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Genetic Characterization of the DNA of the Bacteriophage φX174 70 S Particle

P. J. WEISBEEK, J. H. VAN DE POL, AND G. A. VAN ARKEL

Institute of Genetics, State University, Utrecht, The Netherlands

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The DNA of the 70 S particle of the bacteriophage φX174 has been characterized. By hybridization of RF II DNA of mutants from five different cistrons with DNA from wild-type 70 S particles it is demonstrated that this latter DNA is heterogeneous. Although the molecular weight is about 20% of the intact φX DNA, the 70 S DNA preparation contains nucleotide sequences from all five cistrons tested. The 70 S DNA turns out to consist of the plus strand type.

INTRODUCTION

The 70 S particle of bacteriophage φX174 is a particle that contains only 20% of the normal amount of DNA. It is often found in high concentration in lysates of φX174 (Sinsheimer, 1959). As demonstrated by Bleichrodt and Knijnenburg (1969), it originates from intact 114 S phage after adsorption to cell debris. The phage particle ejects part of its DNA, which is then removed by nuclease action. The resulting 70 S particle differs from the 114 S particle mainly by its low DNA content.

Eigner *et al.* (1963), have studied the physicochemical properties of the DNA of the 70 S particles (the 70 S DNA). They concluded that the formation of 70 S DNA molecules by random degradation of 114 S DNA seems unlikely and that all 70 S DNA molecules may lack at least a common sequence of nucleotides.

The spikes of the phage particle are responsible for the adsorption of φX174 to its host (Hutchison *et al.*, 1967; Sinsheimer, 1968; Brown *et al.*, 1971). Most likely there are twelve identical spikes on each particle (Burgess, 1969; Maclean and Hall, 1962; Tonegawa and Hayashi, 1970). Adsorption may proceed through anyone of the twelve spikes and the ejection may not need to start at a fixed point of the circular viral DNA. In that case the 70 S DNA would consist of random or nearly random fragments of the viral genome. In order to decide between these two possibilities we determined the genetic content of the 70 S DNA. This was done using a method similar to that described by Weisbeek and Van de Pol (1970). Wild-type 70 S DNA was hybridized with mutant RF II DNA.[1] During hybridization the 70 S DNA anneals with its homologous region of the complementary circular strand of the mutant RF II DNA. When the 70 S DNA preparation contains DNA of the cistron in which the RF DNA is mutant, the partially double-stranded DNA molecule is heteroduplex: mutant in one strand and wild-type in the other. After spheroplast infection the resulting partially double-stranded circular structure will be converted into a completely double-stranded RF DNA molecule in which the 70 S DNA fragment is covalently incorporated (Weisbeek and Van de Pol, 1970). The heteroduplex DNA molecules will give rise to progeny that consists of mutant and/or wild-type phage. An increase in the proportion of wild-type particles after hybridization demonstrates

[1] Abbreviations used: RF II DNA, double-stranded replicative form DNA with at least one single-strand break; *ss* DNA, single-stranded DNA; SSC, standard saline citrate, 0.15 *M* NaCl–0.015 *M* sodium citrate, pH 7.0.

that the wild-type 70 S DNA contained the wild-type allele of the RF mutant cistron. By using RF DNA of mutants from different cistrons, the 70 S DNA can thus be tested for the presence of DNA stretches from all over the ϕX genome.

MATERIALS AND METHODS

Media. For phage growth and phage titrations the media described by Borrias *et al.* (1969) were used. Preparation, infection and plating of spheroplasts were done using the media described by Guthrie and Sinsheimer (1963).

Bacterial strains. Escherichia coli C122 (BTCC No. 122, abbreviated C), suppressor negative and *Escherichia coli* K58, kindly provided by Zinder and Cooper (1964) were used as host. The latter strain contains an amber suppressor and is ϕX174 resistant. *Shigella paradysenteriae* Y6R (Y), carrying an amber suppressor was also used.

Phage mutants. Wild-type ϕX174 phage is able to grow on C and Y and at 30° as well as 41°. *Ts*27 is a temperature-sensitive mutant and unable to grow at 41°, but showing normal growth at 30°. *Em*7, *to*8, *am*18, and *am*3 are suppressor-sensitive (*sus*) mutants, able to grow on Y, but not on C. These mutants fall in five different cistrons (Borrias *et al.*, 1969).

Growth and purification of phage. Phages were grown in 5-liter batches, as described by Borrias *et al.* (1969). The phages were purified as described by Jansz *et al.* (1966a). The total yield was 1 to 5 $\times$ 10^{14} plaque-forming particles.

