DNA Replication of Bacteriophage T5. 2. Structure and Properties of the Slow Sedimenting Form of Intracellular T5 DNA

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SUMMARY

The intracellular DNA of bacteriophage T5 contains two major DNA forms distinguishable by sedimentation rate through neutral sucrose gradients. The slow sedimenting form (ssf) which moves at a rate similar to that of DNA extracted from mature T5 virus particles, was prepared free of the fast sedimenting form and capsid-associated DNA by electrophoresis on agarose gels in which it migrated as peak II DNA. The ssf DNA thus obtained was subjected to extensive structural analysis. The number and location of single-stranded regions was studied using the single-strand-specific S1 nuclease and electron microscopy. The frequency and position of the single-stranded regions, which could occur both at internal and terminal locations, was reproducible in the population of ssf DNA molecules as a whole but individual molecules were not identical. After analysis of these results in conjunction with results of studies on the location of single-strand interruptions in ssf DNA it is suggested that single-stranded regions mainly occur at those sites corresponding to the sites of the major nicks in mature T5 virion DNA. When the ssf was isolated by gel electrophoresis and maintained in low ionic strength solutions it was found to be associated with several phage-specific proteins including some which are constituents of the mature virus particle. The presence of these proteins reduced the electrophoretic mobility of the DNA from that expected on the basis of its mol. wt. alone. This property of reduced mobility could be reproduced in vitro using exogenous ssf DNA and crude extracts from phage-infected, but not from uninfected, cells. By contrast, when DNA purified from phage T7 particles was incubated with crude extracts of T5-infected bacteria, the electrophoretic mobility of the T7 DNA was unaltered; thus the effect was due to T5-specific proteins on T5-specific DNA.

The contour lengths of both peak II DNA and ssf were measured by electron microscopy and compared to that of mature T5 virion DNA. Unexpectedly it was found that both were reproducibly about 12% shorter than the mature molecule; therefore it appears unlikely that the ssf is a true intermediate in the pathway of T5 DNA replication. These observations are discussed in relation to the current models of excision from concatamers and encapsulation of mature phage DNA.

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INTRODUCTION

The general pattern of excision of mature length bacteriophage DNA from concatemeric precursors and its encapsulation was considered in the Introduction to the previous paper (Everett & Lunt, 1980). It was stated that one of the unusual properties of T5, and hence its importance in this context, was the existence of a pool of intracellular T5 DNA of similar sedimentation rate to that of mature T5 virion DNA. This intracellular DNA, the slow sedimenting form (ssf), appeared to be a precursor of encapsulated DNA on the basis of pulse-chase experiments and studies using T5 strains defective in DNA replication (Carrington & Lunt, 1973). These observations implied that the excision from concatemers and packaging of T5 DNA may be fundamentally different from that of other similar phages. Therefore we decided that a more detailed study of the structure of the ssf was required. The previous paper described a method for the isolation of the ssf on intracellular T5 DNA in a comparatively pure state (Everett & Lunt, 1979); this paper describes in more detail the structure and properties of the ssf.

METHODS

Bacteriophage T5, bacterial strains and the methods used for growth and assay have been described by Everett & Lunt (1980) and in the references of that paper. The preparation of 32P-labelled T5 virion DNA, of 3H-labelled intracellular T5 DNA and its fractionation on sucrose gradients or by gel electrophoresis were also described in the previous paper.

Preparation of 14C-labelled T5 virion proteins. The method of Zweig & Cummings (1973a) was used. Escherichia coli C3000 was grown in low phosphate medium to a cell density of 5 × 10^8/ml and infected with T5 at a m.o.i. of 10. Twenty min later 14C-leucine was added to give 0.2 μCi/ml and aeration was continued until lysis occurred. The phage was purified by differential centrifuging, banding on a CsCl step gradient and finally by centrifugation through a 15 to 30% sucrose gradient (made in 0.05M-tris-HCl, pH 7.8, 2 mM-MgSO4) at 30000 rev/min for 30 min in the SW50.1 rotor of a Beckman L2-65B centrifuge. The gradient was drop-collected and the phage were located by measuring the radioactivity in the 10 μl portions of the fractions. The purified phage particles were disrupted by three cycles of alternate freezing at −70 °C and thawing at 46 °C. After treatment with pancreatic DNase (200 μg/ml for 2 h at 18 °C), sodium dodecyl sulphate (SDS) was added to give a final concentration of 1% and the preparation was stored at −20 °C.

