Two Groups of Capsule-specific Coliphages Coding for RNA Polymerases with New Promoter Specificities

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SUMMARY

Four bacteriophages (A16, CK235, φ1.2 and K31) which specifically attack different encapsulated strains of Escherichia coli have been shown to be related to bacteriophage T7 (which is unable to grow on encapsulated hosts). The conclusion that phages A16 and CK235 are related to T7 is based on (i) similarities in the pattern of expression of intracellular phage proteins, (ii) early appearance, in infected host cells, of a phage DNA-specific RNA polymerase and (iii) hybridization (albeit to a low extent) of A16 DNA and of CK235 DNA to T7 DNA. The first two criteria also apply to phages φ1.2 and K31 but hybridization of their DNAs with T7 DNA could not be detected. The RNA polymerases of CK235 and A16 have similar template specificities and the same applies to the RNA polymerases of φ1.2 and K31. None of the new RNA polymerases can use T7 DNA as template.

INTRODUCTION

Enterobacteria with lipopolysaccharide capsules are lysed by phages which possess specific hydrolases as virion constituents. By means of these particle-bound enzymes the phages tunnel through the thick layer of capsule material until they find the outer membrane where specific adsorption and DNA injection occurs (Lindberg, 1977). One group of such capsule-specific phages is characterized by an icosahedral head with a diameter of about 60 nm, and a short tail of about 20 nm, to which spikes are attached with sixfold symmetry. These spikes display the capsule-lysing activity (Bessler et al., 1975; Rieger-Hug & Stirm, 1981). Since this particle morphology is reminiscent of that of the classical coliphage T7 (which, however, does not infect capsulated strains), Korsten et al. (1979) examined two such capsule-specific phages, Klebsiella phage K11, and Citrobacter phage ViIII, with regard to their genetic relatedness to T7; they found that indeed the genome structures of T7, K11 and ViIII were similar in various respects. The most striking feature was the synthesis, early in infection by K11, and by ViIII, of a phage-coded RNA polymerase which recognizes only DNA of the homologous phage as a template. This observation closely corresponds to what is known for phage T7, and thus it was hypothesized that, in spite of the lack of heterologous transcription (i.e. transcription of the DNA of one phage by the RNA polymerase of another), there was a phylogenetic relationship between T7, K11 and ViIII. In the meantime Dietz (1985) and Dietz et al. (1985) have confirmed this hypothesis for T7 and K11 by means of base sequence analysis of some segments of K11 DNA and comparison to the corresponding sequences of T7 DNA, as determined by Dunn & Studier (1983), and to the DNA sequences of T3 (which is related to T7) as determined by Fujisawa & Sugimoto (1983) and by McAllister et al. (1983). However, so far no capsule-specific Escherichia coli phages have been shown to be related to T7. Considering this, we have investigated a series of phages which specifically lyse one of three different capsule-producing E. coli strains. We did this mainly by (i) comparison, by PAGE and autoradiography, of the
patterns of intracellular protein synthesis by these phages, (ii) checking for the ability of these phages to induce the synthesis of an RNA polymerase which specifically transcribes their own DNA and (iii) checking for the ability of the DNAs from these phages to cross-hybridize with T7 DNA. Out of 16 phages tested, four were found to be related to T7 as judged by similarity of patterns of genome expression and by coding for phage DNA-specific RNA polymerases. These enzymes could be divided into two groups, according to their patterns of heterologous transcription; however, none of them was able to transcribe T7 DNA.

METHODS

Media. For labelling phage protein with [35S]methionine, host cells were grown in a medium containing, per litre, 1 g NH4Cl, 10 g Na2HPO4, 4.5 g KH2PO4, 0.2 g MgSO4, 5 g glucose, and 30 mg of each amino acid required for protein synthesis, except methionine, which was not added. For all other purposes, nutrient broth was used (per litre, 10 g Difco Nutrient Broth, 10 g NaCl).

