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Localization of Single-Chain Interruptions in Bacteriophage T5 DNA

II. Electrophoretic Studies[1]

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Upon denaturation, T5 DNA yields a large number of discrete, single-chain fragments that can be resolved by agarose gel electrophoresis. The positions of the more prominent of these fragments in the T5 duplex were determined by analyzing their sensitivity to digestion with λ exonuclease and their distribution among *Eco*RI fragments of T5 DNA. These experiments also provide firm evidence concerning the polarity of the strands in T5 DNA. An analogous study was carried out on the fragments produced by treating exonuclease III-degraded T5 DNA with the single-strand-specific SI endonuclease. This procedure yielded over 40 discrete duplex fragments that could be resolved with considerable precision by agarose gel electrophoresis. The positions of most of these fragments were determined by analyzing *Eco*RI fragments of T5*st*(+) and T5*st*(0) DNA. Over 20 sites where single-chain interruptions can occur in T5 DNA were identified, and the distribution of interruptions within the terminal repetition was shown to be identical at both ends of the molecule. A precise value for the size of the terminal repetition in T5 DNA was obtained by analyzing SI endonuclease digests of ligase-repaired, circular T5 DNA in agarose gels. The repeated segment represented 8.3% of the T5*st*(+) DNA. The results of this study also provide information concerning the properties of λ exonuclease. Hydrolysis by this enzyme was not terminated when single-chain interruptions were encountered either in the strand being degraded or in the complementary strand.

The locations of the single-chain interruptions in bacteriophage T5 DNA can be determined by several methods. In the preceding paper (21), the positions of the single-stranded regions in exonuclease III-treated T5 DNA were mapped with an electron microscope. The principal advantage of this method is that intermolecular variations in the distribution of interruptions can be immediately recognized. T5 DNA, however, contains a number of sites where interruptions occur at a low frequency. Accurate mapping of these sites by electron microscopy would require examination of a very large number of molecules.

The sites where interruptions occur in T5 DNA can also be localized by analyzing the fragments produced by denaturation of the duplex molecule. As demonstrated by Hayward and Smith (10), denaturation of T5 DNA results in the formation of over 40 discrete fragments that can be separated by agarose gel electrophoresis. These fragments are all derived from one strand (the "interrupted" strand) of the T5 duplex and are not found in ligase-treated DNA (9, 14). T5 DNA can also be fragmented by cleaving the intact strand at the site of each interruption. Shear-induced cleavage at the sites of the principal interruptions has been demonstrated by Hayward (9). In the present investigation, the single-strand-specific SI endonuclease from *Aspergillus oryzae* was used to cleave exonuclease III-treated T5 DNA. This procedure resulted in cleavage at the site of every interruption and yielded a series of duplex fragments analogous to the single-stranded fragments produced by denaturation.

Fragments of T5 DNA produced by both of the above methods were mapped by determining their distribution among fragments of T5 DNA generated by the *Eco*RI restriction endonuclease. Some of the single-stranded fragments were also mapped by determining their sensitivity to digestion with λ exonuclease. The results of these experiments were combined to identify over 20 sites where interruptions can occur and to specify the polarity of the strands in T5 DNA.

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SI endonuclease can also be employed to cleave the single-stranded regions in circular molecules of T5 DNA formed after treatment with T4 DNA ligase and λ exonuclease (19). The action of SI endonuclease resulted in release of the short duplex segments that represent terminal repetition. These duplex segments were analyzed by agarose gel electrophoresis and found to consist of a uniform population of molecules equal to 8.3% of intact, wild-type T5 DNA.

MATERIALS AND METHODS

Enzyme preparations. The preparations of *Eco*RI endonuclease, T4 DNA ligase, λ exonuclease, and *Escherichia coli* exonuclease III used in this study have been described previously (18, 19, 21). SI endonuclease from *A. oryzae* was purified from α-amylase powder (Sigma Chemical Co., St. Louis, Mo.) through the DEAE-cellulose step of Vogt (27).

Enzyme reaction conditions. T5 DNA was treated with *Eco*RI endonuclease and T4 DNA ligase as described previously (18, 19).

Lambda exonuclease incubations were carried out at 32°C in 0.03 M Tris-hydrochloride (pH 8.0)–0.003 M $MgCl_2$. Hydrolysis was terminated by adding 0.1 volume of 0.1 M EDTA.

SI endonuclease incubations were carried out at 37°C in a solution of 0.03 M sodium acetate buffer (pH 4.8), 0.05 M NaCl, and 0.001 M $ZnSO_4$. Reactions were terminated by extraction with phenol followed by dialysis against 0.01 M Tris-hydrochloride (pH 8.0)–10^{-4} M EDTA.

Exonuclease III incubations were carried out at 37°C in 0.01 M Tris-hydrochloride (pH 8.0)–0.001 M $MgCl_2$. These incubations were terminated by heating at 65°C for 5 min. Subsequent incubations with *Eco*RI endonuclease or SI endonuclease were carried out by diluting the exonuclease III reaction mixture two- to threefold and adding the appropriate ingredients for the second enzyme. Sequential exonuclease III-*Eco*RI endonuclease incubations were terminated by adding 1/5 volume of 0.1 M EDTA and heating at 65°C for 5 min.

Agarose gel electrophoresis. Agarose (Sigma) was dissolved by refluxing in the Tris-phosphate buffer of Hayward (8). Cylindrical gels containing 0.5 to 1.0% (wt/vol) agarose were formed in glass tubes (12 cm long; 0.6-cm ID). Electrophoresis was carried out at 1 to 4 V/cm at room temperature. Horizontal slab gels containing 0.7% agarose were prepared and operated as described by Rogers and Rhoades (20). At the conclusion of each run, the gels were stained in 0.5 μg of ethidium bromide per ml, and the bands were visualized by illumination with short-wavelength UV light. Photographs were taken as described by Sharp et al. (23). Unless otherwise indicated, cylindrical gels were employed to fractionate double-stranded DNA, whereas electrophoresis of single-stranded DNA was carried out in horizontal slab gels. Both kinds of gels were loaded with 0.5 to 1.0 μg of fragments from intact T5 DNA. Proportionally smaller doses were employed for analysis of purified *Eco*RI fragments.

Preparative gel electrophoresis of double-stranded DNA was carried out as described above, except that long-wavelength UV light was used to visualize the bands. DNA was eluted from agarose sections by the electrophoretic procedure of Allet et al. (2).

RESULTS

This paper presents an electrophoretic analysis of two analogous sets of fragments of T5 DNA. Single-stranded fragments were produced by alkaline denaturation of the duplex molecule, whereas double-stranded fragments were produced by sequential treatment with exonuclease III and SI endonuclease. In both cases, the ends of the fragments are defined by the natural single-chain interruptions that occur in T5 DNA. Although denaturation is the simpler of the two processes, the electrophoretic mobilities of single-stranded DNA in agarose gels appear to depend on base composition or secondary structure in addition to molecular weight (10). This effect, which often facilitates separation of complementary single chains (8), precludes accurate measurement of molecular weight. In contrast, the electrophoretic mobilities of double-stranded molecules in agarose gels are almost entirely dependent on molecular weight (26). Although duplex fragments of T5 DNA are more difficult to produce, analysis of these molecules should yield a more accurate map of the single-chain interruptions.