Sodium dodecyl sulphate gel electrophoresis. The method was based on that of Laemmli (1970). The separating gel contained either 10% (w/v) acrylamide, 0.27% (w/v) BIS, 0.1% SDS in 0.375 M-tris-HCl, pH 8.8, or 15% acrylamide, 0.4% BIS, 0.1% SDS in the same buffer. The stacking gel contained 3% acrylamide, 0.63% BIS, 0.1% SDS in 0.125 M-tris-HCl, pH 8.8. All acrylamide solutions were thoroughly degassed before polymerization by addition of TEMED and ammonium persulphate to final concentrations of 0.05%. Gels were made both as slabs (10 × 10 × 0.2 cm) and also in tubes (8.5 × 0.5 cm internal diam.). Samples for analysis were diluted into loading buffer which contained 0.065 M-tris-HCl, pH 6.8, 1% SDS, 5% mercaptoethanol, 10% glycerol and then heated at 100 °C for 2 min. Bromophenol blue was added to 0.005% before loading samples (20 μl) into slots in the stacking gel. Circulation of buffer (0.025 M-tris-HCl, 0.192 M-glycine, 0.1% SDS, pH 8.3) was continuous throughout electrophoresis which was carried out at 60 V until the sample dye had entered the separating gel, and then at 100 V for about 2.5 h until the tracking dye reached the bottom of the gel. After electrophoresis the gels were washed in 10% trichloroacetic acid for 2 h and then either stained for 12 h with Coomassie brilliant blue (0.0125% in methanol/acetoc acid/glycerol/water, 9:2:1:1, by vol.) followed by destaining with 7%
acetic acid, or prepared for detection of radioactive proteins by the fluorography method of Bonner & Laskey (1974). The following protein mol. wt. standards were used: phosphorylase a (Sigma), 94,000; bovine serum albumin (Armour Pharmaceutical, Eastbourne, Sussex), 68,000; catalase, 60,000; fumarase, 49,000; ovalbumin, 43,000; α-chymotrypsinogen A, 25,700; lysozyme, 14,300; cytochrome c, 11,700 (all from Sigma).

SI nuclease digestion. SI nuclease from Aspergillus oryzae is specific for single-stranded DNA (Ando, 1966). The reaction conditions used were those of Shishido & Ando (1975). SI buffer contained 0.04 M-sodium acetate, pH 5.0, 0.2 M-NaCl, 0.0005 M-ZnSO₄; enzyme incubations are described under Results. One unit of enzyme activity is defined as the amount of enzyme required to convert 50% of 1 μg of heat-denatured ³²P-labelled T5 DNA to an acid-soluble form in 15 min at 36 °C.

Electron microscopy. The method used was that of Inman & Schnöss (1970). The hyperphase mixture contained 0.032 M-Na₂CO₃, 0.005 M-EDTA, 5% formaldehyde, 50% formamide, 0.1 mg/ml cytochrome c (Calbiochem; 2× crystallized and lyophilized) and DNA at 1 μg/ml. Samples (50 μl) of this mixture were spread on to a hypophase of double-distilled water; the film was then picked up on carbon-coated copper grids (Vollenweider et al. 1975), dehydrated in ethanol and rotary shadowed with platinum. Specimens were examined in a Philips EM300 microscope and photographed on 35 mm film. To measure the contour length of DNA molecules, prints of the photographs were made on Kodak projection paper (29.7 × 42 cm), the image traced and the tracing measured by a Quantimet 720 image analysing computer. These methods will be described in more detail in a separate communication. Magnification was calibrated using a diffraction grating replica (Agar Aids, Ltd; 2160 lines/mm). Photographs of this replica were taken at frequent intervals during examination of the sample grids. The variation in diffraction grating measurement at the standard magnification used for photographing DNA molecules for contour length determinations was less than 2%.

Radioactive materials and their measurement. The Radiochemical centre, Amersham, Bucks. supplied carrier-free ³²P-phosphate, 6-³H-thymidine (24 Ci/mmol) and U-¹⁴C-L-leucine (348 mCi/mmol). The methods for estimating radioactivity were described by Everett & Lunt (1980).

Reagents and enzymes. Acrylamide, NN'-methylenebisacrylamide (BIS), sodium dodecyl sulphate (SDS) and NNNN’N'-tetramethylenediamine (TEMED) were from BDH (Poole, Dorset). Tris and Triton X-100 were from Sigma (London) Chemical Co. DNase I (EC 3.1.4.5) grade I was from Seravac Laboratories, (Maidenhead, Berks). SI nuclease and egg white lysozyme (EC 3.2.1.17) grade I, were from Sigma. Pronase, grade B, was from BDH.

