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Locations of Bacteriophage T4 Origins of Replication

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Partially replicated bacteriophage T4 DNA containing cytosine was isolated from cells 6.5 and 7 min after infection and cleaved with restriction endonuclease *Bgl*II or *Bam*HI. Positions of replication eyes relative to the cleavage sites were observed by electron microscopy. Four groups of eyes were found. They are consistent with replication from origins located at map positions 34, 60, 73, and 86 kilobases. In individual molecules that contained two or three eyes, the distribution of the eyes agreed with the initiation of replication at more than one of these four assigned origins and possibly at two additional origins located near 15 and 110 kilobases, which were reported by P. M. Macdonald, R. M. Seaby, W. Brown, and G. Mosig (p. 111–116, *in* D. Schlessinger, ed., *Microbiology—1983*, 1983) and M. E. Halpern, T. Mattson, and A. W. Kozinski (Proc. Natl. Acad. Sci. U.S.A. 76:6137–6141, 1979).

Bacteriophage T4 is believed to utilize two modes for the initiation of DNA replication (7, 14). The first, which triggers the onset of DNA synthesis at 5 to 7 min after infection, involves the creation of replication forks at site-specific origins of replication. The second, which follows thereafter, involves the generation of replication forks from recombinational intermediates and thus is not site specific.

Attempts to identify the T4 site-specific origins of replication have resulted in discrepancies in both the number and locations of the origins found by different investigators. In the earliest studies, Mosig (13, 15) examined genetic marker distribution among progeny from coinfections involving normal and incomplete (two-thirds-length) chromosomes. A gradient of relative contribution of markers from the incomplete chromosomes suggested an origin near gene 43 (ca. 30 kilobases [kb] on the 166-kb genetic map [8]). Support for this assignment was provided by Marsh et al. (10), who used transformation to measure marker frequencies in parental and daughter strands of partially replicated incomplete chromosomes. More recently, hybridization of nascent DNA to cloned segments of T4 DNA has indicated a strong origin in the region of 76 to 80 kb on the map and a weaker origin at about 110 to 119 kb (4). In contrast, Macdonald et al. (9) hybridized nascent DNA to blots of total T4 restriction fragments and detected a major origin at 15 to 16 kb; however, a second origin at 111 to 114 kb was detected when cells were exposed to heavy irradiation from ^{32}P before infection. King and Huang (6) also hybridized nascent DNA to blots of total T4 restriction fragments but found origins at 29 to 34 and 62 to 64 kb on the genetic map.

Taking a different approach toward identifying the T4 origins of replication, we used electron microscopy to determine the positions of replication eyes relative to *Bgl*II and *Bam*HI cleavage sites in partially replicated molecules isolated from infected cells at the onset of phage DNA replication. The infecting phage contained deoxycytidine (dC)-DNA instead of the normal glucosylated 5-hydroxymethyldeoxycytidine-DNA to permit cleavage with the restriction endonucleases. Midpoints of the eyes located origins at map positions 60, 73, and 86 kb and provided support for an origin near 34 kb. These multiple assignments appear to resolve many of the discrepancies observed by others.

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MATERIALS AND METHODS

Phage and bacterial strains. Bacteriophage T4 *56*(*am*E51,dCTPase$^-$) *denA*(*nd*28,endoII$^-$) *denB*(*r*IIH23B, endoIV$^-$) *alc*8 (11, 16) and its restriction/modification-negative *Escherichia coli* host strains K803 (*supE hsdS rgl gal met*) and B834 (*sup*$^+$ *rm*$_B$ *gal met*) (18) were provided by L. Snyder. In K803, the T4 *alc* quadruple mutant produces 5-hydroxymethyldeoxycytidine-DNA due to the suppression of the amber mutation in the dCTPase gene; in B834, dC is substituted for about 95% of the 5-hydroxymethyldeoxycytidine (16). A low-thymine-requiring spontaneous derivative of *E. coli* K803 was obtained by trimethoprim selection for *thy* mutants followed by selection for growth on medium containing thymine (1 μg/ml) for *deo* mutants (12).