Fragmentation of T5 DNA. Electrophoresis patterns of the single-stranded fragments of three variants of T5 DNA are shown in Fig. 1. Some of the fragments have been numbered according to the scheme described below (see Fig. 2). Denatured T5*st*(+) DNA (wild type, slot 1) contains four single-stranded species, no. 3, 4, 13, and 18, which have been designated major fragments because they form prominent bands. Although different in molecular weight, fragments 3 and 4 migrate together under these conditions (see Fig. 2). The denatured DNA of the T5*st*(0) deletion mutant (slot 2) lacks major fragments 4 and 13 and contains, instead, a new prominent fragment that migrates behind fragment 3. In the case of a second deletion mutant, T5*st*(124), fragment 13 has been replaced by a new fragment that migrates slightly faster than fragment 18 (slot 3). The patterns of the major fragments of T5*st*(+) and T5*st*(0) DNA are similar to those reported by Hayward and Smith (10). The pattern of T5*st*(124) DNA is similar to that reported by Hayward (9) for T5*b*3 DNA.

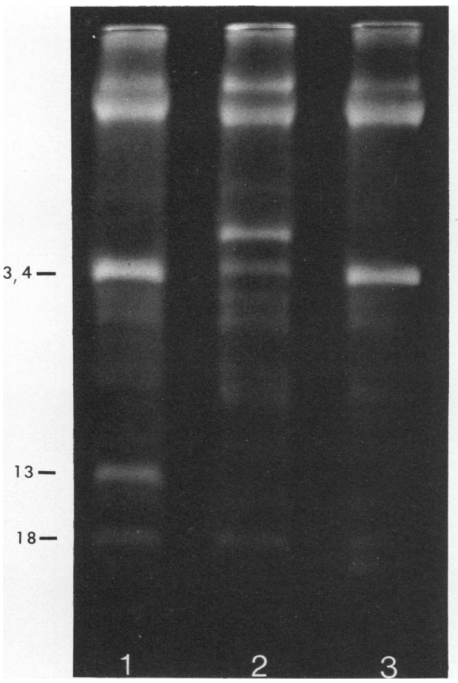

FIG. 1. *Electrophoresis of denatured T5 DNA. T5st(+) DNA (slot 1), T5st(0) DNA (slot 2), and T5st(124) DNA (slot 3) were denatured in 0.1 M NaOH and subjected to electrophoresis in a horizontal 0.7% agarose slab gel. The direction of migration is from top to bottom. The band nearest the origin represents renatured duplex molecules. The strong band immediately preceding the duplex band is the intact strand of T5 DNA. The major single-chain fragments that occur in T5st(+) DNA have been numbered as described in the legend to Fig. 2. The two largest major fragments are not resolved in this gel.*

The production of duplex fragments of T5 DNA by cleavage with SI endonuclease was carried out on molecules that had been degraded to 1% with exonuclease III. This level of hydrolysis represents a loss of approximately 200 nucleotides at each 3'-terminus. The dose of SI enconuclease was empirically adjusted so that an additional 0.8 to 0.9% of the DNA was rendered acid soluble. This dose of SI endonuclease was not sufficient to cleave T5 DNA that had not been pretreated with exonuclease III. Qualitatively similar results were obtained with 10- to 100-fold less endonuclease and with DNA that had been degraded to 2 or 3% with exonuclease III; however, levels of exonuclease III hydrolysis in excess of 1% noticeably decreased the molecular weight of the smaller duplex fragments.

Electrophoretic separations of the fragments produced by exonuclease III-SI endonuclease treatment of T5*st*(+), T5*st*(0), and T5*st*(124) DNA are shown in Fig. 2. Numbers have been assigned, in order of decreasing molecular weight, to the fragments derived from T5*st*(+) DNA. The profiles of the larger fragments, which were resolved in 0.5% agarose gels (Fig. 2A), resemble the profiles of the larger single-stranded fragments produced by denaturation (Fig. 1). In addition to major fragments 4 and 13, T5*st*(0) DNA lacks fragments 7 and 16, whereas T5*st*(124) DNA lacks fragments 7 and 13. Electrophoresis in 1% agarose gels (Fig. 2B) reveals that at least 20 low-molecular-weight duplex fragments are produced by this treatment. T5*st*(+), T5*st*(0), and T5*st*(124) DNA all appear to have identical sets of low-molecular-weight fragments.

Some of the numbered fragments are not visible in the profiles of T5*st*(+) DNA shown in Fig. 2. Fragments 5, 12, and 14 are obscured by major fragments 4 and 13 in T5*st*(+) DNA, but can be seen in the T5*st*(0) profile. Fragments 19 and 20 can be visualized by carrying out electrophoresis in 1% gels for longer times.

The molecular weights of the duplex fragments of T5 DNA were determined from their electrophoretic mobilities relative to reference molecules present in the same gel. *Eco*RI fragments of T5*st*(+) DNA and λ*b*2 DNA were employed for this purpose. The molecular weights of both sets of *Eco*RI fragments have been determined by electron microscopy (2, 18, 26). The results of these determinations are given in Table 1. All of the measurements have been increased by 0.1×10^6 daltons to compensate for the material lost during nuclease treatment.

The results presented in the preceding paper demonstrate that interruptions occur frequently in T5*st*(+) DNA at sites 7.9, 18.5, 32.6, and 64.8% from the left end of the DNA. If the major fragments are defined by these sites, then the molecular weights of these fragments should equal 35.2, 32.3, 14.1, 10.6, and 7.9% of an intact genome. These values are in good agreement with the molecular weights of fragments 3, 4, 13, and 18 (Table 1). The 7.9% fragment would be expected to occur infrequently due to the high density of interruptions at the left end of the molecule (21).

The T5*st*(0) deletion mutant, which has lost the interruption site at 32.6% (21), would be expected to lack fragments 4 and 13. Since T5*st*(0) lacks 7.2% of the wild-type DNA (21), these two fragments should be replaced by a new fragment that equals 39.1% of an intact