Bacteria and phage. E. coli B (strain O1 :K1 :H-), obtained from W. Arber (Biozentrum Basel, Basel, Switzerland) was used as host for phages T3 and T7, which were the reference types from our collection and were referred to earlier by Korsten et al. (1979). The same authors also described phage K11 and its host, Klebsiella spp. sp. 390 (O1 :K11) as well as phage VIII and its host Citrobacter spp. C123. The capsule-forming strains E. coli 09 :K31 and E. coli K235 (O1 :K1 :H-) were obtained from S. Stirm (University of Giessen, Giessen, F.R.G.). Strain E112, which we isolated from a patient, was characterized by F. Orskov (International Escherichia and Klebsiella Centre, Copenhagen, Denmark) as an atypical, yellow-pigmented E. coli, producing serotype O169 lipopolysaccharide. Phages K31 and φ1.2, which grow on strains 09 :K31 and K235, respectively, were also from S. Stirm. Phage φ1.2 has been described with regard to its capsid-bound endo-N-acetylneuraminidase, a novel capsule polysaccharide hydroxide (Kwitowski et al., 1983). We isolated phages A16 and CK235 as well as 12 others, referred to in this study but not specifically named, from sewage, using strains E112 and K235 as indicator bacteria.

PAGE and autoradiography of intracellular phage proteins. For specific labelling of intracellular phage proteins, host cell cultures (3 × 10^8 cells/ml) in methionine-free medium (see above) were u.v.-irradiated (Sylvania germicidal lamp G8T5, 12 cm distance, 2 min) 5 min before infection with phage (m.o.i. 10). The infected culture was divided into 0.3 ml samples and incubated at 30 °C. [35S]Methionine (10 μCi/sample) was added to each sample at different times after infection and incubation continued for 3 min. After 30 μg unlabelled methionine and 2 min later 100 μg chloramphenicol were added to each sample. The infected cells were pelleted, resuspended in 20 μl dissociation buffer (0.1 M-Tris-HCl pH 7, 0.8 M-2-mercaptoethanol, 2 M-SDS, 10% glycerol) and heated (100 °C, 2 min); 5 μl was applied to SDS-polyacrylamide gradient slab gels (10 to 18% polyacrylamide). Basically, the system described by Laemmlı (1970) was used. The gels were 20 cm long. Electrophoresis was at 200 V for 14 h. The gels were fixed, dried at 80 °C under vacuum and autoradiographed for 3 to 5 days at room temperature.

RNA polymerase assay. The procedure for assaying rifampicin-resistant, phage-coded RNA polymerase activities was that of Chamberlin et al. (1970), with minor modifications. As the enzyme source, cell-free homogenates of phage-infected bacteria were used. Exponentially growing host cell cultures (10 ml, 3 × 10^8 cells/ml) were infected with phage (m.o.i. 5) and incubated for 8 min at 30 °C. Chloramphenicol (100 μg/ml) and ice were added and the samples were centrifuged immediately. The packed cells were resuspended in 0.5 ml buffer (50 mM-Tris–HCl pH 8, 20 mM-2-mercaptoethanol, 0.2% bovine serum albumin) and disrupted by ultrasound (Branson Sonifier B15, microtip, lowest intensity, 45 s). From these extracts, 5 μl (corresponding to approx. 10 μg bacterial protein) was used for each enzyme assay.

Hybridization of heterologous DNA to T7 DNA restriction fragments. T7 DNA was cut with HpaI into 18 restriction fragments (Studier, 1979). The digest was divided into six aliquots which were separated electrophoretically on different tracks of a 1.5% agarose gel. The six identical patterns thus obtained were transferred to a nitrocellulose sheet (Southern, 1975). The same authors also described phage K11 and its host, Klebsiella spp. sp. 390 (O1 :K11) as well as phage VIII and its host Citrobacter spp. C123. The capsule-forming strains E. coli 09 :K31 and E. coli K235 (O1 :K1 :H-) were obtained from S. Stirm (University of Giessen, Giessen, F.R.G.). Strain E112, which we isolated from a patient, was characterized by F. Orskov (International Escherichia and Klebsiella Centre, Copenhagen, Denmark) as an atypical, yellow-pigmented E. coli, producing serotype O169 lipopolysaccharide. Phages K31 and φ1.2, which grow on strains 09 :K31 and K235, respectively, were also from S. Stirm. Phage φ1.2 has been described with regard to its capsid-bound endo-N-acetylneuraminidase, a novel capsule polysaccharide hydroxide (Kwitowski et al., 1983). We isolated phages A16 and CK235 as well as 12 others, referred to in this study but not specifically named, from sewage, using strains E112 and K235 as indicator bacteria.

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phosphate buffer pH 7, with 2 mM-EDTA, 0.3 M-NaCl, and 1% SDS. One final washing was done in the same buffer without NaCl and SDS. The air-dried strips were autoradiographed at room temperature without intensifier screens.