Isolation of partially replicated T4 DNA. *E. coli* K803 *thy deo* was grown at 37°C in 50 ml of Tris hydrochloride-buffered (pH 7.8) minimal medium, which contained (per liter) 0.54 g of NaCl, 0.3 g of KCl, 1.1 g of NH_4Cl, 15 mg of $CaCl_2 \cdot 2H_2O$, 203 mg of $MgCl_2 \cdot 6H_2O$, 0.2 mg of $FeCl_3 \cdot 6H_2O$, 87 mg of KH_2PO_4, 22.7 mg of Na_2SO_4, 12.1 g of Tris, and 2 g of glucose added after autoclaving. This medium was supplemented with 1 mg of Casamino Acids, 50 μg of methionine, and 2 μg of thymine per ml. When the medium contained 10^8 cells per ml the bacteria were pelleted by centrifugation, washed with 100 ml of ice-cold Tris hydrochloride-buffered minimal medium supplemented with 1 mg of Casamino Acids, 50 μg of methionine, 200 μg of uracil, 10 μg of 5-fluorodeoxyuridine, and 400 μg of 5-bromodeoxyuridine per ml. The bacteria were grown for another 1.5 generations, pelleted, and suspended in 2 ml of Tris hydrochloride-buffered minimal medium supplemented with 1 mg of Casamino acids, 50 μg of methionine, 200 μg of uracil, 100 μg of tryptophan, 50 μg of 5-fluorodeoxyuridine, and 400 μg of 5-bromodeoxyuridine per ml. The culture was infected with dC-containing bacteriophage T4 *56 denA denB alc*8 at one T4 particle per bacterium and vigorously aerated by shaking in a water bath at 37°C. After 2 min for phage adsorption, the culture was diluted to 30 ml with the same medium; 15-ml samples were withdrawn 6.5 and 7 min later and quickly chilled in the presence of 0.01 M NaCN and 0.05 M EDTA.

The infected cells were pelleted and suspended in 6 ml of 0.1 M Tris hydrochloride–0.01 M EDTA–0.01 M NaCN (pH 7.8). To lyse the cells the following solutions were added

