LARGE SCALE ISOLATION OF THE CAULOBACTER BACTERIOPHAGE ØCb5 AND ITS RNA GENOME

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1. Introduction

Many investigations of current interest, such as those on species specificity of translation in bacterial systems, require the use, in relatively large quantities, of intact natural RNA's as messengers. Hitherto, only RNA genomes from several well known RNA coliphages were used to program protein synthesis in systems containing ribosomes and initiation factors from various non-host bacterial species [1, 2]. RNA-containing phages, excepting coliphages, have been reported for *Pseudomonas aeruginosa* [3] and for the Caulobacter group [4, 5]. The *Caulobacter crescentus* RNA phage ØCb5 was recently shown by Bendis and Shapiro [6] to be similar to RNA coliphages in its physical properties and in its genetic content, thereby making ØCb5 RNA a suitable model for comparative investigations on translocation.

Isolation of phage ØCb5 in milligram quantities by conventional procedures is impossible because the phage is extremely sensitive to high ionic strength [6]; this makes salt precipitation or cesium chloride banding impractical. In addition, phage maturation and release and cell division, continue concurrently in infected cultures [5]. Although high titer lysates (about 10^{11} pfu/ml) have been obtained 20–24 hr after infection, they are heavily contaminated with ribosomes from disintegrated cells.

The method described in this communication is based on the observations [7] that (i) infected cells remain intact for several hr while producing and releasing virions, and (ii) most of the mature virions that remain associated with intact infected cells can be artificially released by enzymic digestion of the cell wall. The problem of ribosomal contamination has been reduced by the following procedures. Firstly, infected cells containing mature virions are concentrated 5.5 hr after infection before the cells begin to disintegrate; this also eliminates the necessity of concentrating phage particles from large volumes. Secondly, the concentrated infected cells are converted to spheroplasts by lysozyme treatment in the presence of an osmotically protective concentration of polyethyleneglycol. This procedure avoids excessive contamination with ribosomes and allows the isolation of more than 90% pure phage particles in a single sucrose gradient sedimentation.

2. Materials and methods

C. crescentus CB180 and phage ØCb5 were kindly provided by Dr. L. Shapiro. The complex medium (Hy) for large scale growth contained 0.2% peptone, 0.2% yeast extract and 10 ml/l of 100X Hutner's base [8]. For the preparation of inoculum this medium was supplemented with 0.1% glucose (Hyg), and for phage adsorption with 5 ml/l of 0.5 M $MgCl_2$ (Hy5). Cells for inoculum were grown at 30° to 1×10^{11} cells/ml ($A_{600} = 0.580$). Lysates for large scale growth were prepared in 21 flasks according to Bendis and Shapiro [6] by allowing about 2×10^{10} cells to grow in Hy5 medium in the presence of about 10^{12} pfu of phage for 20–24 hr at 34°; titers varied from 5×10^{10} to 5×10^{12} pfu/ml. Lysates were clarified by low speed centrifugation and kept for 5–7 days at −20°. Lysates having titers less than 10^{11} pfu/ml were discarded.

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3. Results and discussion

The following is a procedure to grow phage in four 16 l carboys. Six liters of the bacterial culture (A_{600} = 0.60) were centrifuged for 5 min at 5000 rpm under aseptic conditions; the sediment was suspended in Hy5 and centrifugation was repeated. This procedure [9] leaves most of the non-susceptible stalked cells in the supernatant.* The sediment was suspended in 3.0 l of Hy5 medium to 2×10^{10} cells/ml, warmed to 34° and 500 ml of lysate (5×10^{11} pfu/ml, multiplicity of infection 4.1:1) were added. Adsorption was allowed to proceed for 30 min at 34° without agitation. The post-adsorption mixture was divided into 4 equal vol. and added, respectively, to 4 carboys each containing 16 l of Hy medium. Growth was continued for 5.5 hr at 30° with vigorous aeration. The cultures were cooled in ice and spun in a Sharples centrifuge. The Sharples supernatant contained a total of 1.2×10^{16} pfu (free virions) and was discarded. 1.3×10^{16} pfu was retained in the sediment (infected cells). Hereafter, all procedures were carried out at 0°. The sediment was suspended in 50 ml of Hy medium and 150 ml of 0.05 M imidazole buffer, pH 7.0, containing 20% polyethylene glycol (PEG, Carbowax 4000, Fluka). 30 ml of "Versene-Fe3 Specific" reagent (Fisher), diluted 10-fold with H_2O were added and the mixture was immediately centrifuged for 10 min at 12,000 *g*. The supernatant was decanted, the sediment washed with 50 ml of Hy medium and the washing combined with the supernatant. The sediment was again suspended in 150 ml of 0.05 M imidazole buffer containing 20% PEG and incubated for 10 min at 34°, with 15 mg of lysozyme. The mixture was centrifuged and the sediment washed with TM buffer (10 mM Tris-acetate, pH 7.8, 0.2 mM $MgCl_2$). The combined supernatants after Versene-Fe3 and lysozyme treatments were pelleted by high speed centrifugation (3 hr at 50,000 rpm in Spinco Ti 60.1 rotor) and the pellets carefully suspended in a small volume of TM buffer and dialyzed against the same buffer for 4 hr. Sucrose gradient centrifugation of an aliquot of the dialyzed preparation (fig. 1) demonstrated that about 40% of the material sediments in the 80 S region and coincides with the ØCb5 plaque forming band. The other peaks of fig. 1 were ribosomal subunits dissociated in TM buffer.