Isolation of peak II DNA. The procedures described by Everett & Lunt (1980) were followed. After isolation by isopycnic centrifugation on KI gradients, preparations of peak II DNA were dialysed against TES buffer, pH 8, and then treated with self-digested Pronase (200 μg/ml) for 30 min at 37 °C. In control experiments, omission of the Pronase step occasionally resulted in some DNA degradation. The sedimentation properties of peak II DNA were unaffected by the Pronase treatment; on neutral sucrose gradients it sedimented characteristically very slightly slower than T5 virion DNA, and with a slight trailing edge.

RESULTS

Single-stranded regions in peak II DNA: action of SI nuclease

Peak II DNA obtained as already described was dialysed against SI buffer, pH 5.0. Samples (0.1 ml) which contained about 1 μg of DNA were incubated with 8 units of SI nuclease at 37 °C. At different times (10, 20 and 30 min) the reaction in one sample was
stopped by adding 10 μl of a solution of 1 M-tris-base, 0.1 M-EDTA. The products in each sample were then analysed by centrifugation through gradients of sucrose in 0.05 M-tris-HCl, 0.001 M-EDTA, 1 M-NaCl. 32P-labelled virion DNA was included as a sedimentation marker.

The results (Fig. 1) showed that peak II DNA was essentially unaffected by the incubation conditions, there being no apparent increase in the lower mol. wt. material frequently present in the preparations. By contrast, in the presence of S1 nuclease the peak II DNA was cut into several fragments, the reaction appearing to reach completion in 20 min in the conditions described.

Shishido & Ando (1975) have shown that under appropriate conditions of ionic strength
and temperature, S1 nuclease can cleave T5 virion DNA at sites opposite to the nicks. They suggested that transitory melting near to a nick was necessary to expose single-stranded material which would then act as a substrate for the enzyme. Mature virion DNA was not cut by the enzyme at higher ionic strengths (0.2 M-NaCl) when base-pairing would be stabilized. For our experiments with peak II DNA, the S1 buffer contained 0.2 M-NaCl, and using these conditions we confirmed that T5 DNA was not significantly affected by S1 nuclease during a 20 min incubation period at the same enzyme DNA ratio as that used during peak II digestion (Fig. 2).

We therefore concluded that peak II DNA contains single-stranded regions. These regions appear to be located at specific sites since the products of S1 nuclease digestion gave gradient profiles which were reproducible over several independent experiments. A small portion (about 6%) of the peak II DNA remained uncleaved by S1 nuclease (Fig. 1d). This may represent a fraction of peak II DNA that does not contain internal single-stranded regions; therefore it is clear that the distribution of these regions varies between individual molecules of the same preparation.

Pattern of single-strand interruptions in peak II DNA

Separation of the single-strand fragments produced by alkali denaturation of mature T5 virion DNA is conveniently achieved by electrophoresis through 0.6% agarose gels (Hayward & Smith, 1972a). This method gives considerably better resolution than alkaline
Table I. Relative abundance figures for single-strand fragments produced on alkali
denaturation of mature T5 virion DNA and peak II DNA

<table>
<thead>
<tr>
<th>Band</th>
<th>Peak II DNA</th>
<th>Mature T5 virion DNA*</th>
<th>Mature T5 virion DNA†</th>
<th>Peak II RA/ T5 DNA RA‡</th>
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<tr>
<td>I</td>
<td>0.39</td>
<td>0.56</td>
<td>0.52</td>
<td>0.69</td>
</tr>
<tr>
<td>Ia</td>
<td>0.55</td>
<td>0.31</td>
<td>—</td>
<td>1.77</td>
</tr>
<tr>
<td>II</td>
<td>0.53</td>
<td>0.54</td>
<td>0.40†</td>
<td>0.98</td>
</tr>
<tr>
<td>III</td>
<td>0.89</td>
<td>1.17</td>
<td>0.74</td>
<td>0.76</td>
</tr>
<tr>
<td>IV</td>
<td>0.77</td>
<td>0.80</td>
<td>0.64</td>
<td>0.76</td>
</tr>
</tbody>
</table>

* Calculated from experiments performed in the present work.
† Band Ia does not appear in mature T5 virion DNA, but the figure given is calculated by taking the total number of counts in the region of the gel which corresponds to band Ia of peak II DNA.
‡ RA, Relative abundance.
§ The average of the values given for the $1.45 \times 10^8$ and $1.39 \times 10^6$ mol. wt. fragments.

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sucrose gradients, and was therefore adopted to study the number and location of single-strand interruptions in peak II DNA.