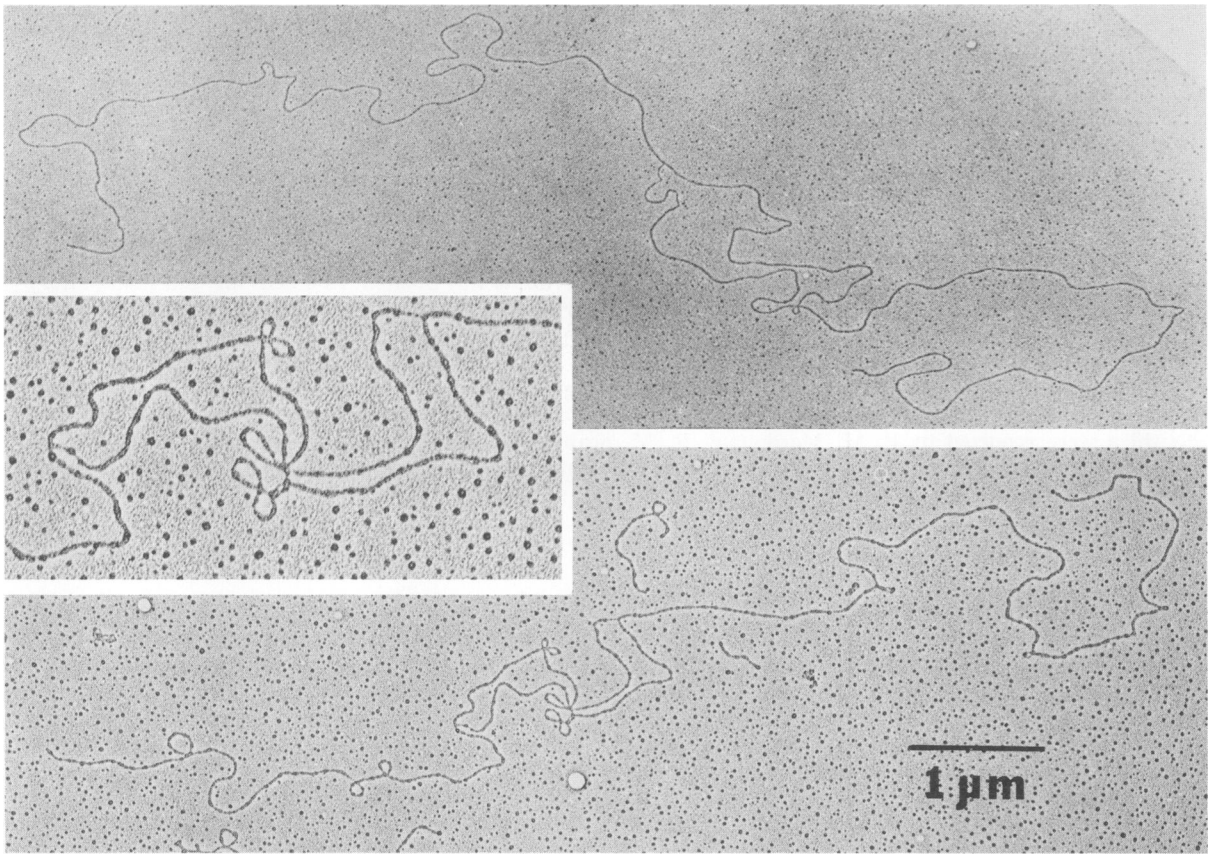

FIG. 1. Electron micrographs of *Bgl*II fragments of partially replicated T4 DNA, which correspond in length to *Bgl*II-1. Inset, Enlargement of the replication eye in the lower fragment, illustrating the single-stranded DNA at one side of each branch point.

sequentially for the indicated incubation times: (i) 0.4 ml of egg white lysozyme (15 mg/ml; Sigma Chemical Co., St. Louis, Mo.) for 60 min at 0°C and then for 10 min at 37°C; (ii) 0.16 ml of 0.5 M EDTA for 5 min at 37°C; (iii) 0.7 ml of 10% sodium lauroyl sarcosinate (Sigma), for 1 min at 37°C; and (iv) 0.16 ml of pronase (10 mg/ml; B grade [free of nuclease]; Calbiochem-Behring, La Jolla, Calif.) for 20 min at 37°C. The lysate was then gradually heated to 65°C and maintained at this temperature for 3 min. After being chilled, the samples were dialyzed overnight at 4°C against 0.05 M Tris hydrochloride–0.02 M EDTA (pH 7.8), and then centrifuged in a CsCl density gradient. T4 dC-DNA ^{32}P labeled by nick translation according to the specifications of Bethesda Research Laboratories, Gaithersburg, Md. was included in the gradients as a density marker.

DNA digestion by restriction endonucleases. Dialysis against 20 mM Tris hydrochloride–0.5 mM EDTA (pH 7.8) was used to remove CsCl from gradient fractions just before digestion with restriction endonucleases (purchased from Bethesda Research Laboratories). A standard reaction mixture was used for all digestions and contained 20 mM Tris hydrochloride–10 mM $MgCl_2$–50 mM NaCl–0.5 mM EDTA (pH 7.8). Sufficient enzyme was added to give complete digestion within 3 h at 37°C. The completeness of the *Bgl*II digestion was monitored by inclusion of the plasmid pSJC102 in the reaction mixture. This plasmid contains three *Bgl*II sites and comprises the large *Eco*RI fragment of R6K inserted into the *Eco*RI site of ColE1; it was constructed and provided by S.-J. Chiang.

Electron microscopy. DNA was spread directly from CsCl gradient fractions or restriction endonuclease digestion mixtures by using formamide-containing phases and the ramp-spreading technique described by Davis et al. (2). Circular pBR322 DNA was included as an internal size standard; it contains 4,362 base pairs (17). Samples were picked up on Parlodion-coated grids, stained with uranyl acetate, and rotary shadowed with platinum. Photographs were taken with a Siemens IA or Zeiss EM 10CA electron microscope, and DNA lengths were measured from projected negatives with a linear integrator (Numonics Graphics).

RESULTS

Isolation of early replicative intermediates of T4 DNA. Density labeling with 5-bromodeoxyuridine and isopycnic banding in CsCl gradients was used to separate early T4 replicative intermediates from the bulk of unreplicated parental T4 DNA and from host DNA. *E. coli* K803 *thy deo* cells grown for 1.5 generations in the presence of 5-bromodeoxyuridine were infected with dC-containing bacteriophage T4 *56 denA denB alc*8 at a multiplicity of 1. Samples were withdrawn at 6.5 and 7 min after infection. The cells were gently lysed, and the DNA was subjected to CsCl density gradient centrifugation. Since no radioactivity was used to label the nascent T4 DNA, a density marker of ^{32}P-labeled T4 dC-DNA was included in the CsCl gradients. The sampling times were chosen based on the kinetics of incorporation of [^3H]thymidine into phage DNA in control

experiments performed under similar infection conditions. In these experiments the onset of T4 DNA replication and rate of label incorporation appeared identical for both wild-type and *56 denA denB alc*8 T4 in *E. coli* K803 *thy deo*.