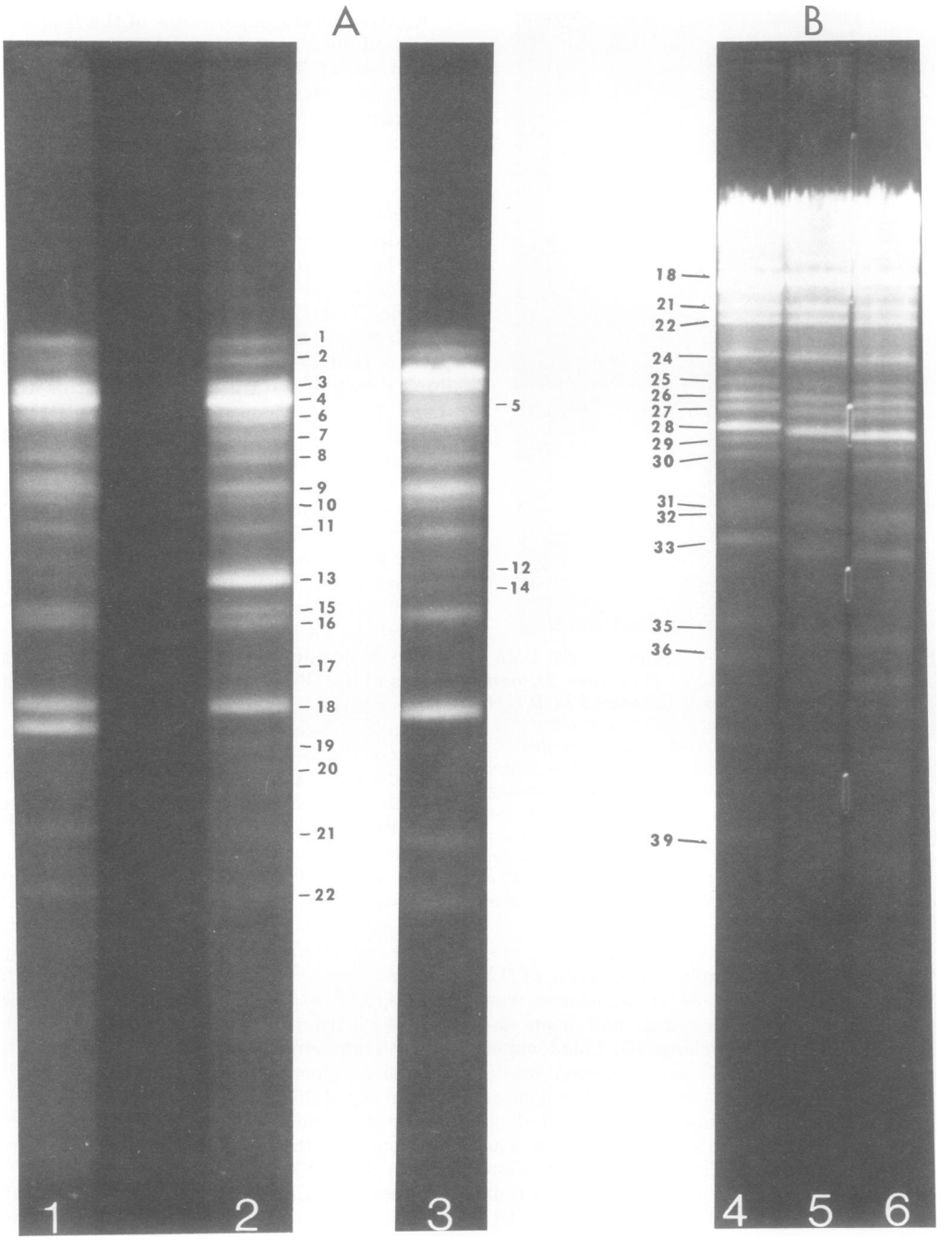

FIG. 2. *Electrophoresis of exonuclease III-SI endonuclease-treated T5 DNA. T5st(+), T5st(0), and T5st(124) DNA were degraded with exonuclease III and endonuclease SI, and the products were analyzed in (A) 0.5% and (B) 1.0% agarose gels. The direction of migration is from top to bottom. Slots 1 and 4, T5st(124) DNA; slots 2 and 6, T5st(+) DNA; slots 3 and 5, T5st(0) DNA. The numbered bands represent fragments that occur in T5st(+) DNA.*

TABLE 1. *Properties of duplex fragments of T5st(+) DNA generated by exonuclease III-SI endonuclease treatment*[a]

Fragment no.	Mol wt	T5st(+) DNA (%)	*Eco*RI fragment	Coordinates
1	46	60		32.6-100.0?
2	35	46		18.5-64.8?
3	26.8	35.0	I	64.8-100.0
4	24.8	32.4		32.6-64.8
5	23.6	30.8	I	64.8-95.8
5.5	22.6	29.5		64.8-94.1
6	21.3	27.8	I	64.8-92.5
6.5	20.7	27.1		
7	19.1	25.0		7.9-32.6
8	16.9	22.1	I	77.9-100.0
9	14.9	19.5		45.3-64.8
10	14.1	18.4	I	81.5-100.0
11	12.6	16.5	I	64.8-81.5
12	11.4	14.9	I	77.9-92.5?
13	10.7	14.0		18.5-32.6
14	10.6	13.9	I	
15	9.9	12.9	I	64.8-77.9
16	9.4	12.3		32.6-45.3
17	8.4	11.0	I	
18	7.7	10.1	II	7.9-18.5
19	6.98	9.1	I, II	
20	6.70	8.8	II	
21	5.95	7.8	II	0.0-7.9
22	5.37	7.0	I, II	0.8-7.9
23	4.43	5.8		
24	4.28	5.6	I, II	2.4-7.9
25	3.65	4.8	I, II	3.3-7.9
26	3.37	4.4	I, II	3.5-7.9
27	3.16	4.1	II	0.0-4.1
28	2.91	3.8	I, II	4.1-7.9
29	2.70	3.5	II	0.0-3.5
30	2.52	3.3	II	0.0-3.3
31	2.12	2.8	II	0.0-2.8?
32	2.05	2.7	II	0.0-2.7?
33	1.90	2.5	II	0.0-2.4
34	1.63	2.1		
35	1.47	1.9		
36	1.23	1.6		
37	0.94	1.2		
38	0.75	1.0		
39	0.62	0.8		0.0-0.8

[a] Molecular weights are given in units of $\times 10^6$. A value of 76.5×10^6 has been used for the molecular weight of T5st(+) DNA. The coordinates of each fragment are given as a percentage of the length of T5st(+) DNA, measured from the left end. "?" indicates tentative assignments. Fragments 5.5 and 6.5 are faint bands that separate from fragments 5 and 6 on high-resolution gels. Fragments 22, 24, 25, 26, and 28 occur at both ends of the molecule. The coordinates for these fragments at the right end can be obtained by adding 91.7 to the values given in the table. The two fragments from T5st(0) DNA that are not found in T5st(+) DNA have molecular weights of 29.8×10^6 and 15.3×10^6. The coordinates of the larger fragment, which migrates between fragments 2 and 3, are 18.5 to 64.8 (wild-type map units). The smaller fragment migrates between fragments 8 and 9 and occurs from 18.5 to 45.3. T5st(124) DNA contains two unique fragments that migrate between fragments 8 and 9, and 18 and 19. The molecular weights of these fragments are 15.5×10^6 and 7.25×10^6. They represent shortened versions of fragments 7 and 13.

genome. T5st(124) DNA lacks a 4.5% segment that maps entirely to the left of the 32.6% interruption (22). In this DNA, fragment 13 should be replaced with a new 9.6% fragment. As indicated in the footnote to Table 1, these predictions agree well with the observed molecular weights of the new major fragments derived from the two deletion mutants.

Analysis of the single-chain fragments. The locations of many of the single-stranded fragments produced by denaturation were elucidated by analyzing *Eco*RI fragments of T5 DNA and by determining the sensitivity of the different single-chain fragments to digestion with λ exonuclease. *Eco*RI digests of T5st(+) DNA contain four large fragments, which represent 37, 25, 21, and 12% of an intact genome. *Eco*RI digests of T5st(0) DNA are similar, except that the third largest fragment equals 17% of the wild-type DNA. The positions of these fragments have been established by procedures that did not require prior knowledge of the locations of the single-chain interruptions (18). The *Eco*RI fragments will be labeled with roman numerals, beginning with the largest, to avoid confusion with the single-stranded fragments.

The four largest *Eco*RI fragments of T5st(+) and T5st(0) DNA were isolated by preparative agarose gel electrophoresis and denatured, and the resulting single chains were analyzed on agarose slab gels. Figure 3 shows the results obtained for fragments I through IV of T5st(+) DNA and fragments I and III of T5st(0) DNA, as well as the unfractionated *Eco*RI digests and intact forms of both DNAs. The electrophoretic separations obtained for intact T5st(+) and T5st(0) DNA are similar to those shown in Fig. 1, except that more of the minor fragments are visible. The unfractionated digests contain the major fragments expected if the restriction enzyme cleavage pattern were superimposed upon the map of the prominent interruptions (Fig. 4). The bands in these two samples (slots 3 and 4) are sharp, indicating that treatment with the restriction enzyme did not introduce additional single-chain breaks.