Uniformly labelled peak II DNA was prepared by the addition of $^{32}$P-phosphate to give $20 \mu$Ci/ml to a culture of E. coli C3000 in low phosphate medium 1 h before infection with phage T5. A 2 ml sample of the culture was removed at 32 min p.i., the bacteria were lysed by the Brij-deoxycholate method and peak II isolated by electrophoresis through two parallel gels. One gel was used to locate the position of peak II, and the corresponding slices from the other gel were retained. The $^{32}$P-labelled DNA in these slices was denatured in situ by incubation of the slices at 4 °C for 6 h in an equal volume of 0.15 M-NaOH. The alkaline extracts were then layered directly on top of 0.6% agarose gels for electrophoresis. Gel slices which contained $^{32}$P-labelled mature virion DNA were prepared and treated similarly, the two sets of samples being run in parallel. After electrophoresis, the gels were sliced and the positions of the single-stranded DNA fragments located by measurement of the radioactivity in the gel slices.

The distribution of the single-strand fragments of peak II DNA was similar to that of T5 virion DNA, but there were some distinct and reproducible differences (Fig. 3). In particular there was a greater proportion of radioactivity in the regions between bands I and II (designated band Ia) in the peak II DNA, while the proportion in what appeared to correspond to the virion DNA intact strand was less than that in virion DNA. In spite of the generally greater heterogeneity of the peak II DNA, the larger major fragments (bands II, III and IV) were well marked. At present we assume that these bands correspond to the larger major bands of the interrupted strand of T5 virion DNA.

Using our data it is possible to provide a rough quantitative comparison between peak II DNA and virion DNA in the form of 'relative abundance' values for each fragment (Table 1). Relative abundance is defined as the ratio of the amount of radioactivity which is actually found in a fragment to the amount which would theoretically be there if the fragment represented a fixed molar fraction of a whole DNA molecule (Hayward & Smith, 1972b). The inherent inaccuracies in this approach are obvious, particularly in view of recent evidence that populations of mature virion DNA molecules display considerably greater heterogeneity than was originally detected (Scheible et al. 1977; Rhoades, 1977). Therefore, although no concrete conclusions can be drawn from the individual figures, the approach is useful in providing a numerical basis for a comparison of the two forms of DNA.

Since peak II DNA not only has internal single-strand regions, but also yields a single-strand fragment pattern similar to that of virion DNA, it follows that some or all of these single-strand regions must be present at positions which correspond to the sites of the nicks in the virion DNA.
Fig. 4. Change in specific activity of the single-strand fragments of peak II DNA during a pulse-chase experiment. Experimental details are in the text: (a) 20 s pulse; (b) 20 s chase; (c) 20 min 20 s chase; (d) 4 min 35 s chase; (e) 10 min chase. Electrophoresis was at 30 V for 14 h. O—O, \(^{3}H\) pulse label; ●—●, \(^{32}P\) uniform label.

'Repair replication' of peak II DNA in vivo

The comparison of the patterns of single-strand fragments of peak II and virion DNA molecules and the fact that peak II DNA contains single-strand regions suggested the possibility that these single-strand regions might be subject to repair replication in infected bacteria. The following experiment was done to explore this possibility.

A T$_5$ phage-infected culture was uniformly labelled with \(^{32}P\)-phosphate as already described and at 32 min p.i. was given a 'pulse' of \(^{3}H\)-thymidine, added to give 10 µCi/ml.
The pulse was terminated after 25 s by the addition of a several thousand-fold excess of non-radioactive thymidine. Samples (2 ml) were removed for Brij–deoxycholate lysis immediately before the start of the ‘chase’ and at intervals thereafter. Peak II DNA was isolated, denatured with alkali and the single-strand fragments resolved by electrophoresis through 0.6 % agarose gels as before (Fig. 4).

The most striking feature of the results is that the ‘intact strand’ of peak II DNA is labelled more slowly than fragments of lower mol. wt. at early times in the labelling period. This result can be explained if the initial incorporation of radioactivity into peak II DNA was predominantly due to repair replication in the ‘interrupted strand’; this is indeed suggested by the prominence of ³H labelling among the fragments which correspond in size to those of the major fragments of the interrupted strand of virion DNA. The results cannot be explained on the basis of semi-conservative replication. The simplest explanation appears to be that repair replication of existing single-stranded regions occurs and that at least some of these regions are located at sites equivalent to the major nick sites of virion DNA. However, the situation must be more complex than this, since a rapidly labelled component (band Ia) of mol. wt. $20 \times 10^6$ to $24 \times 10^6$ appears and could represent a portion of the ‘uninterrupted strand’, although it could also arise by fusion of two or more fragments from the ‘interrupted strand’.

The changes in the specific activities of the single-strand components of peak II DNA reveal a complex situation of fragment turnover during a short pulse of ³H-thymidine. However, after a 10 min chase period the distribution of the pulse label closely follows that of the uniform label.

