The Replication of Bacteriophage K DNA in *Staphylococcus aureus*

By P. J. REES*† A N D B. A. F R Y

Department of Microbiology, University of Sheffield, Sheffield S10 2TN, U.K.

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SUMMARY

Three intracellular forms of K phage DNA were identified by sucrose gradient centrifugation and electron microscopy: (i) a form with sedimentation characteristics similar to mature phage DNA; (ii) a fast-sedimenting form (FSF) and (iii) a rapidly sedimenting complex (RSC). The FSF sedimented in a position intermediate between mature DNA and the RSC and comprised one genome length of phage DNA in association with a small mass of non-DNA material. Each RSC was associated with a discrete mass of non-DNA material about three times the size of that in the FSF and formed a large tangled structure containing up to 30 or more phage equivalents of DNA. The RSC was identified as the major replicative form.

INTRODUCTION

Staphylococcal bacteriophage K is a member of serological group D in the classification of Rountree (1949) and Rippon (1956). This group is distinguished from the other groups in that the phages are virulent and resemble the T-even coliphages in their general morphology (Rosenblum & Tyrone, 1964). In a previous paper we described the morphology of phage K and the effect of infection on the DNA metabolism of the host (Rees & Fry, 1981). In *Staphylococcus aureus* (NCTC 9318) phage K had a latent period of 25 min and an eclipse period of 14 min at 37 °C. Infection caused inhibition of host DNA synthesis and degradation of the bacterial DNA. The nucleotides thus released were subsequently used for the synthesis of viral DNA.

Apart from a recent study of phage 52 HJD, which is a temperate phage in serological group B (Ubelaker & Rosenblum, 1977), no information on the replication of staphylococcal phage DNA has been published. The work below describes the intracellular forms of phage K and their partial characterization.

METHODS

General methods. The phage, its propagating strain, *S. aureus* NCTC 9318, the defined media (DM1 and DM2), the measurement of bacterial growth and the production of K phage with DNA labelled with [3H]methyl thymidine are described in Rees & Fry (1981).

Preparation of 32p-labelled K phage. *S. aureus* was grown in 100 ml of a low phosphate medium containing 2 mCi of carrier-free 32p (as orthophosphate). After growth for 25 min (*A*610 = 0.075; 2 x 10^7 bacteria/ml), K phage was added to give a m.o.i. of 0.1. When lysis was complete (3 to 4 h later), the phage particles were isolated and purified in the same way as 3H-labelled phage (Rees & Fry, 1981). This yielded 5 ml of phage suspension containing 1 x 10^10 to 5 x 10^10 p.f.u./ml with about 9 x 10^-3 d/min/p.f.u.

The low phosphate medium was the same as DM1 but with 6.1 g tris instead of the KH2PO4 and brought to pH 7.4 with 2 m-HCl.

*†* Present address: Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, U.K.
Extraction of DNA. Two procedures were used. (a) Method 1. The bacteria were sedimented, washed and resuspended in 1 to 2 ml 0-05 m-Tris, 0-0012 m-EDTA, 0-145 m-NaCl pH 7-4 (TEN) at 2 x 10^9 bacteria/ml and lysed with lysostaphin (10 units/ml) for 1 h at 4 °C. Subsequent treatment was based on the method of Paetkau et al. (1977). After adding Sarkosyl NL97 and self-digested Pronase [final concn. 1% (w/v) and 2 mg/ml respectively], incubation was continued at 40 °C for 4 h. The resultant clear lysate was cooled and extracted with an equal volume of liquid phenol and washed three times with ether. These conditions gave 98% recovery of phage K ^3H-labelled DNA from infected S. aureus. The prolonged incubation with a relatively high concentration of Pronase was required in order to digest protein material associated with replicating phage DNA. If this was not done a major part of the phage DNA became trapped in the denatured protein and other materials which were precipitated when the bacterial preparation was mixed with liquid phenol (compare Miller, 1975). Milder conditions [0-6% (w/v) SDS and 250 µg Pronase/ml for 1 h at 37 °C] did in fact give quantitative recovery (98%) of ^3H-labelled host DNA from uninfected bacteria, but when applied to bacteria infected with labelled K phage, only 35% of the viral ^3H-labelled DNA was recovered in the aqueous phase: the rest was trapped in the interfacial material produced during the phenol extraction. (b) Method 2. The procedure was based on the work of Miller & Kozinski (1970) who used the non-ionic detergent Triton X-100 to lyse spheroplasts of phage-infected bacteria. The infected staphylococci were treated with lysostaphin as described and then Triton X-100 was added (final concn. 1%, w/v) and lysis allowed to occur at 4 °C overnight. Such lysates were used without further treatment.