**Dot hybridization.** Different amounts (0.1 μg, 0.2 μg, 0.5 μg) of unlabelled DNA from each phage were dotted on a nitrocellulose filter sheet. Denaturation, neutralization, drying and pre-hybridization were as described above. The nitrocellulose sheet was then incubated overnight, at 58 °C, in hybridization buffer containing 32P-labelled T7 DNA obtained by nick translation (Maniatis et al., 1975). Further processing was as described above.

**Other procedures.** Propagation and purification of phage, and extraction of phage DNA were as previously described (Korsten et al., 1979). Autoradiographs were scanned at 595 nm, using a Kontron Spectrophotometer (UVIKON 810) with a slit width of 0.1 mm. For molecular weight estimates (Weber & Osborn, 1969) we used as marker proteins the T7 gene products whose molecular weights were reported by Studier & Dunn (1983).

**RESULTS**

**Screening for T7-like infection strategies by means of PAGE and autoradiography**

In total, 16 capsule-specific coliphages were investigated with regard to their patterns of intracellular phage-coded proteins by means of PAGE and autoradiography. The autoradiograms of the gels were searched for bands of early proteins (start of synthesis: about 6 min after infection, at 30 °C) with molecular weights of about 100000. Such bands have been shown to be a strong indication for the synthesis of phage-coded RNA polymerases (Korsten et al., 1979). Of the 16 phages investigated, four clearly satisfied this criterion (Fig. 1) while the others displayed totally different patterns (not shown).

**Homologous and heterologous phage-directed RNA polymerase activities**

In a second step to characterize the capsule-specific coliphages we looked for the appearance, early in infection, of a template-specific RNA polymerase activity within the infected cells. For this, cell-free extracts of host cells infected for various periods of time were used as the enzyme source. In parallel assays we used as templates the DNA of *E. coli* (as a control) and the DNA of the phage being investigated. For the four phages coding for an early protein in the mol. wt. range of 95 000 to 105 000 we found a new RNA polymerase activity which, at 30 °C, peaked at about 12 min after infection and which was detected only by use of the DNA of the homologous phage; the DNA of *E. coli* was totally inactive as template. Cell-free extracts of cells infected by any of the other 12 phages displayed no new RNA polymerase activity. As in the case of T7 (Chamberlin et al., 1970), the new RNA polymerases were as active in the presence of 20 μg/ml rifampicin as in the absence of this antibiotic. The specific RNA polymerase activities within the infected cells (measured as RNA synthesized with the homologous DNA, per μg protein of cell extract) varied within a factor of 2, as compared to the RNA polymerase activity of T7-infected cells.

In order to characterize further the new RNA polymerases we performed assays with DNAs from heterologous phages as templates. For this, we used the RNA polymerase-inducing capsule-specific coliphages characterized in this study as well as the DNAs of *Klebsiella* capsule phage K11 and *Citrobacter* capsule phage ViIII, described by Korsten et al. (1979). In addition, we used as templates the well characterized DNAs of T7 (Dunn & Studier, 1983) and of T3 (Basu et al., 1984). As shown in Table 1 the RNA polymerases of all four *E. coli* capsule-specific phages were inactive on the DNAs of coliphages T3 or T7 (both non-capsule-specific), or the DNAs from *Klebsiella* capsule phage K11 or *Citrobacter* capsule phage ViIII. However, the heterologous transcription of φ1.2 DNA by phage K31 RNA polymerase was nearly identical to the homologous value, and the reverse assay (K31 DNA and φ1.2 RNA polymerase) showed only a slightly (although reproducibly) diminished activity. The pair A16 and CK235 also showed heterologous transcription; in this case heterologous transcription was clearly less efficient (50 to 70%) than in the homologous systems.