Reconstruction of ‘peak II-like’ material in vitro

If the sedimentation and electrophoresis properties of the peak II are those of a dissociable DNA–protein complex, the origin of the protein and the specificity of its binding are of interest. The following experiments were designed to discover whether the peak II proteins were of phage or host origin, using electrophoretic mobility as an assay of protein binding to the DNA.
Fig. 6. Incubation of T7 virion DNA with T5-infected cell lysates. (a) Control, no incubation; (b) 20 min incubation at 4 °C. Electrophoresis was at 50 V for 15.5 h. ○---○, 3H T7 DNA; ●---●, 32P T5 DNA.

Fig. 7. Incubation of mature T5 virion DNA with T5-infected cell lysates. A sample of T5 virion DNA was incubated with a T5-infected cell lysate at 4 °C for 20 min and immediately before electrophoresis further T5 virion DNA was added. ●—●, 32P radioactivity.

3H-thymidine-labelled peak II DNA was isolated by gel electrophoresis and KI gradient banding as already described. Samples (50 μl) of this preparation were then mixed with 0.2 ml portions of Brij–deoxycholate lysates made from either uninfected bacteria, or from bacteria harvested 30 min p.i. with T5. The mixtures were kept at 4 °C for 20 min and then analysed on agarose gels. Mature T5 virion DNA labelled with 32P was included in each gel as an internal marker.

The results (Fig. 5) showed that incubation with the infected, but not the uninfected cell lysate resulted in the 3H DNA moving with the mobility characteristic of protein-associated peak II DNA. We concluded that the reduction in electrophoretic mobility was caused by association with phage-coded, but not host-coded protein. Furthermore it appears that the
Identification of the proteins in peak II

The phage-specific proteins associated with peak II DNA were detected and identified by polyacrylamide gel electrophoresis in the following way. A culture of *E. coli* C3000 was infected with T5 and 15 min later $^{14}$C-leucine was added to give 3 μCi/ml. A 5 ml sample of the culture was removed 30 min p.i., lysed, and 0.2 ml portions of the lysate subjected to electrophoresis on parallel agarose gels. One gel was used to locate the position of peak II. The corresponding slices from the remaining gels were heated in a boiling water bath and then subjected to electrophoresis using 10% acrylamide gels. Polypeptides 1 to 13: T5 virion proteins. Polypeptides (a), (b) and (c), additional proteins associated with peak II. Electrophoresis through 10% SDS-polyacrylamide gels was as described in the text. The gel was impregnated with PPO and dried before exposure on Kodak RP Royal X-Omat medical X-ray film at −70°C for 6 days.

Fig. 9. Polyacrylamide gel electrophoresis of peak II-associated and mature T5 virion proteins using 15% acrylamide gels. Labelling as in Fig. 8. Polypeptide (d), peak II-associated protein not present in the mature virion. Autoradiography as in Fig. 8.

effect of T5 protein is specific for T5 DNA. For example, incubation of phage T7 DNA with a lysate of T5-infected bacteria caused no apparent change in the electrophoretic mobility of the T7 DNA (Fig. 6). By contrast, the mobility of T5 virion DNA was reduced to that of peak II after a 20 min incubation with T5-infected cell extracts, although if the DNA was added to the cell extract immediately before electrophoresis, the change in mobility was not observed (Fig. 7).
with SDS (final concentration 1%), diluted with an equal volume of loading buffer and then a 50 µl sample of the mixture was subjected to electrophoresis through a 10% polyacrylamide slab gel; 14C-leucine proteins from T5 virions were run on the same gel. After electrophoresis the protein bands were detected by fluorography (Fig. 8).

The results showed that several phage-specific proteins, including virion proteins, were present in these extracts of peak II. The virion proteins identified are polypeptides 5 and 6 (tail proteins; Zweig & Cummings, 1973a) and polypeptides 7, 8 and 10 (head proteins). The major head protein, band 8, was present in very large amounts in peak II material. Polypeptides associated with peak II DNA, but not part of the virion include those labelled (a), (b) and (c) in Fig. 8. Polypeptide (b) is potentially interesting since it is of similar electrophoretic mobility to the precursor which undergoes proteolysis to form the major head protein (Zweig & Cummings, 1973b). Band (c) was also present in large amounts in peak II material. It is of similar mol. wt. to polypeptide E10, suggested to be a DNA binding protein, similar to the gene 32 product of phage T4 (Chinnadurai & McCorquodale, 1974a).

Polypeptides of mol. wt. lower than about 18000 were not resolved in these experiments since they migrated at the dye front. Therefore the experiment was repeated using 15% polyacrylamide gels. The result, shown in Fig. 9, indicated the presence of two further proteins associated with peak II DNA: one, polypeptide (d), is not found in mature virions, while the other, polypeptide 13, corresponds to a protein which Zweig & Cummings (1973a) were unable to assign to either the heads or tails of mature phage particles.