The T4 DNA banded in the middle of the CsCl gradients and was well separated from host DNA, which was located at or near the bottoms of the tubes due to extensive incorporation of 5-bromodeoxyuridine and its greater percentage of G · C base pairs. Electron microscopy of DNA spread directly from fractions just to the denser side of the T4 dC-DNA marker showed the presence of molecules with one or more eyes, each eye consisting of two branch points linked by two arms of equal length. Forked and more highly branched molecules were also observed. Altogether, these replicative and probably some recombinational intermediates represented about 1% of the molecules in the denser fractions. No such molecules were found in the main band of parental T4 dC-DNA.

Frequently, a single-stranded connection was observed at one or both branch points of an eye (Fig. 1). When single-stranded connections were visible at both branch points, they were always arranged in a *trans* configuration, as expected for bidirectional replication with the proximal Okazaki fragments not yet initiated on the lagging strand at each fork or having been lost during isolation of the DNA.

A total of 75 molecules with eyes but no other branch points were observed. Of these, 63 contained a single eye. The eyes varied in size from 1.5 to 61 kb and averaged 13 kb. The average total length of these single-eye molecules was 117 kb. This was a reduction from the full chromosome size of 170 kb (5, 8), reflecting breakage either in vivo or during the isolation of the T4 DNA molecules. Eight molecules showed two eyes and one molecule had three eyes, with the eyes distributed along the DNA. The average length of these molecules was 135 kb.

The remaining three molecules exhibited a secondary eye within one branch of the primary eye. In one case the secondary eye was symmetrically positioned in the middle of the primary eye. In the others the secondary eyes occurred off center. Similar structures were observed by Delius et al. (3) and Dannenberg and Mosig (1). These structures suggest reinitiation of replication from the same origin and further support overall bidirectionality of replication, with some variation in the timing of initiation or rate of replication in both directions.

Location of origins of replication on the T4 genetic map. Gradient fractions containing DNA molecules with eyes were dialyzed, and defined ends were generated on the otherwise circularly permuted T4 DNA molecules by cleavage with restriction enzyme *Bgl*II. Electron microscopy showed that as a result of digestion the average size of molecules with eyes dropped from 117 to 39 kb.

A total of 29 molecules with eyes were found, and none were excluded from the following analysis. Nine of the molecules apparently corresponded in size to the 56.5-kb fragment *Bgl*II 1 (8). These nine molecules measured 52.6 to 61 kb and averaged 55.1 kb, which was within the standard error of ±5% we observed for the measurement of DNA length by electron microscopy. Two of these molecules are shown in Fig. 1. Because T4 chromosomes are circularly permuted and fragment *Bgl*II 1 is so long (one-third of the T4 genome), only two of three full-length T4 chromosomes would yield a full-sized *Bgl*II-1. With the reduction in size to an average of 117 kb as measured for the undigested T4 DNA molecules containing eyes, the yield of full-length *Bgl*II-1 would be even lower. About one molecule out of three would yield a full-length *Bgl*II-1. Therefore, the low fraction of full-length *Bgl*II-1 was to be expected.

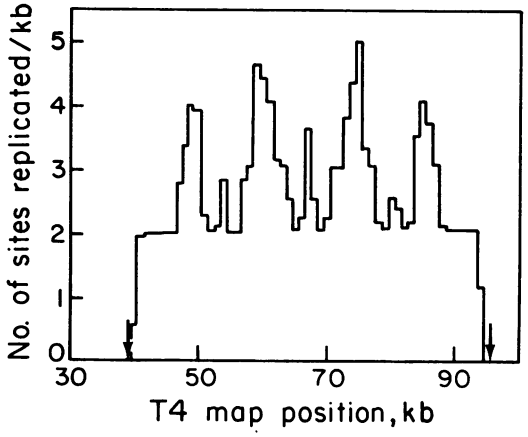

FIG. 2. Histogram of sites replicated in the nine T4 DNA fragments which contained replication eyes and corresponded in length to *Bgl*II-1. Because the left and right ends of the fragments could not be distinguished, each fragment is included twice, in both possible orientations on the genetic map. The histogram is not absolutely symmetrical because the 1-kb intervals used to construct the histogram correspond to the kilobase intervals of the T4 map, whereas fragment *Bgl*II-1 starts at 39.4 kb and ends at 95.9 kb (5). Arrows indicate the *Bgl*II cleavage sites. Each ordinate value represents the sum of the fractions of DNA replicated per 1-kb segment of all nine fragments.