The single-chain fragments present in intact T5st(+) and T5st(0) DNA occur primarily within *Eco*RI fragments I and II (Fig. 3). *Eco*RI fragment I appears to contain fragments 3, 5, 6, 8, 10, 11, and 15 (slots 5 and 10), whereas *Eco*RI fragment II contains single-chain fragment 18 (slot 6). Both of these *Eco*RI fragments also contain a number of smaller single chains that are not visible in this photograph. *Eco*RI fragment III from T5st(+) DNA contains two fairly

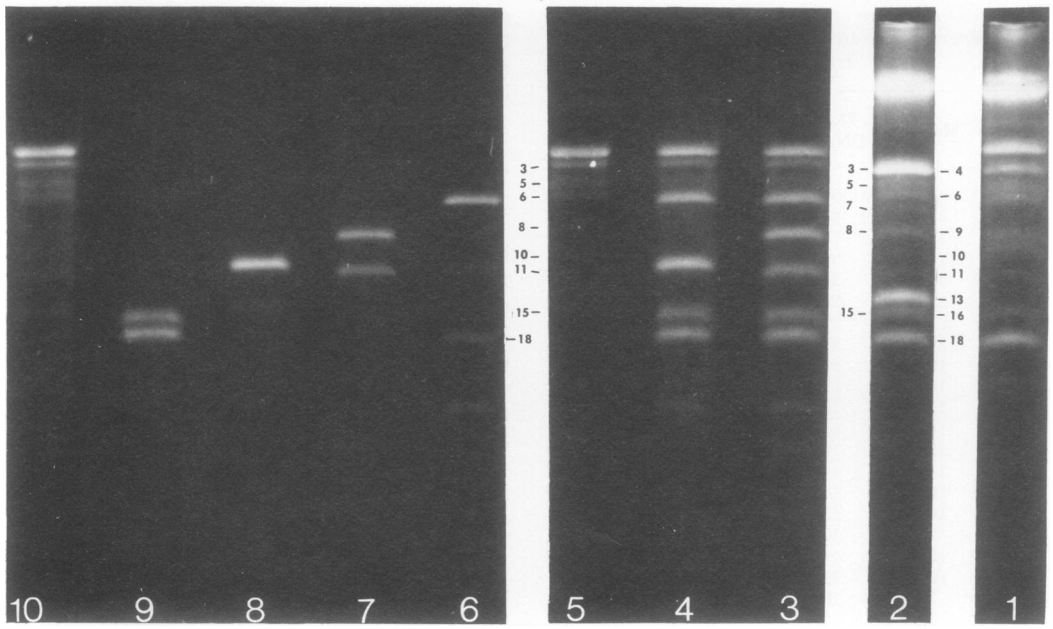

FIG. 3. *Electrophoresis of denatured EcoRI fragments of T5 DNA. T5st(+) and T5st(0) DNA were degraded with EcoRI endonuclease, and the four largest fragments were isolated by preparative gel electrophoresis. The purified EcoRI fragments were then denatured in alkali and analyzed in a 0.7% agarose slab gel. The direction of migration is from top to bottom. All slots contain denatured DNA. Slot 1, Intact T5st(0) DNA; slot 2, intact T5st(+) DNA; slot 3, EcoRI digest of T5st(+) DNA; slot 4, EcoRI digest of T5st(0) DNA; slot 5, EcoRI fragment I from T5st(+) DNA; slot 6, EcoRI fragment II from T5st(+) DNA; slot 7, EcoRI fragment III from T5st(+) DNA; slot 8, EcoRI fragment III from T5st(0) DNA; slot 9, EcoRI fragment IV from T5st(+) DNA; slot 10, EcoRI fragment I from T5st(0) DNA. The preparations of EcoRI fragments II and III from T5st(+) DNA used in this experiment show a low level of cross-contamination.*

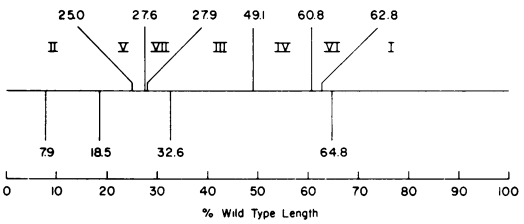

FIG. 4. *Maps of the EcoRI cleavage sites and the prominent single-chain interruptions in T5st(+) DNA. The points of cleavage by EcoRI endonuclease are indicated in the upper part of the figure. The EcoRI fragments are identified with roman numerals. The cleavage sites at 27.6 and 27.9% are missing in the T5st(0) and T5st(124) deletion mutants. The four sites where single-chain interruptions occur frequently in T5st(+) DNA are indicated in the lower part of the figure. The site at 32.6% is missing in T5st(0) DNA.*

strong bands that are missing in fragment III from T5*st*(0) DNA, reflecting the presence of the interruption at 32.6% (slots 7 and 8). These two gels also contain a faint band that, as discussed below, indicates the presence of an interruption at 45%. *Eco*RI fragment IV appears to be free from interruptions. The two bands seen in this gel (slot 9) represent the intact, complementary strands contained in the duplex form of fragment IV.

Additional information concerning the locations of the single-chain fragments has been obtained by digesting T5 DNA with λ exonuclease, an enzyme that specifically degrades external 5′-termini in double-stranded DNA (5, 17). Treatment of T5 DNA with λ exonuclease should therefore result in degradation of the single-chain fragments that occur at the 5′-end of the interrupted strand. At the same time, the single-chain fragments located at the 3′-end of the interrupted strand should be released from the duplex molecule as the 5′-end of the intact strand is degraded.

T5 DNA was digested to various extents with λ exonuclease under conditions that permit uniform degradation of all duplex termini. Due to the processive nature of λ exonuclease (5), this was accomplished by employing a twofold excess of enzyme (19). The single chains that were sensitive to λ exonuclease digestion were identified by analyzing the reaction products,