To confirm that the polypeptides (b) and (c) have similar electrophoretic mobilities to the major head protein precursor and polypeptide E10 respectively, the peak II-associated proteins were directly compared with T5-specific proteins synthesized at different times during infection. T5-specific proteins were labelled with 14C-leucine by procedures similar to those of Zweig & Cummings (1973a). A culture of bacteria in minimal medium was grown to 5 × 10^8 cells/ml and infected with T5. At intervals after infection, 2 ml samples were removed from the bulk culture and incubated at 37 °C with 0.25 µCi 14C-leucine for 4 min before being poured on to 1 ml of frozen 0.1 M-NaCl. The bacteria were collected, resuspended in loading buffer and then heated at 100 °C for 2 min. The proteins were separated by electrophoresis through 10% polyacrylamide slab gels; extracts of uninfected bacteria, T5 virion proteins and peak II proteins were analysed in parallel.

The electrophoretic patterns of the proteins synthesized in this experiment resembled those demonstrated for T5 infections by McCorquodale & Buchanan (1968) and Zweig & Cummings (1973a). Since infection was not synchronous under these conditions, host proteins were still being synthesized between 3 and 7 min p.i. However, polypeptide PE-1 (gene A1 product; Beckman et al. 1971) was easily distinguishable. Later samples contained predominantly 'early' proteins (11 to 15 min) or 'late' proteins (20 min onwards). Protein E10 is easily identifiable since it is produced in large amounts and also because, although it is an 'early' protein, it is still made in appreciable quantities late in infection (Chinnadurai & McCorquodale, 1974a). It is clear that polypeptide E10 has the same electrophoretic mobility as polypeptide (c) of the peak II DNA-associated protein.

The precursor to the major head protein is easily seen as a prominent late protein band immediately below that of the major tail protein, band 6 (Fig. 10; Zweig & Cummings, 1973b). The intensity of band (b), the peak II component with mobility identical to that of the major head protein precursor, was sometimes poor in these preparations of peak II (Fig. 10). This is probably caused by the 15 min delay between the addition of 14C-leucine and the time at which the labelled culture sample was removed, during which cleavage of the 3H-precursor would be expected to occur. If the labelling period with 14C-leucine was reduced to 4 min before preparation of peak II during the period of 'late' protein synthesis there was much enhanced intensity of polypeptide (b); in these instances the major head protein and its precursor were found in approximately equal amounts.
Table 2 lists the protein components found in T5 virions and the peak II material. Evidence for the T5-specific origin of the peak II proteins has been given so the question which now arises is whether they have any functional significance in vivo, or whether their presence is merely a post lysis artefact. For some of the peak II proteins a functional role appears plausible For example, if E10 is indeed a DNA binding protein analogous to the T4 gene 32 product, then it would be associated with single-strand regions in vivo and would probably be required for DNA repair synthesis. The electrophoretic mobility of band (a) is similar to that of protein E2, mol. wt. 96000 of Chinnadurai & McCorquodale (1974b) and which they tentatively identified as T5 DNA polymerase. We have not pursued this interesting possibility, but if it is correct, the presence of the polymerase is consistent with the repair processes detected in the peak II DNA.

It is more difficult to envisage a role for the structural proteins found associated with peak II. Pulse-chase experiments (Everett, 1978) produced labelling patterns consistent with the possibility that the peak II-associated major head protein could be a precursor of that found in phage heads and mature virions. However, the labelling patterns could have
Table 2 Summary of T5 virion and peak II binding proteins as identified by polyacrylamide gel electrophoresis

<table>
<thead>
<tr>
<th>Polypeptide*</th>
<th>Mol. wt.</th>
<th>Mature virion</th>
<th>Peak II</th>
<th>Role 1†</th>
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<tr>
<td>1</td>
<td>140,000</td>
<td>+</td>
<td>−</td>
<td>Tail</td>
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<td>2</td>
<td>128,000</td>
<td>+</td>
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<td>Tail</td>
</tr>
<tr>
<td>3</td>
<td>125,000</td>
<td>+</td>
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<td>(a)</td>
<td>95,500</td>
<td>−</td>
<td>+</td>
<td>E1/E2</td>
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<tr>
<td>4</td>
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<td>6†</td>
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</tr>
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<td>+</td>
<td>NJII</td>
</tr>
<tr>
<td>13</td>
<td>15,500</td>
<td>+</td>
<td>+</td>
<td>?§</td>
</tr>
</tbody>
</table>

* Numbering of virion polypeptides and their mol. wt. according to Zweig & Cummings (1973b).
† Identification of head or tail proteins according to Zweig & Cummings (1973a). Numbering of other polypeptides according to Chinnadurai & McCorquodale (1973).
‡ Position of polypeptide 8(a) was masked by polypeptide 8 in all experiments.
§ Not identified as head or tail protein by Zweig & Cummings (1973a).
|| Not identifiable compared with the results of Chinnadurai & McCorquodale (1973).

resulted from events in which the protein did not remain associated with DNA throughout the period of apparent precursor to product conversion.