**Cross-hybridization of capsule-specific phage DNAs with T7 DNA**

Although, as postulated earlier (Korsten et al., 1979), phylogenetic relationships among different phages may be inferred from similarities in the patterns of phage-coded intracellular proteins even in the absence of cross-annealing of the respective nucleic acids, such cross-
Fig. 1. Pulse-chase labelling of intracellular phage proteins. Host-coded protein synthesis was inhibited by u.v. irradiation of exponentially growing bacteria. These were then infected with phage and incubated at 30 °C. At different times after infection, 3 min pulses of [35S]methionine were given to different samples of the infected cultures and the cells prepared for SDS–PAGE on a 10 to 18% polyacrylamide gradient slab. Dried gels were autoradiographed to monitor the times of synthesis of phage-coded proteins. The numbers above the autoradiographs refer to the times of chasing (i.e. the end of the 3 min pulse). The letters r and p correspond to samples in which [35S]methionine was present throughout the whole latent period; p refers to the phage investigated and r to T7, used as a reference. Numbers at the left refer to genes coding for proteins whose molecular weights are as follows: gene 16 product (internal coat protein), 143800; gene 1 product (RNA polymerase), 98100; gene 10 product (major coat protein), 36400; gene 6 product (exonuclease), 40000; gene 0.3 product (anti-restriction protein), 13700. (a) Phage CK235; (b) phage A16; (c) phage φ1.2; (d) phage K31.
**RNA polymerases of capsule-specific phages**

Fig. 2. Dot hybridization of $^{32}$P-labelled T7 DNA with the DNAs of the phages investigated. Increasing amounts of DNA (from left to right: 0.1 μg, 0.2 μg, 0.5 μg) were dotted on nitrocellulose, denatured and incubated with nick-translated radioactive T7 DNA. The extent of hybridization was visualized by autoradiography. The dots contained DNA from the following sources: (a) T7, (b) T3, (c) K11, (d) A16, (e) CK235, (f) φ1.2, (g) K31, (h) plasmid pZmC134 (control).

Table 1. Template specificity of phage-coded RNA polymerases*

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<tr>
<th>Source of RNA polymerase</th>
<th>Source of template DNA</th>
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<td>T7</td>
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<td>T3</td>
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<td>K11</td>
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<td>ViIII</td>
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<td>A16</td>
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<td>CK235</td>
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<td>K31</td>
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<tr>
<td>φ1.2</td>
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* Rifampicin-resistant RNA polymerase activity was measured as described by Chamberlin et al. (1970). As templates, different phage DNAs were used and the activity with the homologous template (RNA polymerase and template DNA from the same phage) taken as the norm (100%) to which heterologous activities refer. A dash indicates a heterologous activity of less than 5% of the homologous one. All values represent averages of at least three assays.

Annealing, whenever observed, is a strong indication of relatedness. Thus, we checked for heterologous hybridization, with T7 DNA, of the DNAs of the phages investigated here. As a first step we monitored overall hybridization by dotting different amounts of phage DNA on nitrocellulose filters under denaturing conditions, followed by incubation with radioactive nick-translated T7 DNA. The filters were then washed and autoradiographed. As shown in Fig. 2, the DNAs of phages φ1.2 and K31, as well as that of plasmid pZmC134, used as a control, did not detectably hybridize with T7 DNA, whereas the DNAs of phages A16 and CK235 hybridized to an extent comparable to that of K11. [The DNAs of T3 and of K11 were used for comparison because Korsten et al. (1979), applying the C0t technique of Britten & Kohne (1968), had found...
Fig. 3. Hybridization of various radioactive DNAs with HpaI restriction fragments of bacteriophage T7. A HpaI restriction digest of T7 DNA was divided into six aliquots which were resolved by electrophoresis on an agarose slab and transferred to a nitrocellulose sheet. A strip of it, corresponding to one aliquot, was then incubated with 32P-labelled DNA obtained by nick translation of DNA from (a) T7, (b) T3, (c) K11, (d) A16, (e) CK235 and (f) plasmid pZmC134 (control). Nick translation of all DNAs was under identical conditions. Notice hybridization with bands F-H and G which together cover the whole of gene 1, coding for RNA polymerase (Studier & Rosenberg, 1981). [Due to a small deletion, fragments F and H are fused in our T7 reference type (Studier, 1979).]

these DNAs to be about 35% and 8% homologous to T7, respectively. The somewhat aberrant intensities on row d of Fig. 2 were probably due to the non-homogeneous distribution of the DNA baked on the nitrocellulose.]