On a preparative scale, the phage were isolated by sucrose gradient sedimentation in the Spinco SW 25.2 rotor (10–30% sucrose in TM buffer, centrifugation for 12 hr at 19,000 rpm), yielding a total of 384 A_{260} units of 95% pure ØCb5 particles. The sedimentation coefficient of the phage (Spinco, Model E) in TM buffer was 86 S. This value is higher than that reported by Bendis and Shapiro (70 S, [6]) for ØCb5 released from spontaneously lysed cells. The sedimentation coefficients of RNA coliphages varied from 79 S (ØMS2) to 83 S ($ØQ_\beta$, [11]). Phage ØCb5 RNA was isolated by phenol extraction in the presence of 2% dodecyl sulfate according to published procedures [10].

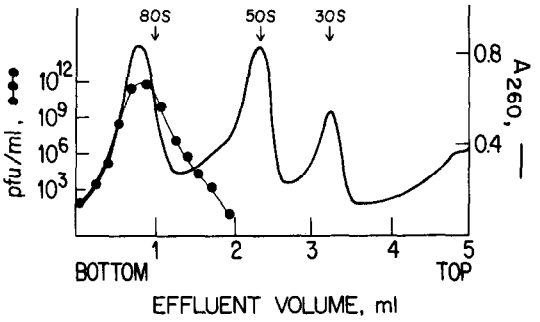

Fig. 1. Sucrose density gradient analysis of ØCb5 preparation after dialysis in TM buffer (for details, see text). 4.5 A_{260} units in 0.125 ml of TM buffer were layered on 5 ml of a linear gradient (15–30% sucrose, w/v) and centrifuged at 50,000 rpm at 3° for 110 min. —: Optical density 260 nm; •—•: infectivity, pfu/ml.

It will be seen from table 1 that the chemical and physical properties of phage Cb5 RNA are remarkably similar to those of coliphage MS2 RNA. Phage Cb5 RNA is easily degraded by pancreatic ribonuclease. Preliminary experiments on *in vitro* translation indicate that ØCb5 RNA is active as messenger in both the *C. crescentus* CB1 80 and in the *E. coli* ribosomal system, as is MS2 RNA; however, each ribosomal system displays a distinct preference for binding and translation of its specific messenger.

* Only one form of the dimorphic host, flagellated and piliated swarmer but not stalked cells, is susceptible to infection [5].

Table 1
Chemical and physical properties of Cb5 RNA and MS2 RNA.

RNA	$S_{20,w}$ (0.2 M NaCl)	Tm (0.1 M NaCl)	Hypochromicity (%)	Base composition A	U	G	C
ØCb5	31	59°	19.5	24.6	23.2	28.6	23.6
ØMS2	29	60°	18	23.7	24.4	27.1	24.8

Sedimentation coefficients were measured at pH 7.5 in a Spinco Model E ultracentrifuge using the UV absorption system. UV-melting profiles were run in a Beckman DU-2 Spectrophotometer equipped with a thermostated cuvette compartment. Base composition was determined according to Uziel et al. [12]. Base composition of MS2 RNA was taken from [10].

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