Preparation of intracellular forms of phage K DNA. S. aureus was grown in DM1 to a cell density of 5 x 10^8 bacteria/ml, harvested and resuspended in the same volume of medium and infected with phage K (m.o.i. = 5). Samples were taken at intervals as required and immediately frozen, using a bath of ethanol and solid CO_2. Alternatively, in pulse-labelling experiments, the bacteria were harvested and resuspended in DM2 and samples were taken and pulse-labelled for 30 s with [^3H]methyl thymidine (18 to 25 Ci/mmol: The Radiochemical Centre, Amersham; 5 µCi/ml final concn.) and then frozen immediately. Samples were subsequently thawed to 4 °C and the bacteria disrupted using the required extraction method (method 1 or 2 above). This basic type of procedure was used throughout these experiments. In some cases the phage used in the initial infection contained ^32P-labelled DNA so that the fate of the parental phage DNA could be followed. Separation of the DNA components in the lysates was by rate zonal centrifugation in neutral sucrose gradients in either (i) the SW41 rotor (Beckman) with 11 ml of a linear 5 to 20% sucrose gradient over a 1 ml cushion of 50% sucrose or (ii) the SW39 rotor (Beckman) with 3.5 ml of a linear 5 to 20% gradient over 1 ml of 50% sucrose. Sucrose gradients were prepared and fractionated as described by Rees & Fry (1981).

Electron microscopy. DNA from sucrose gradients was taken directly for electron microscopy and mounted on grids by the micro version of the diffusion procedure of Lang & Mitani (1970), stained with uranyl acetate and shadowed with Pt-Pd (Rees & Fry, 1981).

RESULTS

Sedimentation analysis of replicating phage K DNA

In phage K-infected bacteria the synthesis of host DNA is inhibited (Rees & Fry, 1981). Consequently, when infected bacteria are pulse-labelled with [^3H]thymidine, only DNA actively replicating at the time of the pulse should become labelled. When DNA was extracted (using prolonged Pronase digestion, method 1) and centrifuged through a sucrose gradient, most of the labelled DNA was in material which sedimented like, or slightly faster than
Replication of staphylococcal phage K DNA

Fig. 1. Sedimentation of intracellular phage DNA after phenol extraction of lysed infected bacteria. At 15 min after infection, a sample of the culture was pulse-labelled with [3H]thymidine, the bacteria subsequently lysed using lysostaphin, Sarkosyl and Pronase (method 1) and extracted with phenol. A sample of the aqueous phase was mixed with marker 32P-labelled DNA (phenol-extracted from mature K particles) and centrifuged through a sucrose gradient (SW41 rotor at 150000 g for 3.5 h at 4 °C). ○, Pulse-labelled 3H-labelled DNA; ●, marker phage 32P-labelled DNA; △, sucrose concn. Other experimental details are given in the text.

marker DNA isolated from mature phage particles (Fig. 1). It was noticeable that the peak of intracellular phage DNA material was much broader than the peak of marker DNA, indicating that it was heterogeneous and probably contained viral DNA in molecules other than simple genome lengths. There was also a smaller peak of 3H-labelled material which passed into the sucrose cushion at the bottom of the gradient. This material was called the fast-sedimenting form (FSF) of the viral DNA. The FSF may have contained complexes in which the protein was hidden from or resistant to the Pronase and consequently a conglomerate of replicating viral DNA molecules remained in a high mol. wt. complex. Alternatively, it could have been formed by aggregation of the lighter component and an artefact produced during the extraction procedure. Essentially the same type of sedimentation profile was obtained irrespective of the time of sampling in the latent period. These and other results indicated that phage K replication did not involve a well-defined temporal sequence of molecular types of viral DNA, as found with phage λ (Kaiser, 1971).