Isolation of the 70 S particles. 70 S particles occur in high amounts in cultures that have been infected with an m.o.i. = 10 and that have been aerated for 5 hr after addition of the phage (Sinsheimer, 1959). The purification of the 70 S particles was done in a manner similar to that used for the normal phage. In the last purification step, the RbCl density centrifugation, the 70 S particles were visible as a diffuse white band approximately 1 cm below the meniscus, whereas the phage was concentrated in a narrow white band in the lower half of the tube.

Preparation of DNA. *ss* DNA and 70 S DNA were prepared by extraction with phenol (Sinsheimer, 1959) of the purified virus and the purified 70 S particle, respectively. After extraction the water layer was freed from phenol by gel filtration (Jansz *et al.*, 1966a) by means of a Sephadex G-25 column (2.5 $\times$ 45 cm), equilibrated with 0.1 $\times$ SSC. The DNA fractions were concentrated in a rotary evaporator to an absorbance at 260 nm of 2. The *ss* DNA prepared in this way and stored at −20° retained its biological activity for months.

RF DNA was prepared according to the method of Jansz *et al.* (1966b), except for the Sephadex G-100, which was replaced by Sepharose 2B. The RF II DNA was isolated from the RF DNA preparation by means of a CsCl gradient containing ethidium bromide by the procedure of Radloff *et al.* (1967). Sephadex and Sepharose were products of Pharmacia, Uppsala, Sweden.

Assay of infective ϕX174 DNA. Infective phage DNA was assayed in spheroplasts of *E. coli* K58. Spheroplasts were prepared as described by Guthrie and Sinsheimer (1960), except for the use of PAM medium (Guthrie and Sinsheimer, 1963) instead of nutrient broth. For the assay, 1 volume of DNA was mixed with 1 volume of spheroplasts. After adsorption for 15 min at room temperature, 8 volumes PAM medium was added and the suspension was shaken for 3 hr at 30°.

When the spheroplasts were infected with mutant *ss* DNA at saturating DNA concentrations, about 10^7–10^8 phage particles/ml were formed. For mutant RF DNA the yield was about 1 $\times$ 10^6 particles/ml at saturating DNA concentrations.

Hybridization. Equal volumes of 70 S DNA or *ss* DNA fragments (A_{260nm} = 0.05; 2 $\times$ SSC) were mixed with RF II DNA or *ss* DNA (A_{260nm} = 0.01; 2 $\times$ SSC). As selfings we used 1:1 mixtures of RF II DNA or *ss* DNA with 2 $\times$ SSC. The DNA solution was denatured by placing the DNA in boiling water for 5 min (Marmur and Doty, 1961). Next the DNA was immediately incubated in a 64° water bath for renaturation. Samples were diluted 10-fold in cold 0.05 *M* Tris, pH 8.1 and stored in ice until assayed in spheroplasts.

Fragmentation of wild-type ss DNA with deoxyribonuclease. Wild-type *ss* DNA was

made 1.0 in absorbance at 260 nm in 0.01 *M* potassium phosphate + 2 m*M* $MgCl_2$, pH 7.0. The deoxyribonuclease (EC 3.1.4.5) was dissolved in the same buffer (0.1 μg/ml). To 1 volume DNA 0.05 volume of deoxyribonuclease was added and the mixture was incubated at 25°. In order to stop the enzyme action the DNA solution after 90 min enzyme action was mixed with 0.1 volume of 0.1 *M* sodium citrate. This solution was made 2 × SSC with an absorbance at 260 nm of 0.5. The fragment preparation obtained was no longer infective. The fragments had an average $s_{20,w}$ of 2.9 S.

Sucrose gradient centrifugation. This was done in the SW 25 rotor of a Spinco Model L 50 preparative ultracentrifuge at 4°. The neutral sucrose solution contained 1 *M* NaCl–0.05 *M* sodium citrate, pH 7.0, whereas the alkaline sucrose solution was 1 *M* NaCl–0.05 *M* sodium citrate, pH 12.4. One to two milliliters of DNA solution were layered on 28 ml of sucrose (5–20%). After centrifugation the tubes were punctured, the contents were analyzed at 254 nm with a LKB 4701 A Uvicord ultraviolet absorptiometer, and 0.7-ml fractions were collected.

Sedimentation analysis. Velocity sedimentation was performed in a Spinco Model E analytical ultracentrifuge equipped with an ultraviolet optical system with photoelectric scanner. Concentrations of DNA used for sedimentation were 20 μg/ml. The sedimentation coefficients were corrected in the usual way to obtain $s_{20,w}$ values.