Therefore it is possible that some of the proteins are DNA-associated in vivo and could be involved both in DNA synthesis and in events concerned with capsid assembly. However, if the latter is correct, it is difficult to envisage a role for the tail proteins associated with peak II since it has been shown that the pathways of head and tail assembly are independent of one another (Zweig & Cummings, 1973a).

Electron microscope visualization of peak II DNA

Samples of peak II DNA were prepared for electron microscopy by a method based on the formamide technique of Inman & Schnöss (1970). All peak II DNA molecules were linear; no loops or branch points were detected. However, most molecules (about 70%) contained single-stranded regions either at the ends, or internally, or both. Some of these regions were surprisingly long, of the order of about 1 μm (Fig. 11). The detection of single-stranded regions is consistent with the results of S1 nuclease digestion (Fig. 1) and also with the observation that 86% of a preparation of the ssf bound to nitrocellulose (Carrington & Lunt, 1973).

A number of apparently intact peak II DNA molecules were photographed and their contour lengths measured using the Quantimet image analysing computer. All preparations of peak II DNA contained a variable number of small fragments; these were probably shear products of larger molecules which would be especially fragile because of the single-stranded regions. Molecules which were obviously fragments were not measured.

The contour length of peak II DNA was compared to that of mature T5 virion DNA prepared for electron microscopy by exactly the same method. The results (Table 3) showed that the contour length of peak II DNA varied over a relatively wider range and was also
Fig. 11. Electron micrograph of a typical peak II DNA molecule. Arrows indicate single-stranded regions.
**Table 3. Contour length of peak II DNA, the slow sedimenting form and mature T5 virion DNA**

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Mean length (μm) ± s.d.</th>
<th>No. of molecules measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature T5 DNA</td>
<td>42.13 ± 0.81</td>
<td>25</td>
</tr>
<tr>
<td>Peak II DNA</td>
<td>37.35 ± 2.22</td>
<td>18</td>
</tr>
<tr>
<td>Slow sedimenting form</td>
<td>36.80 ± 2.74</td>
<td>9</td>
</tr>
</tbody>
</table>

* The contour length of mature T5 virion DNA prepared by the formamide method is significantly greater than that prepared by the aqueous method. Our results gave a contour length of 36.40 ± 0.89 μm for mature T5 DNA prepared by the aqueous method of Davis et al. (1970), compared with published values of 36.2 ± 0.8 μm (Lang, 1970) and 37.4 ± 0.9 μm (Lang et al. 1976).

approx. 12% shorter than that of mature T5 virion DNA. To check that the preparation of peak II DNA did not result in enrichment of shorter than unit length molecules from the ssf, the contour length of ssf DNA was also measured. All experimental conditions were the same as for peak II DNA measurements, except that the ssf was isolated from a Brij-deoxycholate lysate of infected bacteria (Carrington & Lunt, 1973; Everett & Lunt, 1980). The contour length of the ssf was similar to that of peak II DNA, and approx. 12% shorter than that of mature T5 virion DNA (Table 3).

**DISCUSSION**

From their similar sedimentation rates and other properties, we consider that peak II DNA and the slow sedimenting form of intracellular T5 DNA are equivalent (Carrington & Lunt, 1973; Everett & Lunt, 1980). It was previously postulated that the slow sedimenting form was an intermediate in the conversion of concatemers to mature virion DNA (Carrington & Lunt, 1973). The experiments described here have investigated this hypothesis in detail.

The structure of the ssf has been shown to be complex and not identical in all molecules of a population. The presence of single-stranded regions originally suggested by the nitrocellulose binding of the ssf was confirmed by electron microscopy and susceptibility to S1 nuclease. Although sucrose gradient analysis of the S1 nuclease products of peak II DNA gave rather poor resolution the results were reproducible (Fig. 1). Comparison of these results with those of agarose gel analysis of the single-strand components of peak II DNA (Fig. 3) allows a general but simplified picture to be drawn of the structure of peak II DNA. Since the pattern of single-stranded interruptions in peak II DNA is similar to that of mature T5 virion DNA and most peak II DNA molecules are cut at least once by S1 nuclease, it follows that peak II DNA contains interruptions (which may be either simple nicks or single-stranded regions) at locations equivalent to the sites of the major nicks in mature T5 virion DNA. This hypothesis is supported by the correlation between the sizes of the S1 nuclease products of peak II DNA (Fig. 1c) and those of its single stranded components (Fig. 3; Table 1).