In the previous experiments, the prolonged Pronase digestion was expected to break up any structures involving protein holding K DNA molecules together in a large replicating complex. In order to preserve such complexes a less drastic method was required to lyse the bacteria and make the DNA available for sedimentation analysis. The second extraction method (method 2) avoided proteolytic digestion and used a non-ionic detergent to lyse the bacteria. To allow for the expected more rapid sedimentation of DNA associated with subcellular components, the sucrose gradients were centrifuged at a slower speed. Consequently the sedimentation profiles cannot be compared directly with those obtained when the first extraction procedure was used.

When the fate of the parental phage 32P-labelled DNA was examined in bacteria taken 10 min after infection and lysed by the milder procedure (method 2) the parental DNA was located in three components: two sedimented close together near the top of the gradient
Fig. 2. Sedimentation of intracellular phage DNA after extraction using Triton X-100. Bacteria were infected with K phage containing 32P-labelled DNA. A sample was taken 10 min after infection, and lysed using lysostaphin and Triton X-100 (method 2) and a sample of the lysate sedimented through a sucrose gradient (SW39 rotor at 75000 g for 50 min at 4 °C). Arrows indicate position of peak maxima. RSC, Rapidly sedimenting complex; FSF, fast-sedimenting form (114S); MP, 'mature' phage DNA (73S). ○, 32P-labelled DNA; ○, sucrose concn.

Changes in intracellular forms of phage K DNA during the latent period

In a double-labelling experiment, S. aureus was infected with K phage containing 32P-labelled DNA and then [3H]thymidine was added to the culture 4-5 min after infection in order to label the viral DNA synthesized during the latent period (Fig. 3). Immediately after infection the 32P-labelled parental phage DNA was found in a broad peak of material between fractions 30 and 34 and this presumably contained K phage DNA with the sedimentation characteristics of mature DNA and FSF. It was not clearly resolved into two peaks. In addition, some labelled material sedimented to the bottom of the gradient and there was a small peak around fraction 5 (the RSC position). By 5 min post-infection the RSC peak was more pronounced and the 32P in the upper part of the gradient was partially resolved into two peaks, at fractions 30 and 34. The distribution of the parental viral DNA was similar at 10 min after infection, but by 15 min the peak of FSF was reduced and the mature phage peak was more pronounced. In subsequent samples there was no obvious peak of parental DNA in the FSF position. Material containing tritium was found at 5 min post-infection mostly at
Replication of staphylococcal phage K DNA

Fig. 3. Sedimentation of parental and newly synthesized phage DNA. S. aureus was infected with 32P-labelled K phage and 4.5 min later, [3H]thymidine (2 μCi/ml) was added to the culture. Samples were taken at intervals, lysed and analysed as in Fig. 2. ■, Newly synthesized 3H-labelled DNA; ○, parental K 32P-labelled DNA.

the bottom of the gradient, co-sedimenting with the parental phage DNA: a small 3H peak was also observed at fraction 31, possibly in FSF. By 10 min, the 3H-labelled components sedimented as a broad heterogeneous peak between fractions 1 and 15 and also in a well-defined peak in the FSF position. After the 10th min the amount of newly synthesized DNA detected in the FSF position was much reduced, and the two major 3H peaks seen in samples taken from 15 min onwards co-sedimented with the parental 32P label in the positions of RSC and mature phage DNA. From 15 min onwards, a distinct shoulder of slower
The replicative form of K phage DNA

