by treatment of the filters with RNAase. Results are means of three determinations.

Fig. 4. Effect of rifampicin on RNA metabolism of (a) non-induced cultures and (b) 10 min induced cultures. The germinating (7 h) cultures were labelled with [3 H]uridine for 3 min and then rifampicin ($200 \mu\text{g ml}^{-1}$) was added. The radioactivity in TCA-insoluble material was determined after 30 min on ice. Results are means of three determinations.

Fig. 5. Hybridization of pulse-labelled DNA from non-induced and induced cultures of *S. coelicolor* O1 to ϕ C31 (■) or *S. coelicolor* 1915 chromosomal DNA (□) immobilized on filters. DNA was used at 25 and $100 \mu\text{g}$ per filter, respectively, and radioactive DNA at $10 \mu\text{g}$ (3.5×10^3 c.p.m. μg^{-1}) per filter. The filters were treated with sheared denatured herring sperm DNA before use so as to avoid unspecific binding. Hybridization was done at 67°C for 20 h in $2\times$ SSC. Results are means of three determinations.

synthesis had already started at the moment of adding this antibiotic, followed by a progressive decrease in the amount of TCA precipitable radioactivity until a plateau was reached, around 10 min after the addition of rifampicin. The amount of stable RNA in these conditions was 60% and the half life of the unstable RNA was around 5.3 min. In cultures pulse-labelled 10 min after induction (Fig. 4b), the addition of antibiotic revealed that virtually no stable RNA was synthesized and that the half life of the unstable RNAs produced was around 1 min. These data indicate not only that the amount and specificity of the RNAs synthesized by induced and non-induced cultures differed, but also that the host RNA synthesis had qualitatively changed as a consequence of phage development.

Hybridization analysis of DNA synthesized by *S. coelicolor* O1 cultures

The result of the hybridization of pulse-labelled DNA, extracted from non-induced and induced cultures of *S. coelicolor* O1, with ϕ C31 DNA immobilized on nitrocellulose filters is shown in Fig. 5. Before induction a significant proportion (17% of the counts applied to the filters) hybridized. This value increased 10 min after induction, indicating that phage DNA synthesis began at this time, thus marking the onset of the late period of development of the

phage. The hybridized counts gradually increased, reaching a maximum of 60% at 30 min of the latent period, and remained at this level until the beginning of cell lysis. Cellular DNA synthesis remained at the same level in non-induced and induced cultures for at least 30 min, after which it gradually decreased in induced cultures.

DISCUSSION

In order to study the lytic cycle of phage ϕ C31, we have constructed a thermoinducible lysogen which allows synchronous development of the phage upon exposure of the cultures to a short high-temperature pulse.

The changes in the overall pattern of macromolecular synthesis after induction of the cultures, and mainly the reduction in RNA and protein biosynthesis, clearly seem to reflect an interaction between phage development and host metabolism. In some other phages, host metabolism proceeds unchanged until lysis and no effect on bulk macromolecular synthesis is seen (Schachtele *et al.*, 1972; Kolebrander *et al.*, 1972). It can be seen in Fig. 2 that RNA synthesis is inhibited approximately fourfold immediately after induction, whereas there is only a minor decrease in overall protein synthesis throughout the developmental cycle. This differential inhibition could be due to a selective effect on 'non translatable' RNA (i.e. mainly ribosomal and transfer RNA, both of cellular origin) and this would be consistent with the absence of stable RNA synthesis by induced cultures (Fig. 4). Alternatively, there may be a general inhibition of cell RNA synthesis and protein synthesis, but efficient translation of the abundant phage mRNA (phage specific RNA synthesis increases after induction to reach values of about 50 times the level of synthesis in non-induced cultures). The enormous synthesis of phage DNA 10 min after induction could, at least in part, explain the preferential biosynthesis of phage RNA simply by the increase in the proportion of phage templates. Nevertheless, since inhibition of gross RNA synthesis is detected before phage DNA replication starts, there may be active inhibition of host RNA formation. An interesting possible mechanism for selective inhibition of host RNA synthesis would arise if ϕ C31 exploited the presence in the host of more than one class of sigma factor (Westpheling *et al.*, 1985). Conceivably, the phage could inactivate a major vegetative sigma factor required for transcription of rRNA genes and itself use a different host factor. This would be compatible with the observation of specific transcription of ϕ C31 DNA *in vitro* by an RNA polymerase fraction enriched for sigma factor conferring specificity for promoters distinct from the major prokaryotic consensus type (Chater, 1984).

A surprising finding is the very high level of hybridization found between the DNA extracted from non-induced cultures and ϕ C31 DNA, namely 17% of the counts applied to the filters. This may be due to the presence of free ϕ C31 genomes in *S. coelicolor* O1 non-induced hyphae; when restriction fragments of the DNA extracted from non-induced cultures of *S. coelicolor* O1 were run in gels, discrete ϕ C31 bands were revealed (data not shown).

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