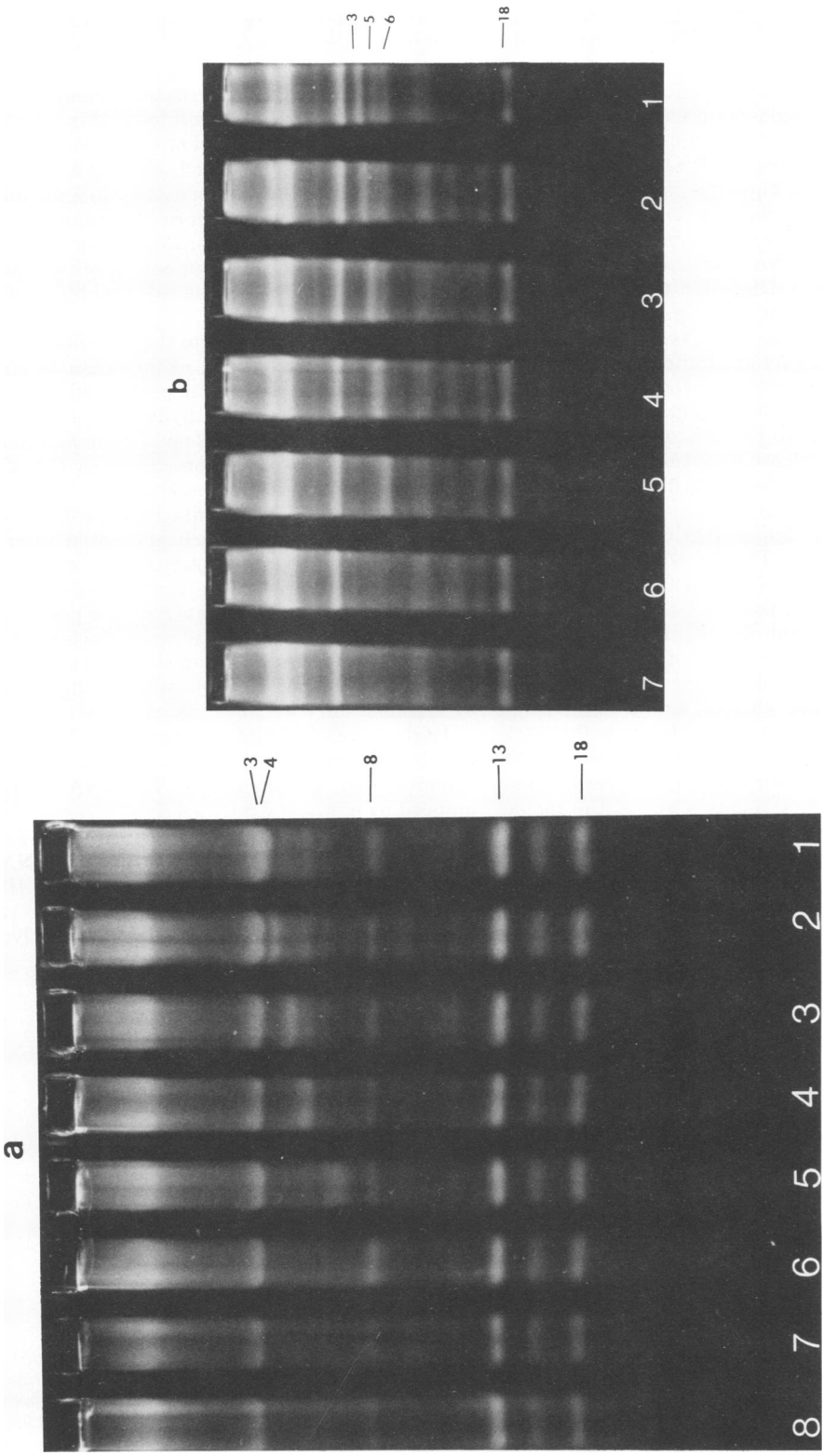

FIG. 5. *Effect of λ exonuclease digestion on the electrophoretic patterns of denatured T5st(+) and T5st(0) DNA. Samples of native DNA were hydrolyzed with λ exonuclease as described in the text. At various times, portions were removed from the incubation mixtures, denatured in alkali, and analyzed in 0.7% agarose slab gels. (a) T5st(+) DNA: slot 1, 0% digestion; slot 2, 3.0% digestion; slot 3, 6.0% digestion; slot 4, 8.7% digestion; slot 5, 10.7% digestion; slot 6, 13.8% digestion; slot 7, 16.4% digestion; slot 8, 18.4% digestion. (b) T5st(0) DNA: slot 1, 0% digestion; slot 2, 2.0% digestion; slot 3, 4.6% digestion; slot 4, 7.0% digestion; slot 5, 9.4% digestion; slot 6, 17.2% digestion; slot 7, 23.6% digestion.*

after denaturation, on 0.7% agarose slab gels. Typical results obtained for T5*st*(+) and T5*st*(0) DNA are shown in Fig. 5.

The initial stages of λ exonuclease digestion appear to affect single-chain fragments 3 and 8. The degradation of fragment 3 is evident in both T5*st*(+) and T5*st*(0) DNA. The degradation of fragment 8, however, is evident only in T5*st*(+) DNA (Fig. 5a, slot 2). Fragment 8 is obscured in T5*st*(0) by a fragment that is absent in T5*st*(+) DNA and migrates between fragments 8 and 9. Digestion past 7% leads to loss of fragments 5 and 6 (Fig. 5b). In contrast to fragment 3, these two fragments are not affected by 2% digestion, indicating that their 5'-termini do not extend to the end of the duplex. Most of the other fragments visible in these gels appear to be resistant to the initial stages of λ exonuclease digestion, although loss of some of the less prominent fragments would be difficult to detect.

An experiment demonstrating the release of single-chains from T5*st*(0) DNA during the course of λ exonuclease digestion is shown in Fig. 6. In this case, the reaction products were not denatured prior to electrophoresis. The bright band near the origin of each gel represents the partially degraded T5 duplex. As digestion proceeds, various single-chain fragments begin to appear. Digestion from 3 to 14% releases many of the low-molecular-weight fragments (slots 2 through 5), whereas digestion from 16 to 19% releases one of the major fragments number 18 (slots 6 and 8). To prevent hydrolysis of the released single chains (25), this experiment must be performed with a ratio of active enzyme molecules to duplex termini of less than two.

The results of these two experiments, when combined with the analysis of the *Eco*RI fragments presented earlier, unequivocally demonstrate polarity of the interrupted strand in T5 DNA. The external 5'-terminus of this strand must occur at the right end of the molecule, as defined by *Eco*RI fragment I, since the single-chain fragments that are sensitive to λ exonuclease are contained within *Eco*RI fragment I. Similarly, the 3'-terminus must occur at the left, since single-chain fragment 18, which lies within *Eco*RI fragment II, is released by λ exonuclease digestion.

These results also indicate that the degradation of duplex DNA by λ exonuclease is not terminated by the presence of preformed single-chain interruptions. The hydrolysis of fragments 5 and 6 (Fig. 5b) clearly demonstrates that the progress of λ exonuclease is not affected by single-chain interruptions in the strand being degraded. Similarly, the release of fragment 18 (Fig. 6), which is located internally, reveals that interruptions in the complementary strand are unable to stop hydrolysis. These experiments do not exclude the possibility that interruptions cause λ exonuclease to dissociate from the DNA and that this event is followed by the rapid reinitiation of digestion by a second enzyme molecule.

Analysis of the duplex fragments. The duplex fragments generated by exonuclease III-SI endonuclease treatment of T5 DNA were mapped by analyzing purified *Eco*RI fragments in a manner similar to that described above for the single-stranded fragments. T5*st*(0) DNA was treated sequentially with exonuclease III and *Eco*RI endonuclease, and the four largest *Eco*RI fragments were isolated by preparative

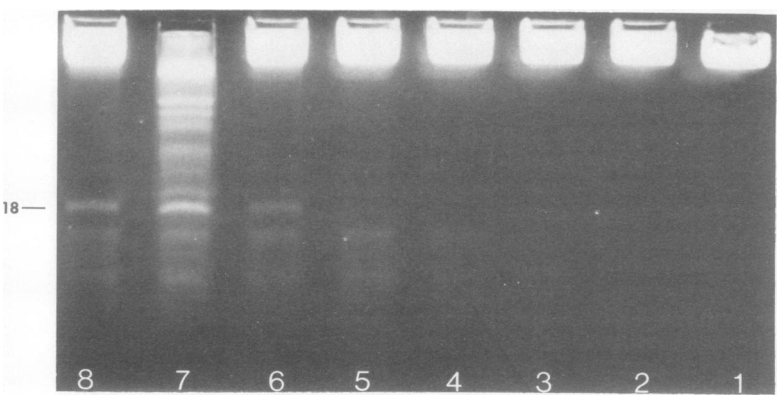

FIG. 6. *Release of single chains from T5 DNA during the course of digestion with λ exonuclease. T5st(0) DNA was hydrolyzed with λ exonuclease as described in the text. Samples were removed from the incubation mixture at various times and analyzed, without denaturation, in a 0.7% agarose slab gel. Slot 1, 0% digestion; slot 2, 2.7% digestion; slot 3, 6.2% digestion; slot 4, 8.8% digestion; slot 5, 13.7% digestion; slot 6, 15.9% digestion; slot 7, denatured intact T5st(0) DNA; slot 8, 19.5% digestion.*

gel electrophoresis. The purified *Eco*RI fragments were then treated with SI endonuclease, and the resulting products were analyzed in agarose gels. The electrophoretic profiles of *Eco*RI fragments I through IV in 1.0% gels and fragment I in a 0.5% gel are shown in Fig. 7.