In the previous experiment, the amount of the marker ($^3$H) for newly synthesized DNA was especially pronounced in the FSF position during the first 10 min of the latent period, but thereafter it became much less significant. This observation, coupled with its sedimentation properties, could be taken to indicate that the FSF was an early replication intermediate between the initial infecting phage DNA and a later more complicated replication form, the sedimenting $^3$H-labelled material, centred on fraction 11, was found on the lighter side of the RSC. The large peak of $^3$H in the RSC position at 25 min presumably is $^3$H-labelled DNA in mature phage particles because these have been shown to be unaffected by the extraction procedure and to sediment to this position in the experimental conditions used here. When mixed with an RSC preparation, intact K phage particles co-sedimented with the RSC material irrespective of the rotor speed and time of centrifugation.

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Fig. 5. The sedimentation of the RSCs of phage K DNA in (a) an extended sucrose gradient and (b) a sucrose gradient containing Triton X-100. Ten min after infection the bacteria were pulse-labelled, lysates prepared (lysozyme and Triton X-100) and samples (0.2 ml) layered on to either (a) a 4.5 ml linear 5 to 50% sucrose gradient or (b) a 3.5 ml linear 5 to 20% gradient over a 1 ml 50% sucrose cushion and in which the sucrose solutions all contained 1% (w/v) Triton X-100. Gradients were centrifuged (SW39 rotor) at 75000 g for 50 min at 4 °C. ○, Pulse-labelled 3H-labelled DNA; ○, sucrose concn.

RSC. Alternatively, the FSF could be formed as a result of degradation of RSCs particularly at early times post-infection when the RSCs may be less stable.

In order to provide further information on the true replicative form of K phage DNA, samples of infected bacteria were taken at appropriate times, given a short pulse of [3H]thymidine and then examined to see whether the newly synthesized (i.e. pulse-labelled) DNA occurred in the FSF or the RSC. Fig. 4 shows the results for bacteria taken at 7 and 15 min after infection. These two times have been chosen because the earlier one was within and the other shortly after the end of the eclipse period. The majority of the pulse-labelled material at 7 min sedimented with the 32P parental phage DNA in the RSC position: only a minor amount was in the FSF position. At 15 min the sedimentation of the pulse-labelled components was similar, but the material in the RSC position was more heterogeneous. The RSC was thus identified as the major replicative form of the phage DNA.

Is the RSC an aggregate?

The possibility was considered that the rapid sedimentation of the RSC form of K phage DNA could have been due to aggregates of replicating DNA structures being formed during or after extraction. Thus, when subjected to centrifugation through a sucrose gradient, they appeared to be a homogeneous species because they sedimented through the gradient until they were held up at or near the boundary with the 50% sucrose cushion. To test this possibility steeper, linear gradients of 5 to 50% sucrose, with no cushion at the bottom, were used for the sedimentation of pulse-labelled RSCs (Fig. 5a). The complexes formed a single, sharp peak near the bottom of the gradient, reflecting homogeneity of the RSCs. If the RSCs
were the consequence of non-specific aggregation it is most unlikely that the aggregates
would be homogeneous in size and therefore sediment in a single sharply defined peak.

Another possibility was that the RSCs were artefacts formed during the centrifugation and
caused by aggregation of protein associated with the replicating DNA, this aggregation
resulting from transfer from the detergent-rich environment of the lysate to a detergent-free
gradient. Accordingly, a lysate of infected bacteria was prepared in the usual way in Triton
X-100 by method 2 and sedimented through a sucrose gradient formed over a 50% sucrose
cushion and in which all the sucrose solutions contained 1% (w/v) Triton X-100. The labelled
DNA material again produced a sharp peak near the bottom of the gradient (Fig. 5 b),
indicating that passage into a detergent-free environment was not a cause of aggregation and
RSC formation.