To determine the positions of the eyes on the T4 genetic map, the nine molecules assigned to *Bgl*II-1 were normalized to a length of 56.6 kb and used to construct the histogram of replicated sites shown in Fig. 2. Because the left and right ends of these molecules could not be distinguished, each molecule was included twice in the histogram in both possible orientations on the T4 genetic map. The eyes of these nine molecules fell into four groups, indicating the existence of a maximum of four origins of replication in *Bgl*II-1 and a minimum of two, given the double representation of each molecule. The four possible origin sites were at positions 49, 62, 74, and 86 kb on the T4 genetic map, with at least one of the inner sites and one of the outer sites being origins of replication. The small peaks between the four possible origins are due to the overlap of the largest eyes centered at adjacent possible origins.

Among the 20 remaining *Bgl*II-digested molecules with eyes, 16 were longer than the second largest *Bgl*II fragment (17.5 kb [8]), but they were shorter than *Bgl*II-1. They can be regarded, therefore, as segments of *Bgl*II-1, with one end generated by *Bgl*II cleavage and the other end representing either a natural chromosome terminus or a point where the molecule had been broken. Although four molecules were shorter than *Bgl*II-2, they did not correspond in length to any of the smaller T4 *Bgl*II fragments; thus, they may also represent portions of *Bgl*II-1.

If all 20 fragments represent portions of *Bgl*II-1, it should be possible to position each on the genetic map with one end fixed at one of the termini of *Bgl*II-1 and have the eyes overlap one of the putative origins. Line diagrams of the 20 molecules are shown arranged this way in Fig. 3; only the putative origins at map positions 74 and 86 kb have been used for Fig. 3 since the other two sites are symmetrically positioned within *Bgl*II-1 and thus would yield the same results. In all but three cases the *Bgl*II-digested molecules

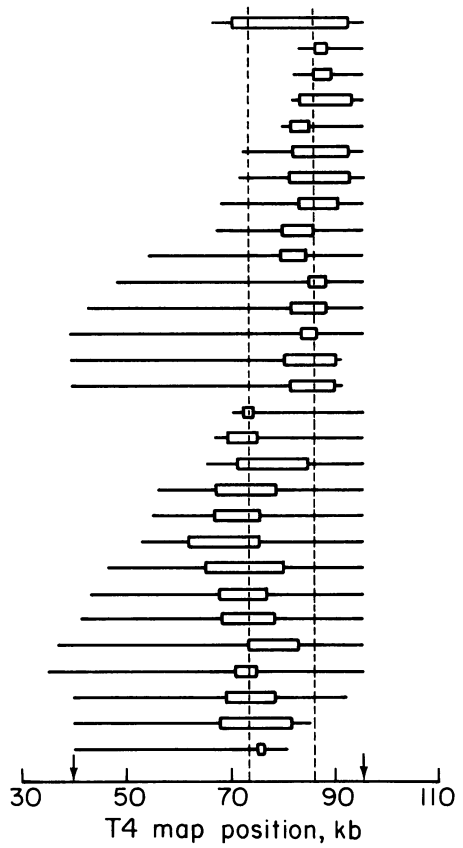

FIG. 3. Line diagrams of all *Bgl*II fragments which contained replication eyes. They are positioned to maximize overlap of the eyes with the putative replication origins at map positions 74 and 86 kb while maintaining one end of each fragment fixed at a terminus of fragment *Bgl*II-1. Arrows indicate the *Bgl*II cleavage sites at the termini of *Bgl*II-1. Dashed lines indicate the average midpoints of the two groups of replication eyes.

could be positioned with eyes overlapping the possible origins, and the three exceptions lay very close by. Even with the smaller eyes the fit was good. Thus, all eyes in this population of partially replicated molecules can be accounted for by origins within *Bgl*II-1. Line diagrams of the nine full-length *Bgl*II-1 fragments with eyes are also included in Fig. 3. In this case their lengths were not normalized to that of *Bgl*II-1 as in Fig. 2.