The absence of interruptions within *Eco*RI fragment IV is indicated by the resistance of this fragment to SI endonuclease cleavage (Fig. 7, slot 1). *Eco*RI fragment III from T5*st*(0) is also largely resistant to SI endonuclease, although two faint bands representing cleavage products are formed (slot 2). The molecular weight of the larger of these products has been estimated to be 10.0×10^6.

*Eco*RI fragment II appears to contain most of the low-molecular-weight duplex fragments produced by exonuclease III-SI$^-$ endonuclease treatment of intact T5 DNA (slot 3). Included in this group are fragments 18 through 33 and possibly some of the smaller fragments. Fragment II also contains a strong band between fragments 22 and 24, which represents the interval between the prominent interruption at 18.5% and the *Eco*RI cleavage point at 25.0% (Fig. 4).

*Eco*RI fragment I contains many of the large duplex fragments. As indicated in the 0.5% gels shown in Fig. 7 (slots 6 and 7), this group includes fragments 3, 5, 6, 8, 10, 11, 12, 14, 15, 17, and 19. Fragment I, however, also contains some of the low-molecular-weight fragments. As indicated in the 1.0 and 0.5% gels (slots 4 and 7), fragments 22, 24, 25, 26, and 28 are probably present in both *Eco*RI fragments I and II, whereas fragments 29 and 30 and possibly 21, 27, 31, and 33 appear to be absent from fragment I. The fast-migrating band in the 1%

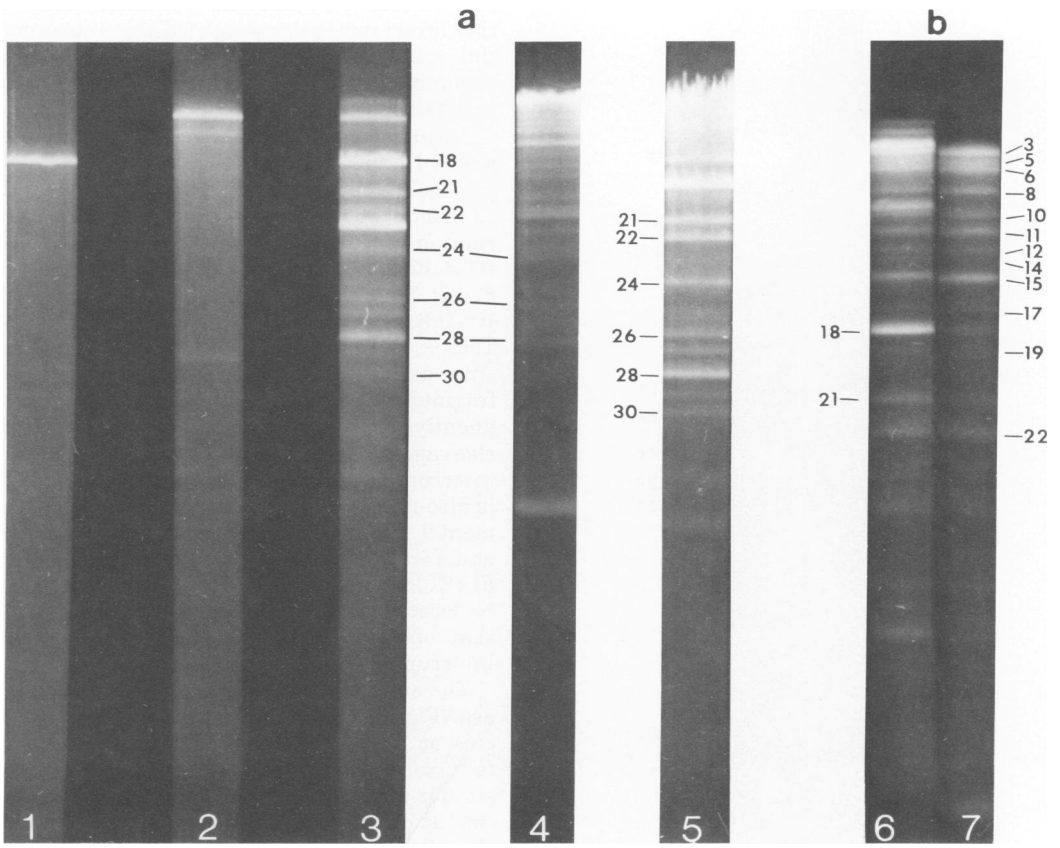

FIG. 7. *Analysis of SI endonuclease digests of exonuclease III-treated EcoRI fragments of T5 DNA. Exonuclease III-treated T5st(0) DNA was degraded with EcoRI endonuclease, and the four largest fragments were isolated by preparative gel electrophoresis. The purified fragments were then degraded with SI endonuclease, and the products were analyzed in (a) 1.0% and (b) 0.5% agarose gels. Slot 1, EcoRI fragment IV; slot 2, EcoRI fragment III; slot 3, EcoRI fragment II; slots 4 and 7, EcoRI fragment I; slots 5 and 6, intact T5st(0) DNA.*

gel of fragment I (slot 4) represents the interval between the *Eco*RI cleavage point at 62.8% and the prominent interruption at 64.8% (Fig. 4).

The identities of the low-molecular-weight fragments derived from *Eco*RI fragment I were confirmed by electrophoresis in 0.7% agarose slab gels. A comparison of the duplex fragments derived from *Eco*RI fragment I and intact T5*st*(0) DNA (Fig. 8) indicates that *Eco*RI fragment contains fragments 22, 24, 26, and 28 and lacks fragments 20, 21, 27, 29, and 30. The identity of the faint bands in *Eco*RI fragment I that migrate close to the positions of fragments 18 and 21 is not known.

The electron microscopic observations presented in the preceding paper and the experiment measuring the release of single chains from T5 DNA by λ exonuclease digestion (Fig. 6) indicate that most of the low-molecular-weight fragments occur at the ends of the DNA, within the terminal repetition. The presence of many of these fragments at both ends of the DNA implies, therefore, that the pattern of interruptions is identical within both copies of the terminal repetition. This observation can be exploited, as demonstrated below, to map the sites where interruptions occur within the terminal repetition.

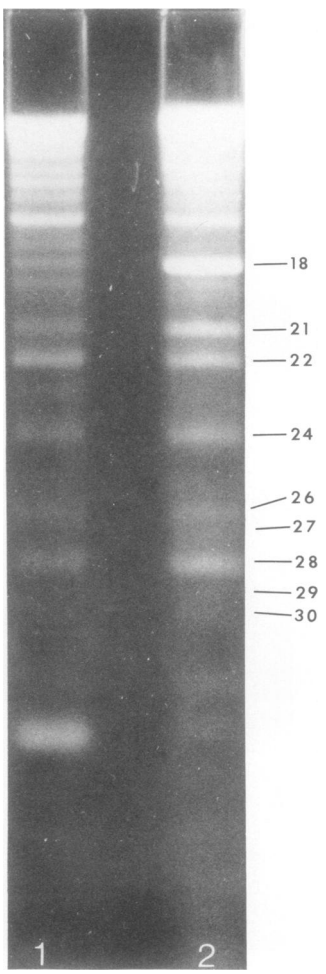

FIG. 8. *Comparison of the duplex fragments derived from intact T5st(0) DNA and EcoRI fragment I by treatment with exonuclease III and endonuclease SI. Electrophoresis was carried out in a 0.7% horizontal agarose slab gel. Slot 1, EcoRI fragment I; slot 2, intact T5st(0) DNA.*

Location of the single-chain interruptions. The results presented in the preceding section provide additional evidence that the ends of the major fragments of T5 DNA are defined by the principal single-chain interruptions, as identified in the preceding paper (21). Fragment 3, for example, occurs within *Eco*RI fragment I, is sensitive to λ exonuclease digestion, and has the correct molecular weight (Table 1) to occupy the space between 64.8% and 100%. Similar arguments can be made for fragments 4, 13, and 18.