Electron microscopy of intracellular forms of K phage DNA

In order to gain further information about the structure of the various intracellular forms of
phage K DNA, regions of the sucrose gradients containing peaks of 3H- and/or 32P-labelled
material were sampled and prepared for electron microscopy. The DNA in the peak nearest
to the top of the sucrose gradients co-sedimented with marker DNA extracted from phage
particles and electron microscopy confirmed that this intracellular DNA was linear, with a
contour length similar to that of mature DNA. Linear molecules of identical contour length
were also observed in samples taken from the PSF region, but in this case the DNA was
always associated with a small mass of other material. Since the DNA in the FSF was linear
and had the same contour length as mature phage DNA, it seemed probable that its faster
sedimentation rate was due to this attached material.

In contrast, the RSCs comprised large complex structures with loops and several
free-ended strands of DNA emanating from a dense central region of aggregated material.
The size of these cores was generally about three times larger than those in the FSF structures
and they were of irregular shape and varied in average diam. from 0·2 to 1·0 µm. Although
some short pieces of linear DNA were also seen on the grids, by far the major portion of the
DNA strands were in complex structures. The number of complexes per grid square (of side
250 µm) was low, usually less than one or two, such that fortuitous overlaps of DNA from
different complexes cannot explain their intricate appearance.

A precise estimate of the total length of DNA in an RSC was not possible because of the
tangling of the various molecules. By inspection, together with some calculation, the RSCs
obtained at 10 min post-infection were estimated to each contain about 30 phage equivalents
of DNA. Photographs of these complexes were screened for the unambiguous presence of
DNA strands longer than mature DNA, but no such strands could be found. It was, however,
recognized that the tangled nature of the RSCs could be obscuring the presence of long DNA
molecules. The rapid sedimentation of the complexes was presumably due to the large
amount of DNA contained in them, their compact nature, and the influence of the mass of
associated material.

At the times when intracellular phage particles are being assembled in the infected bacteria
a distinct ‘shoulder’ or trailing peak was found immediately behind the RSC position in
sucrose gradients (e.g. the samples from 15 min onwards in Fig. 3). Electron microscopic
examination of the material in these fractions revealed the presence of linear DNA molecules,
each approx. one genome in length and associated with a structure very similar in size and
shape to a K phage head. It is not clear whether this DNA is in the process of being packaged
into phage heads or whether it comes from immature phage particles which have released
their DNA during the extraction procedure or during the isolation and mounting for electron
microscopy. It can, however, be concluded that Triton X-100 has no effect on mature phage
particles, since when the RSCs prepared from infected bacteria late in the latent period (20
and 25 min) were examined in the electron microscope the specimens contained not only DNA complexes but also phage particles which appeared to be complete and undamaged.

**DISCUSSION**

It is clear that sedimentation through sucrose gradients enables three forms of viral DNA to be identified in K phage-infected bacteria. The slowest sedimenting form is mature phage DNA. The other two forms were designated the fast-sedimenting form (FSF) and the rapidly sedimenting complex (RSC). Both of the latter have small masses of other material attached to the DNA. We have evidence (P. J. Rees & B. A. Fry, unpublished results) that this matter contains lipids derived from the cytoplasmic membrane and proteins. The RSC form of K DNA was not found in preparations extracted using Method 1. On the other hand, some FSF was still present in such extracts (e.g. Fig. 1) and contained attached material. It cannot be ruled out that the processing may have removed at least some protein and/or lipid from these structures. Since some material remained after this treatment it is concluded that the compounds which were left were for some reason relatively resistant to the digestion procedure employed.