To distinguish which of the four possible sites in *Bgl*II-1 represent actual origins, a second set of T4 replicative intermediates was cleaved with *Bam*HI. *Bam*HI cleaves T4 DNA at a unique site at map position 85.4 kb (8). This is near one of the possible origins in *Bgl*II-1. A total of 27 molecules with replication eyes were observed. Their lengths ranged from 16 to 132 kb with an average of 60 kb; the eyes averaged 8.7 kb. The wide distribution of lengths of *Bam*HI-cleaved molecules was expected due to the circular permutation of T4 chromosomes. Figure 4A shows a histogram of the distribution of eyes about the *Bam*HI site. All molecules with eyes were placed on the left side of the *Bam*HI site so that the eyes would fall within the region covered by *Bgl*II-1. Since the *Bam*HI end of the molecules could not be distinguished from the chromosome end, each molecule has been represented twice, oriented with each end alternatively at the *Bam*HI cleavage site. Five possible sites of replication initiation stand out as peaks above the background. Two are especially prominent. These two sites are centered at map positions 60 and 73 kb and are concordant with two possible origins defined by digestion with *Bgl*II, i.e., at 62 and 74 kb on the T4 genetic map. Of the remaining two possible origin sites established by the analysis with *Bgl*II, the one at 49 kb can be eliminated, leaving the symmetrically disposed site at 86 kb as a third T4 origin of replication. This origin was not detected with the *Bam*HI-cleaved molecules because it coincides almost exactly with the *Bam*HI cleavage site and apparently produced fragments with forks, all of which were excluded from the present analysis. The three small peaks distal to the *Bam*HI cleavage site represent (in all but three cases) eyes in molecules which when positioned in the reverse orientation permit the eyes to overlap the origins of replication at 60 and 73 kb. This can be seen with the line diagrams of all *Bam*HI molecules shown in Fig. 4B. The three eyes that do not overlap the origins at 60 and 73 kb probably reflect an additional origin near 34 kb.

Distribution of multiple eyes on individual DNA molecules. If the presence of two or three eyes on the same DNA molecule were due to initiation of replication at defined

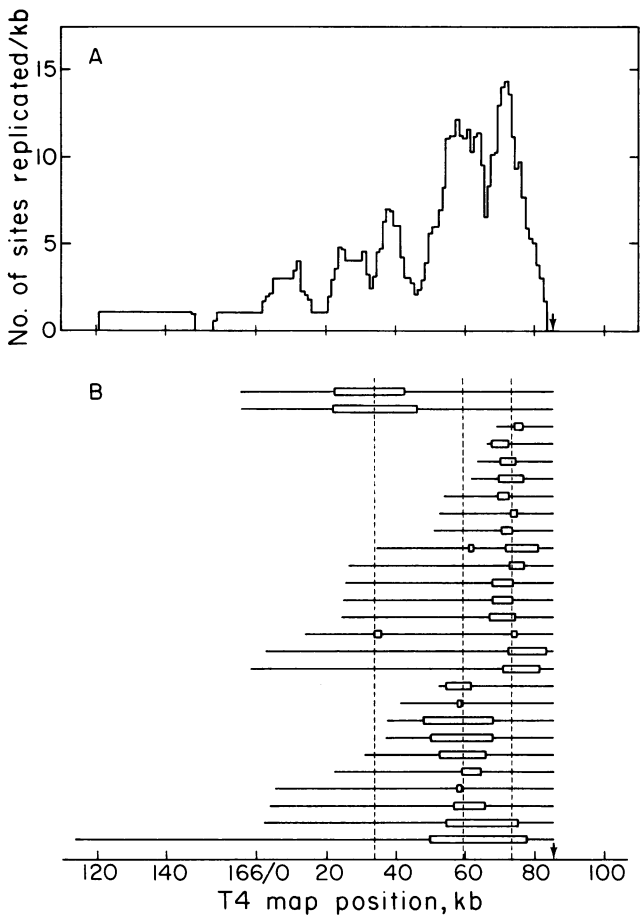

FIG. 4. Histogram of sites replicated (A) and line diagrams (B) for all partially replicated T4 DNA molecules cleaved with *Bam*HI. Arrows designate the *Bam*HI cleavage site. Each molecule is represented twice in the histogram, oriented with each end alternatively at the *Bam*HI cleavage site. The line diagrams are positioned to maximize overlap of the eyes with putative replication origins while maintaining one end of each fragment fixed at the *Bam*HI cleavage site.