As indicated by the analyses of *Eco*RI fragments III and IV, the central region of T5 DNA is largely free from interruptions other than the 32.6 and 64.8% sites. The existence of an interruption at 45.3% can be inferred from the 10.0 × 10^6-dalton fragment derived by SI cleavage of *Eco*RI fragment III of T5*st*(0) DNA. The 10 × 10^6-dalton fragment, which equals 13.1% of T5*st*(+) DNA, must span the site of the deletion since an identical fragment is not derived from fragment III of T5*st*(+) DNA (Fig. 3). Consequently, it must be located between the *Eco*RI cleavage site at 25% in T5*st*(0) DNA and an interruption at 45.3%. An interruption at 45.3% is also necessary to specify the left end of fragment 9 (Fig. 9). This fragment occurs in T5*st*(+) and T5*st*(0) DNA and is not found in any of the *Eco*RI fragments. As a result, fragment 9 must be located between 45.3 and 64.8%, assuming that one end occurs at one of the principal interruptions.

The sensitivity of fragment 8 to λ exonuclease (Fig. 5) indicates that an interruption occurs at 77.9%. Fragment 15, which is resistant to λ exonuclease, fits between 64.8 and 77.9%. A similar argument can be made for an interruption at 81.5% specified by fragments 10 and 11 (Fig. 9). The assignment of fragment 10 to the interval between 81.5 and 100% is based on the apparent greater resistance of fragment 11 to λ exonuclease digestion. If the positions of these fragments are reversed, the interruption would occur at 83.4%.

At least five of the duplex fragments of T5

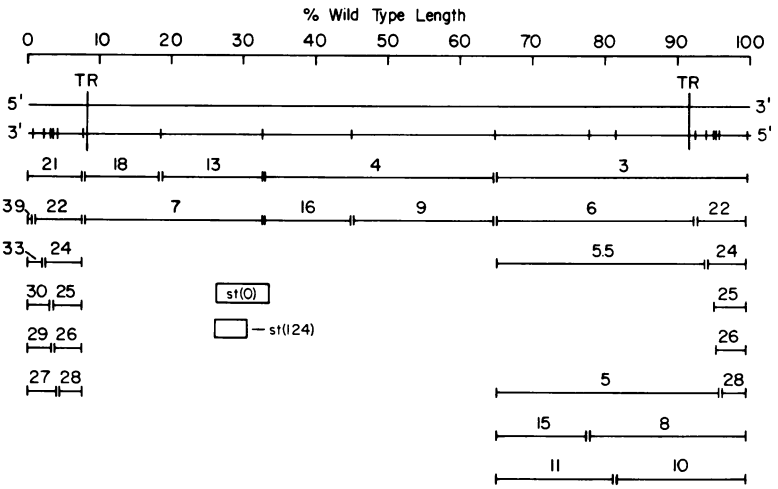

FIG. 9. *Map of the sites where single-chain interruptions occur in T5st(+) DNA. The two strands of the T5st(+) duplex are shown at the top of the figure. The positions of the single-chain interruptions are indicated by short vertical lines in the strand whose 3'-terminus occurs at the left end of the DNA. The positions of the fragments of T5 DNA that define these sites are given in the lower part of the figure along with the locations of the T5st(0) and T5st(124) deletion mutations. The boundaries of the terminal repetition are indicated by "TR." The positions shown for most of the fragments were determined as described in the text. In a few cases, such as fragments 5.5 and 39, the assignments were based solely on size considerations. Precise values for the positions of the single-chain interruptions are given in Table 1.*

DNA generated by exonuclease III-SI endonuclease treatment appear to occur within both copies of the terminal repetition (Table 1). These fragments must be located internally, with both ends specified by single-chain interruptions. Conversely, fragments that occur only in *Eco*RI fragment II must extend to the left end of the duplex molecule. The right ends of these fragments thus define interruptions within the repeated segment at the left of T4 DNA. The internally located fragments are likely to have at least one end specified by a principal interruption. At the left end of the DNA, these fragments probably extend leftward from the interruption at 7.9% (Fig. 9). At the right end, however, this set of fragments must specify an interruption that occurs extremely close to the end of the duplex molecule. As demonstrated below, the terminal repetition in T5 DNA is equal to 8.3% that of the wild-type genome. A site analogous to the 7.9% interruption would therefore occur at 99.6%.

A physical map of T5 DNA that summarizes the results obtained in this study is shown in Fig. 9. Only six interruptions have been included within the terminal repetition, although additional sites undoubtedly occur in this region. Tentative locations for two of these sites are given in Table 1.

Size of the terminal repetition in T5 DNA. In an earlier study (19), the terminal repetition in T5 DNA was visualized in an electron microscope by preparing circular molecules from T5 DNA treated with DNA ligase and λ exonuclease. If λ exonuclease digestion proceeds past the repeated segment at each end of the DNA, the resulting circular molecules contain an internal duplex segment that represents the terminal repetition. The lengths of these segments were estimated by electron microscopy to equal 6.4×10^6 daltons.

In the present study, circular molecules prepared in the same manner were degraded by SI endonuclease, and the digestion products were analyzed by agarose gel electrophoresis. SI endonuclease would be expected to cleave the two single-stranded regions in each circular molecule and release the internal duplex segment. The result of an experiment in which T5*st*(124) DNA was treated with ligase, digested to 12% with λ exonuclease, annealed, and then treated with SI endonuclease is shown in Fig. 10. The effect of SI treatment is to produce two bands, the faster of which migrates at the rate expected for the repeated segment of T5 DNA. This fragment is not seen in the absence of SI treatment. As determined in gels where reference molecules were present, the molecular weight of the terminal repetition is estimated to be 6.35×10^6, or 8.3% of the T5*st*(+) DNA.

DISCUSSION

As originally demonstrated by Hayward and Smith (10), denaturation of T5 DNA yields a

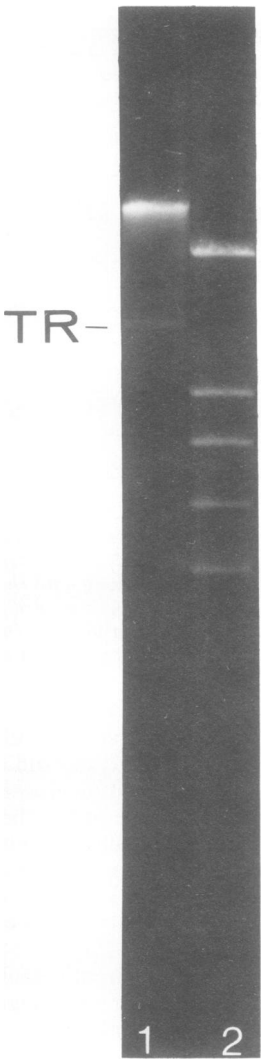

FIG. 10. *Electrophoresis of SI endonuclease-treated circular T5 DNA. Circular molecules of ligase-repaired T5st(124) DNA were formed by annealing DNA after digestion to 12% with λ exonuclease. The preparation of circular molecules was then degraded with SI endonuclease. The products of this digestion are shown in the gel in slot 1. Slot 2 contains an EcoRI digest of λb2 DNA. The band labeled "TR" in slot 1 represents the terminal repetition in T5 DNA.*

series of discrete, single-stranded fragments that can be fractionated by agarose gel electrophoresis. An analogous series of duplex fragments can be obtained, as demonstrated in the present study, by treatment of T5 DNA with exonuclease III and SI endonuclease. In both cases, the ends of the fragments are defined by natural single-chain interruptions. Consequently, experiments that determine the positions of these fragments can be used to locate the interruptions.