The FSF could be an essential feature of the sequence of events after entry of the parental K DNA into the staphylococci, e.g. a precursor of the RSC. Thus, the FSF only occurs in significant amounts in samples derived from bacteria taken during the first half of the latent period (Fig. 3), and its properties suggest it is a lighter or less complicated structure than the RSC. However, the RSC is the main replicative form of the viral DNA and this is formed immediately or soon after entry of the parental DNA. The pulse-labelling results indicated that the major part of the \(^{3}H\) incorporated co-sedimented in the RSC position (Fig. 4). The amount found in the FSF position was much smaller and often there was little or no \(^{3}H\) sedimenting in this region of the gradient (e.g. the profile in Fig. 5 b). At later times in the latent period, after the beginning of phage assembly, the RSCs appeared to be more heterogeneous, possibly because of a wider variation in the number of phage equivalents of DNA in the various complexes. Alternatively, the larger complexes may have been more susceptible to degradation during the processing of the infected bacteria. We suggest that although the RSC is the main replicative form, it is unstable and can be broken down into FSF during the processing of the infected material. The result in Fig. 5 (b) perhaps provides a clue to the instability. In that experiment, Triton X-100 was present in the sucrose gradient as well as in the bacterial lysate and little, if any, FSF was detected. This may indicate that maintenance of the RSCs which involve membrane components is aided by Triton X-100 in an analogous fashion to the stabilization of various membrane enzymes in aqueous environments (Singer, 1974).

The RSCs isolated at 10 min after infection contained about 30 phage equivalents of DNA and this is about half the DNA required for the phage yield (60 p.f.u./bacterium; Rees & Fry, 1981). Thus, there may be a limited number, perhaps only one or two, of these RSCs per infected bacterium. This idea is supported by an examination of the sedimentation profile of the DNA extracted from bacteria when the m.o.i. was greater than 1. A considerable proportion of the infecting viral DNA remained in the 'mature' form and was not converted to FSF or RSC (Fig. 3). However, more data will be required before a precise quantification can be made of the replicating and non-replicating DNA in the bacteria and the way this is influenced by differences in multiplicity of infection.

The general appearance of the K phage RSCs resembles the rapidly sedimenting replicating DNA structures previously described for bacteriophages T4 (Huberman, 1968) and T7 (Serwer, 1974; Paetkau et al., 1977). As in the K phage experiments, these complexes were associated with other material. However, the presence of the latter depended on the extraction procedure employed and its removal did not appear to alter the sedimentation of these
replicating forms of T phage DNA. Center (1972), in his study of the intracellular forms of T7 DNA, also found an early intermediate form of replicating DNA but did not investigate the nature and role of this intermediate.

The precise molecular details of the replication of phage K DNA are not yet known. No evidence was found for the presence of circular forms of the viral DNA in samples taken at any time in the latent period, irrespective of which extraction method was used in their preparation. Replication therefore takes place on a linear template. If the replication origin was internal, then perhaps structures with replication 'eyes' should be seen in K phage complexes. Irrespective of the exact position of the replication origin in the genome, one could expect to find Y-shaped molecules at some stage as the replication fork proceeded along the parental DNA molecule. However, in spite of extensive searches under the electron microscope and examination of photographs, such structures were not found. Inspection of much-enlarged micrographs of RSCs also failed to reveal DNA molecules greater than one genome in length. The available evidence therefore suggests that K phage DNA replicates as linear, non-concatemeric molecules. However, these results have been obtained from pulse-labelled, and therefore actively replicating, DNA molecules, and so cannot rule out the possibility of concatemer formation by recombination after replication, as proposed for the DNA of bacteriophages T4 and T7 (Miller, 1975).

In a study on the replication of another staphylococcal bacteriophage, a lytic variant of the serological group B phage 52, Ubelaker & Rosenblum (1977) found that infection caused repression of host chromosomal replication, but did not result in the degradation of the bacterial DNA. Three forms of intracellular phage DNA were isolated: open circles, closed circles and linear molecules. The extraction procedure employed would have excluded long linear molecules and membrane-bound DNA. Therefore, they were unable to reach definite conclusions as to the mode and site of phage DNA replication. Their results contrast with those found here and in a previous study (Rees & Fry, 1981) of phage K.

In a later paper we will give further details of the properties of the rapidly sedimenting complexes of replicating K DNA and evidence for replication taking place at a membrane site.

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Replication of staphylococcal phage K DNA


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