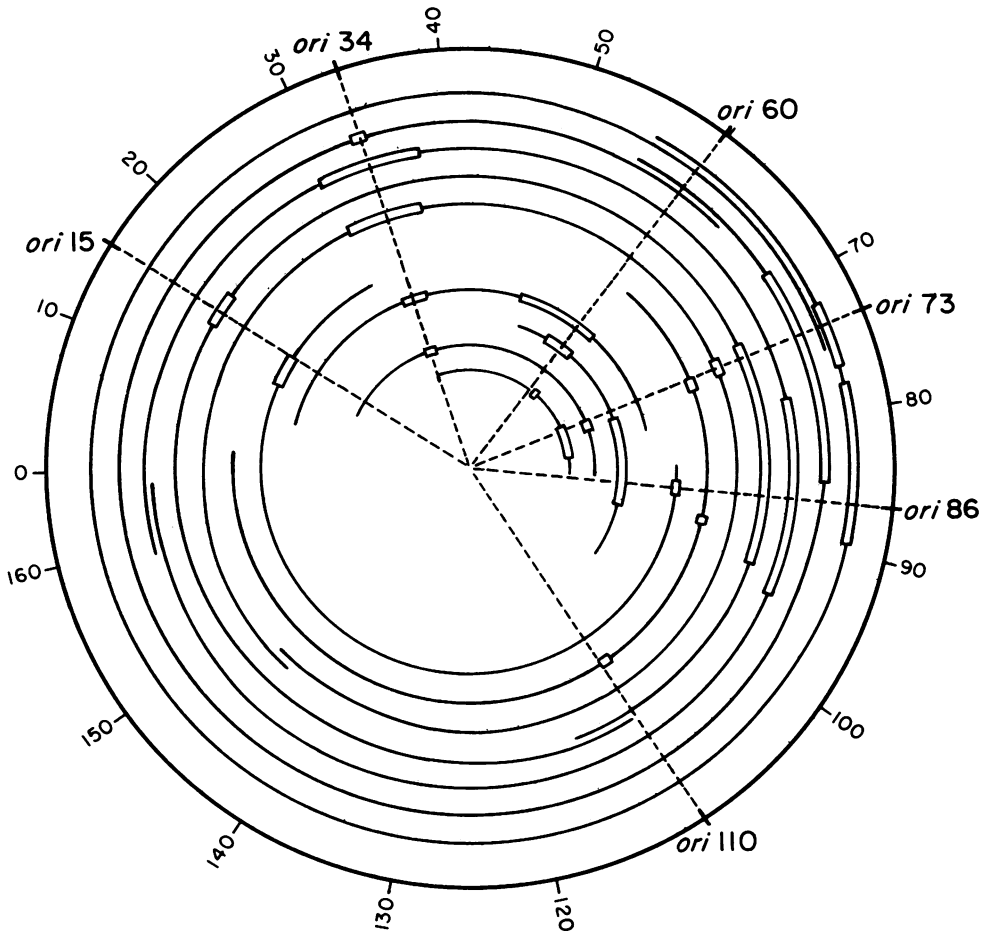

FIG. 5. Correlation between the distribution of multiple replication eyes on individual DNA molecules and the positions of reported replication origins on the T4 genetic map (outer circle). The two innermost arcs are line diagrams of *Bam*HI-cleaved molecules; the remaining nine arcs represent uncleaved molecules.

origins, it should be possible to account for the distribution of most or all of these eyes on the basis of the distances separating the known origins. For the two *Bam*HI-cleaved molecules with two eyes and the nine undigested molecules which were observed to contain more than one eye, a near-perfect correlation exists between the distribution of the eyes on the molecules and the locations of origins of replication on the T4 genetic map (Fig. 5), provided that two additional origins are located at 15 to 16 and 110 to 119 kb, as reported by Macdonald et al. (9) and Halpern et al. (4). For several of the molecules in Fig. 5 alternative arrangements can be drawn to permit overlap or near overlap of eyes and origins. The alignments of replication eyes and origins shown were chosen on the basis of best fit to the origins at 34, 60, 73, and 86 kb and, second, to the proximities of the midpoints of the eyes to the origin loci.

DISCUSSION

The distribution of replication eyes relative to *Bgl*II and *Bam*HI cleavage sites indicates that T4 origins of replication exist at 60, 73, and 86 kb on the genetic map and support the assignment of a fourth origin near 34 kg. The results further indicate that under our experimental conditions the initiation of T4 DNA replication can occur at about the same time at more than one of the several origins on the T4 genome, possibly including the previously reported origins near 15 and 110 kb.

Of these origin assignments, the one at 86 kb was identified with the greatest certainty. It was detected within fragment *Bgl*II 1 as one of two alternative sites (the other being at 49 kb) depending upon which way *Bgl*II-1 fragments with replication eyes were oriented on the genetic map; but the results with *Bam*HI-cleaved molecules clearly eliminated the 49-kb site as a possibility. The other pair of possible origin sites in *Bgl*II-1, at 60 and 73 kb, appeared to be functional when all *Bam*HI-cleaved molecules were placed to the left side of the *Bam*HI site. This placement was prompted by our inability to find eyes in *Bgl*II fragments other than *Bgl*II-1. However, the possibility exists that some of the eyes at these two sites or all eyes at one of the sites were actually generated by origins to the right of the *Bam*HI site, at either 98 or 111 kb.