RESULTS

70 S DNA Purification and Characterization

A preparation of DNA from purified 70 S particles contained considerable intact viral DNA as was judged from the infectivity on spheroplasts. Much of this was removed by centrifugation in a neutral sucrose gradient. The residual infectivity could be removed only by centrifugation in an alkaline sucrose gradient (Fig. 1). The infectivity was then completely removed from the extinction peak that contained the 70 S DNA. This noninfective

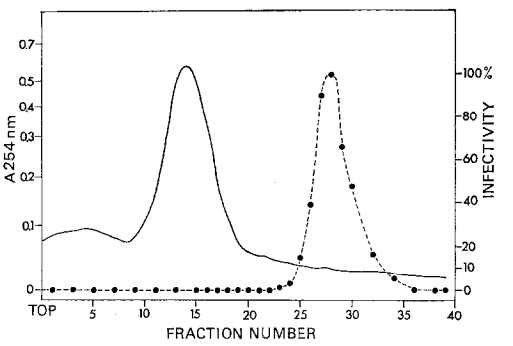

FIG. 1. Alkaline sucrose gradient centrifugation of wild-type 70 S DNA. The 70 S DNA in 1 *M* NaCl–0.05 *M* sodium citrate was made 0.1 *N* NaOH. Two milliliters of the alkaline DNA solution was layered on top of 28 ml of sucrose solution (5–20% sucrose in 1 *M* NaCl–0.05 *M* sodium citrate, pH 12.4) and spun for 39 hr at 24,000 rpm. The tubes were punctured and 0.7 ml fractions were collected. The fractions were neutralized with 0.25 ml of 1 *M* potassium phosphate, pH 7.0. ——, absorbance at 254 nm; ●-----●, infectivity on spheroplasts.

DNA preparation was used in the hybridization experiments.

A sedimentation coefficient, $s_{20,w}$ = 9.0 S was found for wild-type 70 S DNA. From this the molecular weight was calculated to be 3.8×10^5 (Studier, 1965). This value is in reasonable agreement with that of 4.2×10^5 as found by Eigner *et al.* (1963).

Conditions of Hybridization

Denaturation of RF DNA increases its infectivity several times (Sinsheimer, 1968; Pouwels *et al.*, 1968). This increase is due to a higher efficiency of *ss* DNA in the spheroplast assay as compared with RF DNA. During renaturation the complementary strands anneal and the infectivity drops to about the value it had before denaturation. During hybridization of 70 S DNA with RF DNA, this reannealing of RF strands competes with the annealing of the much smaller 70 S DNA and the RF DNA strands. To minimize the annealing of the RF strands we used low RF DNA concentrations (A_{260nm} = 0.01). In order to favor hybridization with 70 S DNA we used a large excess of 70 S DNA (A_{260nm} = 0.05).

TABLE I

WILD-TYPE:MUTANT RATIO[a] DURING HYBRIDIZATION OF WILD-TYPE 70 S DNA AND WILD-TYPE SS DNA FRAGMENTS WITH RF II DNA OF MUTANTS FROM FIVE CISTRONS

Mutant		Wild-type 70 S DNA		Wild-type *ss* DNA fragments		Selfing[b]	Reversion frequency × 10^4	(1)[d]:(3)
		Wild-type: mutant × 10^4	Increase[c] (fold)	Wild type: mutant × 10^4	Increase[c] (fold)	Wild-type: mutant × 10^4		
		(1)	(2)	(3)	(4)	(5)	(6)	(7)
*am*3 RF	A[e]	<2.0		<5.4		<3.1		
	B	46	46	22	22	<1.0	0.2	2.1
	C	54	45	25	21	<1.2		2.2
*to*8 RF	A	<2.7		<2.7		<2.9		
	B	230	135	22	13	1.7	2.0	10.5
	C	87	36	24	10	2.4		3.6
*am*18 RF	A	<4.8		<3.2		<7.1		
	B	26	26	9.2	9	<1.0	0.3	2.8
	C	48	48	14	14	<1.0		3.4
*em*7 RF	A	<2.2		<2.6		<3.5		
	B	590	2458	220	917	<0.24	0.1	2.7
	C	460	1315	330	943	<0.35		1.4
*ts*27 RF	A	<1.5		<1.6		<1.3		
	B	36	450	5.8	73	<0.08	0.03	6.2
	C	24	160	9.2	61	<0.15		2.6

[a] The wild-type:mutant ratio of the *sus* mutants is calculated from the plaque numbers on C at 37° and on Y at 37°, and for the *ts* mutants from those on C at 41° and on C at 30°. If the sign < is used, no wild-type plaques were found if plated undiluted.