Those phage DNAs which to some degree hybridized with T7 DNA were then analysed in more detail against individual T7 HpaI restriction fragments. The aim of this was to estimate the relative degree of base sequence homology with respect to the genome regions covered by these fragments. The relative efficiencies of hybridization of different phage DNAs with specific T7 HpaI fragments (Fig. 3) were evaluated semi-quantitatively by means of scanning profiles (not shown). This confirmed the impression from Fig. 3, that in some instances there were clear differences in the extent of base sequence homology to T7 along the genomes of the phages investigated here and that the genome segments with a maximum or a minimum of DNA homology to corresponding T7 segments are different for each phage. (For instance, compare the relative intensities of bands B and C, or K and L.)
RNA polymerases of capsule-specific phages

DISCUSSION

Of the four capsule-specific coliphages for which we here describe a new phage-coded RNA polymerase activity, CK235 and A16 are certainly related to T7 since they show a low but definite base sequence homology with many fragments of T7 DNA, including in particular the gene 1 fragments (F–H and G). Moreover, phage-encoded 95K to 105K polypeptides appear early in cells infected with CK235 and A16, with the same kinetics of synthesis as the 98-1K T7 RNA polymerase (Studier & Dunn, 1983). It seems obvious that these are responsible for the new phage-coded RNA polymerase activities which we have demonstrated.

In the case of phages φ1.2 and K31, where no base sequence homology with T7 could be detected, and where the electrophoretic patterns of intracellular phage-coded proteins differ more from T7 than those of CK235 or A16 do, the circumstantial evidence for associating the new RNA polymerase activities with the bands of the early 95K proteins is not as straightforward as for CK235 and A16. It is still strong, however, in view of the fact that there are only two or three other protein bands appearing early enough to account possibly for the early RNA polymerase activity, and these bands have such low molecular weights (see Fig. 1) that it is difficult to imagine their being endowed with such a complex property as a highly template-specific RNA polymerase activity. [These early proteins, however, are good candidates for being homologous with the products for gene 0.3 (anti-restriction protein, mol. wt. 13700), or gene 0.7 (protein kinase, mol. wt. 41000) of T7 (see Studier & Dunn, 1983).] The fact should also be considered, that in the 12 other capsule phages where no new RNA polymerase was detected, no early phage-coded polypeptides in the mol. wt. range 80K to 120K were synthesized. The lack of detectable base sequence homology between φ1.2 and K31 on the one hand, and T7 on the other, although indicating that the first two phages are not closely related to the latter, is by no means an argument against a common ancestry of all three phages, since nucleotide sequences may diverge very extensively without corresponding amino acid substitutions (e.g. Yanofsky & Van Cleemput, 1982). Considering the similarities between φ1.2 and K31 with regard to the patterns of intracellular proteins and with regard to the template specificity of their RNA polymerases, there can be no doubt, however, that in spite of their totally different host ranges (no host range mutants of either phage for the host of the other could be found), phages φ1.2 and K31 are related; the same applies to A16 and CK235.

One crucial problem refers to the evolution of different promoter specificities of T7-related RNA polymerases from one common ancestor. In the case of T7 and T3 it has been shown that the differences in template specificity of their RNA polymerases lie in only three or four base changes in the corresponding ‘late’ (i.e. phage RNA polymerase-specific) promoters (McAllister et al., 1983; Basu et al., 1984). A similar observation has been made by Dietz (1985) who showed that the K11-specific RNA polymerase recognizes a promoter which differs by only three bases from the consensus sequence of T7 promoters and by four bases from the consensus sequence of T3 promoters (nevertheless, K11 RNA polymerase will not transcribe T3 and it transcribes T7 DNA very poorly; see Table 1). Thus, one has to postulate a divergent co-evolution of RNA polymerases and promoter sequences in order to explain the differences in template specificities found today among the expanding variety of T7-related phages. In the course of evolution, deleterious mutations in enzyme specificities were obviously not lost by selection so fast as to preclude adaptive promoter mutations, and vice versa. As shown in Fig. 3, fragments H–F and G which together cover the whole of gene 1, coding for RNA polymerase (Studier & Rosenberg, 1981), show a fairly pronounced homology with all heterologous DNAs. This indicates that in spite of different promoter specificities of the polymerases, the corresponding genes have remained relatively conserved. This is in agreement with Hyman et al. (1974) who in a comparison between T3 and T7 observed a high sequence homology in gene 1.

The molecular basis of differences in promoter recognition by phage RNA polymerases is not yet understood. However, since these RNA polymerases are relatively simple (they consist of only one polypeptide), and since intragenic recombination of genes coding for RNA polymerases with different specificities occurs (Hausmann & Tomkiewicz, 1976), there is a promising possibility for investigating that question. (The available range of genetically related capsule phages offers a further opportunity for studies on the evolution of enzymes, namely the
investigation of the different capsid-associated hydrolases which serve the purpose of breaking down the different capsular polysaccharides of the respective hosts.)

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