The discrepancies in the literature regarding the locations of T4 origins of replication are resolved in large part by the present observations. The origins at 73 and 86 kb would explain the initial replication detected by Halpern et al. (4) in the area of genes 50 to 5, which is bracketed by these origins. Halpern et al. hybridized radioactively labeled, newly synthesized DNA to various cloned T4 DNA fragments and detected the strongest signal with a fragment which extends from 76 to 83 kb on the map. Such increased labeling of the

region would be expected when eyes that were initiated on different chromosomes at 73 or 86 kb became superpositioned in the intervening region. Halpern et al. (4) also reported the existence of a possible weak origin in the region of 112 to 118 kb on the map. Its usage would explain the position of a replication eye near 110 kb in one of the molecules we observed which contained two eyes. Furthermore, some of the eyes on the *Bam*HI-cleaved molecules would fall in this interval if placed on the right rather than the left side of the *Bam*HI site, as discussed above.

The origin we assigned to 60 kb is undoubtedly responsible for the initial replication detected at 62 kb by King and Huang (6) in nitrocellulose blots of *Eco*RI fragments probed with ^{32}P-labeled nascent DNA. These investigators assigned the origin to a 1.9-kb *Eco*RI fragment at 62 kb, but another *Eco*RI fragment of similar size spans the 60-kb position (8) and may actually be the origin-containing fragment.

King and Huang (6) also reported an origin at 29 to 34 kb based on hybridization of the ^{32}P-labeled nascent DNA to a 5.4-kb *Eco*RI fragment. In support of an origin in this region, we observed three overlapping eyes centered at 34 kb.

The present data provide some support for an origin of replication in the region between genes *dda* and *dam* at 15 to 16 kb as reported by Macdonald et al. (9), its usage possibly accounting for the patterns of replication eyes seen in two of the uncleaved molecules with two eyes. The restriction enzymes chosen for our analysis probably precluded a more definitive observation. If the origin lies too near a *Bgl*II site, the resultant eyes could have been cleaved to produce forked molecules, which were eliminated from analysis. Additionally, the *Bam*HI cleavage site is so distant that most molecules covering the *dda*-to-*dam* region will end before the *Bam*HI site is reached, preventing the generation of defined ends on the molecules. By analogy, the distance from the *Bam*HI site is the probable reason for not observing more molecules with eyes initiated at the origin near 34 kb on the map. Cleavage of replicative intermediates with enzymes which cut T4 DNA infrequently but closer to the putative origin near 15 kb is needed to obtain a definitive answer.

Altogether, there is evidence for six origins of replication, which are located at intervals around the T4 genome. With dC-containing T4 DNA and no radioactive labeling, we clearly detected the use of four of these origins and have some evidence that the remaining two origins were also functional. Why have other investigators detected different subsets of these origins? The answer may be a combination of differences in strains and cultural conditions and in the means of detection. That Halpern et al. (4) did not detect origins near 15, 34, and 60 kb can be attributed to their not having tested for hybridization to cloned fragments from these areas. This, however, does not apply to the studies of Macdonald et al. (9) or King and Huang (6), who hybridized newly synthesized DNA to blots of total T4 restriction fragments and only detected origins near 15 and 110 kb and near 34 and 60 kb, respectively. In these studies different origins may have been preferentially activated by different growth and labeling conditions. Morris and Bittner (personal communication) have shown that such differential activation can occur. Similarly, Lin and Mosig (personal communication) have shown that defects in T4 DNA topoisomerase or host DNA gyrase can lead to differential usage of the T4 origins.

What advantage would be derived in having origins of replication spaced around the T4 genome? A possibility is that they contribute to the rapid acceleration of DNA synthesis that occurs at the onset of replication at 5 to 7 min after infection. First, initiation could occur at more than one origin per chromosome, as appeared to be the case for 12% of the molecules with eyes examined before restriction enzyme cleavage. Second, and perhaps more importantly, if initiation occurred preferentially at the origins which lie closest to the ends of the circularly permuted T4 chromosomes, the replication forks would soon reach the ends of the molecules, leaving 3′ single-stranded termini on the lagging strand templates. As has been proposed by Mosig and supported by several lines of evidence (14), these single-stranded ends could invade homologous regions on coinfecting chromosomes or the terminal redundancies of the individual molecules to create recombinational intermediates from which secondary replication forks can be initiated. These additional forks would accelerate the rate of DNA synthesis.

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