[b] In the selfings, the mutant RF DNA is mixed with an equal volume of 2 × SSC.

[c] Columns (2) and (4) give the minimal increase (after hybridization) of the wild type:mutant ratios of the hybridization mixtures as compared with those of the selfings.

[d] Column (7) gives the ratios of the columns (1) and (3). This represents the difference in ratio obtained after hybridization with the 70 S DNA and with the *ss* DNA fragments.

[e] A gives the ratio after mixing, but before denaturation. B gives the ratio after denaturation, but before renaturation. C gives the ratio after denaturation and 15 min renaturation.

Hybridization of Wild-Type 70 S DNA or Wild-Type ss DNA Fragments with Mutant RF DNA

Wild-type 70 S DNA was hybridized with RF II DNAs of mutants from five different complementation groups (Borrias *et al.*, 1969): *am*18, *am*3, *to*8, *em*7, and *ts*27. The results are summarized in Table 1. They show that hybridization of wild-type 70 S DNA with mutant RF DNA gives a large increase in wild type:mutant ratio. The hybridization is so rapid that immediately after denaturation (0 min renaturation) most of the wild-type activity is already present. If compared to the selfings the difference in wild type:mutant ratio is even greater (26–1300 times, column (2), Table 1). The increase relative to the selfings is exclusively due to an increase in wild-type particles (growth on C or at 41°) because the total yields on Y (of *sus* mutants) or at 30° (of *ts* mutants) of the hybridization mixtures do not differ from the yields of the corresponding selfings.

In addition each mutant RF DNA was also hybridized with a preparation of randomly fragmented wild-type *ss* DNA. Because this preparation contained fragments from every part of the genome, it had to give partially double-stranded heteroduplexes, and thus an increase in wild-type particles after hybridization with the RF DNA. Moreover, the wild type:mutant ratio for each mutant can be com-

pared with the ratio found after hybridization with 70 S DNA. This comparison gives information on the relative frequency of each cistron in the 70 S DNA preparation.

From the increase in wild-type particles after hybridization it is evident that the wild-type 70 S DNA hybridizes at least as well with the RF DNA as do the wild-type *ss* DNA fragments.

From the fact that the wild-type 70 S DNA is capable of producing wild-type particles after hybridization with RF DNA of mutants from five different cistrons we conclude that the 70 S DNA is not a homogeneous small part of the DNA but rather consists of fragments of the majority of the ϕX174 genome.

In the hybridization experiments with the five mutant RF DNA's, the ratios obtained after hybridization with 70 S DNA are equal or slightly higher than the ratios with the *ss* DNA fragments. So, because the *ss* DNA fragments are random fragments, it is reasonable to conclude that the 70 S DNA preparation consists of random or nearly random DNA fragments.

Hybridization of Wild-Type 70 S DNA and Wild-Type ss DNA Fragments with Mutant ss DNA

If the 70 S particles originate from intact 114 S particles, the DNA of the 70 S particle will be of the viral (+) strand type. This implies that the 70 S DNA will not be complementary to intact viral (+) DNA and cannot anneal with it. This was tested by hybridization of the wild-type 70 S DNA with *am*18 and *am*3 *ss* DNA. The same was done with wild-type *ss* DNA fragments instead of 70 S DNA. Indeed the mixture of wild-type 70 S DNA or *ss* DNA fragments with mutant *ss* DNA gives no measurable increase in wild-type:mutant ratio after hybridization. Hence it may be concluded that there has been no annealing.

Hybridization of ts27 70 S DNA with ts27 RF DNA and with am18 RF DNA

A control for the specificity of the annealing process was to hybridize *ts*27 S DNA with *ts*27 RF DNA and with *am*18 RF DNA. Correct pairing of the *ts*27 70 S DNA with *ts*27 RF DNA will result in a partial double-stranded DNA molecule which is *ts*27 in both strands and therefore no increase in wild-type particles is to be expected. On the other hand, an increase in wild type: mutant ratio would indicate an aspecific hybridization. A test for the usefulness of the *ts*27 70 S DNA is hybridization with *am*18 RF DNA. The increase in wild-type progeny should be the same as with wild-type 70 S DNA. The results are shown in Table 2. No increase in wild-type particles is found with *ts*27 RF DNA and *ts*27 70 S DNA, whereas with *am*18 RF DNA and *ts*27 70 S DNA the increase is large and well comparable with the increase when wild-type 70 S DNA is used. Hybridization of 70 S DNA with RF DNA can therefore be considered to be a specific process.