Most of the information concerning the precise locations of the single-chain interruptions was obtained from analysis of the duplex fragments generated by exonuclease III and SI endonuclease. In contrast to that of single-stranded DNA, the electrophoretic mobilities of duplex molecules in agarose gels depend almost exclusively on molecular weight. An indication of the potential differences between molecular weight estimates based on single- and double-stranded DNA is provided by the largest major fragment in T5*st*(0) DNA. Using calibrated gels, Hayward and Smith (10) obtained a value of 17.2×10^6 daltons for the single-stranded form of this fragment. The same fragment was estimated in the present study to have a duplex size of 29.8×10^6 daltons. This fragment occurs between the interruptions at 18.5 and 64.8% in T5*st*(0) DNA. The molecular weight of this interval, as determined in an electron microscope (21), is 29.9×10^6.

The principal disadvantage in using duplex fragments of T5 DNA is that their production depends on the action of two nucleases. As a result, each fragment will be smaller, by approximately 0.2% of T5*st*(+) DNA, than the corresponding segment in the intact molecule. Although this effect is of little consequence for most of the fragments, it does preclude accurate estimation of the size of the smallest fragments. The possibility also exists that treatment with exonuclease III and SI endonuclease will cleave sites other than single-chain interruptions. It has been reported (24) that high doses of SI endonuclease will cleave duplex DNA at adenine-plus-thymine-rich regions. The level of SI endonuclease employed in this study, however, was not sufficient to cleave T5 DNA in the absence of prior treatment with exonuclease III. Moreover, the size and location of many of the duplex fragments are in generally good agreement, as indicated by the analyses of *Eco*RI fragments, with the size and location of the single-stranded fragments produced by denaturation. Since the origin of the single-stranded fragments is less questionable, this result argues that the duplex fragments are not artifacts.

The results presented in the preceding paper (21) demonstrated that single-chain interruptions occur with variable frequencies at a large number of localized sites in T5 DNA. These conclusions were confirmed and amplified by the present study. The large number of sites where interruptions can occur is indicated by the large number of bands detected by electro-

phoresis. The interruptions mapped in this study are those that specify the more abundant fragments. Increasing the length and running time of the gels reveals a number of less abundant fragments, which could, in principle, be used to map additional sites.

The sharpness of the electrophoretic bands suggests that interruptions occur at precisely defined locations in T5 DNA. The band widths of the smaller duplex fragments represent size differences of less than 50 base pairs. An even lower limit, approximately 20 base pairs, can be obtained from the smallest fragments of denatured T5 DNA observed by Hayward and Smith (10). Neither of these studies, however, demonstrated that interruptions invariably occur at the same place in the nucleotide sequence.

The conclusion (21) that even the principal interruptions are occasionally missing is supported by the existence of minor fragments that span these sites. Fragment 7, which can be identified in both the single- and double-stranded fragments, illustrates this situation. The size of the fragment and the fact that it is missing in T5*st*(0) DNA suggest that it occurs between either 7.9 and 32.6% or 18.5 and 45.3% (Fig. 9). Although the fit is better at the first location, both alternatives indicate the absence of a principal interruption. The existence of minor fragments that are larger than any of the major fragments also reveals the occasional absence of one of the principal interruptions.

The conclusion (21) that interruptions occur at both ends of T5 DNA is also supported by the present study. In fact, interruptions appear to occur at identical sites within the two copies of the terminal repetition. Although the frequency of interruptions may be higher at the left end of the molecule (21), the positions of these sites clearly appear to be specified by the nucleotide sequence of T5 DNA.

In an earlier report from this laboratory (19), it was suggested that interruptions occur at only one end of T5 DNA. This conclusion was based on the observation that one of the major fragments (no. 3) appeared to extend to the right end of the duplex molecule. It was assumed that this fragment occurred in most of the population. In reality, as demonstrated here and in the preceding paper, additional interruptions frequently occur between 64.8% and the right end of the DNA. The relatively strong band formed by fragment 3, and hence its designation as a major fragment, is thus due mostly to its high molecular weight.

The interruption at 7.9% is of interest because it occurs extremely close to the right end of the terminal repetition. Several lines of evidence indicate that this interruption lies within the repeated segment. The size of the interval between 0 and 7.9% has been measured at 7.9% by electron microscopy (21) and 7.8% by gel electrophoresis (fragment 21). The size of the terminal repetition has been estimated at 8.4% by electron microscopy (19) and 8.3% by gel electrophoresis (Fig. 10). Both methods suggest that an interval of 0.4 to 0.6% of T5*st*(+) DNA occurs between these two sites. In addition, Rhoades and Rhoades (19) observed that the frequency of circle formation of unrepaired T5 DNA exceeds 50% even after digestion of more than 10% of the DNA with λ exonuclease. Since the 3'-end of the interrupted strand occurs at the left of the DNA, this observation indicates that a principal interruption cannot occur at or immediately to the right of the boundary between the repeated and unrepeated segments. If an interruption did occur at this position, the entire terminal repetition would be lost as the 5'-end of the intact strand is degraded. In fact, cyclization of unrepaired T5 DNA becomes impossible only after λ exonuclease digestion proceeds past the interruption at 18.5% (unpublished data).

The magnitude of the interval between the interruption at 7.9% and the end of the terminal repetition specifies the position of an interruption at approximately 99.6%. As discussed in Results, the interruptions at 7.9 and 99.6% define the right ends of fragments that, due to the terminal repetition, occur two times per molecule. It could be argued, by analogy with the 7.9% site, that interruptions occur frequently at 99.6%. However, a preliminary analysis of the single-chain fragments derived from the right end of T5 DNA has indicated that interruptions occur infrequently at this site (unpublished data).

The significance of the positions where interruptions occur in T5 DNA is unclear. Single-chain interruptions have been postulated to serve as initiation points for DNA replication (7). The principal origin of the initial rounds of T5 DNA replication occurs close to the center of the genome (3), within *Eco*RI fragment IV (N. Hamlett and M. Rhoades, unpublished data). This region is completely free from detectable interruptions. Initiation of T5 DNA replication also occurs less frequently at several secondary sites (3). These sites have not been mapped, and it is possible that they correspond to natural interruptions.

Single-chain interruptions might also serve as either initiation or termination points for transcription. Most of the sites where T5-specific RNA is initiated in vivo, however, are not

located close to the principal interruptions (11, 12). Furthermore, the template properties of T5 DNA for transcription in vitro are not detectably altered by repair of the interruptions with DNA ligase (15).

The interruption at 7.9% has been postulated to be the signal that separates the first-step-transfer segment of T5 DNA from the rest of the genome (4). The injection of T5 DNA into a bacterial cell occurs in two discrete steps, with only 8% of the DNA being transferred in the absence of viral protein synthesis (16). The fact that most T5 DNA molecules have additional interruptions between 0 and 7.9%, however, indicates that interruptions per se are not sufficient to stop the initial stages of injection.

Finally, it has been suggested (1) that single-chain interruptions are foci for high levels of site-specific recombination and are responsible for the four linkage groups that comprise the T5 genetic map (6, 13). However, the fact that only 29 T5 genes have been mapped (13) might indicate the existence of large, unmarked segments of the genome. Since T5 crosses generally exhibit high levels of recombination, it is possible that these unmarked regions are responsible for the separate linkage groups.

The observation that none of the interruptions in T5 DNA occurs in all of the population raises the question as to whether any of these sites perform a vital role in the T5 life cycle. This problem is currently being investigated by the isolation of T5 mutants that lack one or more of the natural interruptions.

ACKNOWLEDGMENTS

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