Our most unexpected finding was that the contour lengths of peak II DNA and the slow sedimenting form were on average about 10 to 12% shorter than that of mature T5 virion DNA. Although internal DNA molecular length standards were not included in these experiments, all preparations were processed identically and the magnification was regularly re-calibrated. The intrinsic errors of measurement (less than 2%) using the Quantimet image analyser were considerably less than the length difference between peak II and T5 virion DNA. The total length of single-stranded regions in peak II DNA was on average less than 2% of the whole, and therefore insufficient to produce an apparent length difference of the magnitude observed. The fractionally slower sedimentation rate of peak II DNA
compared to that of T5 virion DNA (Everett & Lunt, 1980) gives a mol. wt. of approx. $65 \times 10^8$ for peak II DNA (using the equation of Burgi & Hershey, 1963) again about 12% less than that of the mature T5 virion DNA (about $74 \times 10^8$; McCorquodale, 1975). This calculation assumes a negligible effect of the single-stranded regions on the sedimentation rate of peak II DNA. The mol. wt. difference between peak II and T5 virion DNA would not be detectable by agarose gel electrophoresis under our conditions.

The origin and role of the slow sedimenting form remains unclear. Since vigorous pipetting was required to reduce concatemers to virion-sized DNA, and concatemeric DNA exhibited different behaviour to the ssf during pulse-chase experiments (Carrington & Lunt, 1973), shear degradation of larger precursors appears an unlikely source of the ssf. It is also unlikely that breakdown of partially formed head structures releases the ssf during lysis since our experiments (Fig. 4) indicate that peak II DNA is undergoing repair, an improbable activity for an encapsulated component. Enzymic breakdown of concatemers or virion-sized DNA after cell lysis would yield a spread of DNA sizes, not the relatively narrow range observed (Everett & Lunt, 1980; Table 3). In addition, T5 virion DNA showed no sign of degradation after mixing with lysates prior to centrifugation or electrophoresis.

We believe the ssf is a genuine intracellular intermediate, although its length precludes it from being a direct precursor of virion DNA. Metabolically it behaves as a product of concatemers, and an amber mutant which cannot make phage heads cannot make the ssf (Carrington & Lunt, 1973). Hence it could arise as a result of faulty excisions during abortive packaging, or as a by-product of normal packaging.

In other phage systems, concatemer cutting occurs concomitantly by processive encapsulation of contiguous ‘headfuls’ of DNA (Murialdo & Becker, 1978). The mechanisms involved are not known: they may be different for λ whose unique DNA sequences are generated by cutting at specific cos sites, and for phages whose virion DNA is both unique and terminally redundant. Thomas (1967) proposed such redundant genomes could be produced by the nicking of concatemers at the opposite ends of each redundant region, polymerase invasion and strand displacement then separating the resultant duplex segments. Concatemeric T7 DNA contains single-strand regions at approximately genome-length intervals (Schlegel & Thomas, 1972), suggesting they may have a role in maturation, perhaps by a variation of Thomas’ mechanism. Concatemeric T5 DNA contains single-stranded regions, some of which occur at ‘mature-length’ intervals (Everett, 1978). However, each redundant region of T5 DNA is about 30 times longer than that of T7 DNA, so extensive repair type synthesis would need to accompany T5 virion formation by Thomas’ model. Indeed, the main problem in envisaging the maturation of T5 virion DNA lies in its possession of both a unique sequence and a very large terminal redundancy. This combination may require modification of the sequential ‘headful’ mechanism and perhaps some wastage of concatemeric DNA.

At present we do not know whether the ssf DNA consists of random or specific sequences, or whether its peculiarities, including association with T5 proteins at low ionic strength, are partly responsible for its formation, or whether these features result from it being a transient intermediate of concatemer processing. The ssf does not accumulate (Carrington & Lunt, 1973): presumably it could be lost by recombination, by degradation or some may appear in aberrant heads or particles. Examples of the latter could include the T5 ‘del’ particles described by Saigo (1976) in which the ‘right-hand’ terminally redundant region of virion DNA is missing, or the defective particles described by Labedan & Legault-Demare (1974). Related to these observations may be the finding that shorter than ‘mature length’ T7 DNA molecules accumulate in certain bacterial mutants where host defects block virus assembly (Yamada et al. 1978).
Thus our results describe yet another unusual feature of T5 replication. It seems evident that the ssf is not a productive intermediate in virion formation and that clearly any explanation of T5 concatemer processing must account for its formation.

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