TABLE 2

WILD-TYPE:MUTANT RATIO DURING HYBRIDIZATION OF *ts*27 70 S DNA WITH *ts*27 RF II DNA AND WITH *am*18 RF II DNA[a]

Mutant		*ts*27 70 S DNA		Selfing	
		wild type: mutant $\times 10^5$	Increase (fold)	Wild-type: mutant $\times 10^5$	Reversion frequency $\times 10^5$
*ts*27 RF	A	<2.8		<3.1	
	B	<0.7		<0.7	0.3
	C	<1.4		<1.5	
*am*18 RF	A	<9.2		<7.8	
	B	1100	458	2.4	3.0
	C	810	162	5.0	

[a] For explanations see the legend to Table 1.

DISCUSSION

Wild-type particles are formed upon hybridization of wild-type 70 S DNA with mutant RF DNA (Table 1). The absence of wild-type particles after hybridization of *ts*27 70 S DNA with *ts*27 RF DNA (Table 2) demonstrates that hybridization is a process that involves specific pairing of the fragment with the RF DNA. In this respect this hybridization is in complete agreement with the hybridization between RF DNA fragments with intact *ss* DNA (Weisbeek and Van de Pol, 1970).

The absence of wild-type particles after hybridization of 70 S DNA with *ss* DNA shows that the 70 S DNA is of the viral (+) strand type.

The results presented in Table 1 clearly demonstrate that the wild-type 70 S DNA hybridizes with the mutant RF II DNA as well as the wild-type *ss* DNA fragments do with the mutant RF DNAs. The ratios with the 70 S DNA are higher than with the *ss* DNA fragments. This may be due to the difference in size. The molecular weight of the 70 S DNA is 3.8×10^5, whereas the molecular weight of the *ss* fragments is about 1 to 4×10^4.

The hybridization rate between RF DNA fragments and intact *ss* DNA (Weisbeek and Van de Pol, 1970) is much lower than the rate of hybridization of 70 S DNA with RF DNA obtained in the present experiments. Whereas in the latter experiments the maximum hybridization level was reached immediately after denaturation, the hybridization between RF DNA fragments and intact *ss* DNA required at least 10 min incubation at 64° to reach its maximum. This difference may be a consequence of the fact that fragments of the minus strand of the RF DNA can hybridize with viral (+) *ss* DNA, but also with its own plus strand fragments (present in high concentration), by which the actual concentration of the minus strand is lowered. In the case of the 70 S (+) DNA, however, the fragments can only anneal with the minus strand of the RF DNA.

The wild-type 70 S DNA preparation yields wild-type progeny after hybridization with RF II DNA of mutants from all five different cistrons tested. This means that the wild-type 70 S DNA preparation is a heterogeneous population of DNA fragments. Every fragment contains nucleotide sequences from one or more different cistrons. All five cistrons are present in the fragment preparation.

When the wild type:mutant ratios after denaturation and renaturation (C) of the wild-type 70 S DNA with the mutant RF DNAs are compared for different mutants, large differences are observed. The ratio varies between 2.4×10^{-3} and 4.6×10^{-2} (nearly 20-fold). The same spread is observed for the ratios after hybridization of the wild-type *ss* DNA fragment with the mutant RF DNAs; between 9.2×10^{-4} and 3.3×10^{-2} (36-fold). However, when for each mutant the ratio for 70 S DNA is compared with the ratio for the *ss* DNA fragments, they differ only by a factor 1.4–3.6. As the wild-type *ss* DNA fragments were random fragments, we may conclude that in the wild-type 70 S DNA preparation the five cistrons tested are nearly equally frequent present. The five cistrons are spread over the genome (Hutchison *et al.*, 1967; Hayashi and Hayashi, 1970) and include about 60% of the viral DNA. Fragments with a molecular weight of 20% of the viral DNA appear to be randomly distributed over 60% of the genome. In view of this it is reasonable to conclude that the 70 S DNA consists of random fragments of the total DNA. Adsorption of 70 S particles to cell debris (Bleichrodt and Knijnenburg, 1969) is thus followed by random ejection of the greater part of the DNA.

Adsorption of the phage particle to the cell wall or to debris in the lysate leads to concerted rearrangement of the structure of the phage coat. This permits the viral DNA to escape through its phage coat. After adsorption to intact cells, the viral DNA enters the cells, presumably through interaction with host cell components; whereas with cell wall fragments, the DNA becomes exposed to degradation, leaving a random viral DNA fragment in the phage coat.

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