JOURNAL OF VIROLOGY, Dec. 1975, p. 1375-1379
Vol. 16, No. 6

Printed in U.S.A.

Alkali Lability of Bacteriophage φW-14 DNA

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Received for publication 17 June 1975

The molecular weight of bacteriophage φW-14 DNA, determined by velocity sedimentation in neutral sucrose gradients, was 92 ± 6 × 10^6. The DNA showed marked fragmentation in alkaline sucrose gradients. This fragmentation was not a consequence of preexisting single-strand interruptions in the DNA, since thermal denaturation of DNA yielded intact single strands. The α-putrescinylthymine groups in φW-14 DNA appeared to be labile; some, or parts of some, of these groups were cleaved from the DNA in alkali.

The DNA of several viruses yields fragments in alkaline sucrose gradients. In bacteriophage T5 DNA, each molecule yields an intact strand and several fragments from the complementary strand (2, 5, 7, 11). In phage SP50 DNA (16), murine cytomegalovirus DNA (15), and herpes simplex virus type 1 DNA (20), some molecules yield intact complementary strands and the remainder yield a heterogeneous mixture of fragments derived from both strands. In the case of murine cytomegalovirus DNA, the fragmentation pattern varies from preparation to preparation, the most carefully prepared samples showing the least fragmentation (15).

T5 does not contain single-strand interruptions; one of the strands contains short RNA sequences that make it alkali labile (17). Herpes simplex virus type 1 DNA also contains ribonucleotides (1, 6).

Phage φW-14 DNA contains the hypermodified pyrimidine α-putrescinylthymine (putThy) (10). When tested as a substrate for the T4 UV-damage-specific nuclease (17), φW-14 DNA, from its behavior in alkaline sucrose gradients, appeared to contain single-strand interruptions before treatment with the enzyme (A. W. Kozinski, personal communication).

This paper describes the effects of alkali on φW-14 DNA. There are no single-strand interruptions in the DNA before treatment with alkali, nor does the DNA appear to contain ribonucleotides.

MATERIALS AND METHODS

Media and buffers. Casamino Acids-mannitol medium (10) and H broth were used for the preparation of stock lysates of φW-14 and T4, respectively. φW-14 was stored in phage buffer (4), and T4 was stored in 0.01 M Tris-hydrochloride–0.15 M NaCl (TN) buffer, pH 7.4. Tris-Casamino Acids-glucose medium (9) with normal phosphate was used to prepare [^{3}H]thymidine-labeled T4. The same medium, but with 0.2% succinate instead of glucose and only 0.2× normal phosphate, was used to prepare ^{32}P-labeled φW-14.

Bacterial and phage strains. φW-14 was grown on *Pseudomonas acidovorans* strain 29 (10). T4BOr was grown on *Escherichia coli* B23.

Preparation of labeled phages. (i) For [^{32}P]φW-14, $H_3{}^{32}PO_4$ was added to an exponentially growing culture of *P. acidovorans* (1.5 × 10^8 cells/ml) to give a final specific activity of approximately 1 μCi per μg of phosphorus. When the culture density reached 3 × 10^8 cells/ml, φW-14 was added at a multiplicity of infection of 10 and incubation continued until lysis.

(ii) [^{14}C]uracil-labeled φW-14 was prepared as described previously (8).

(iii) φW-14 with the putrescinyl groups of putThy labeled was prepared by using [^{3}H]ornithine (R. A. J. Warren, manuscript in preparation).

(iv) [^{3}H]thymidine-labeled T4 was prepared as described previously for T7 (12) and purified by differential centrifugation.

Purification of φW-14. After lysis, DNase was added (final concentration, 5 μg/ml) and the lysate was shaken gently at 30 C for 30 min. Then Pronase (previously self-digested at 37 C for 30 min) was added (final concentration, 20 μg/ml), and incubation continued for 2 h. The phage was purified by two cycles of low- and high-speed centrifugation at 4 C in a Sorvall SS-1 rotor (10 min at 3,000 × *g*; 30 min at 25,000 × *g*). The final phage pellet was resuspended in 1 ml of TN buffer. The concentrated suspension was layered onto a discontinuous CsCl gradient (2.0 g of a solution of density 1.4 g/ml over 2.0 g of a solution of density 1.6 g/ml). The gradient was centrifuged for 20 min at 30,000 rpm in a Beckman SW-50.1 rotor. The densest, phage-containing band was removed through the side of the tube with a syringe and dialyzed for 4 h at 4 C against 4 liters of TN buffer.

DNA extraction. The phage suspension was made 0.01 M in EDTA. The DNA was extracted from phage by gentle rotation of the suspension with an equal volume of water-saturated phenol. The

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aqueous layer was washed several times with ether to remove residual phenol.

Sucrose gradients. DNA solutions were layered on 4 ml of 5 to 20% sucrose gradients in silicone-treated polyallomer tubes. Alkaline gradients contained 0.2 M NaOH. Gradients for the analysis of thermally denatured DNA contained 50% formamide. Gradients were centrifuged at 35,000 rpm for 3 h in a Beckman SW50.1 rotor. The tubes were punctured and fractions were collected directly onto glass-fiber filter disks. The disks were dried and overlaid with toluene-based 1,4-bis-[2]-(5-phenyloxazolyl)benzene and 2,5-diphenyloxazole scintillant, and the radioactivity was determined in an Isocap 300 scintillation spectrometer.

Hydroxyapatite chromatography. Hydroxyapatite was prepared according to Miyazawa and Thomas (14). The column was run at room temperature, using a gel bed 0.6 cm deep and 0.7 cm in diameter. All buffers were potassium phosphate, pH 7.0, containing 1% formamide. A mixture of T4 and ϕW-14 DNA was heated for 3 min at 80 C in 0.05 M buffer containing 50% formamide, cooled on ice, and then dialyzed for 4 h at 4 C against 0.05 M buffer containing 1% formamide. DNA samples were applied to the column and washed in with about 3 ml of 0.05 M buffer to allow the column to equilibrate, and the DNA was eluted with a gradient of buffer (0.05 to 0.35 M). The gradient was generated with an ISCO Dialagrad gradient apparatus, using an auxiliary mixing chamber to reduce the dead volume between the pumps and the column to approximately 0.5 ml. The flow rate was 10 ml/h for 4 h. Fractions of 0.3 ml were collected during the washing in of the DNA to determine the amount of applied radioactivity retained by the column. Fractions of 1.0 ml were collected during the running of the gradient. Fractions were collected by using a Gilson FC 80H collector. The refractive index of every fifth fraction was measured to determine the gradient profile. Samples of each fraction were spotted on Whatman GFA disks for the determination of radioactivity. Recovery of applied radioactivity was always greater than 90%.

Treatment of DNA with alkali. For the determination of the release of radioactivity from ϕW-14 DNA by exposure to alkali, the DNA solution was made 0.2 M in NaOH and then incubated for 4 h at room temperature or 20 min in a boiling water bath. Triplicate samples of treated and untreated solutions were spotted on Whatman 3MM filter paper squares. The squares were washed twice in ice-cold 5% trichloroacetic acid, twice in 95% ethanol, and once in ether and dried in air, and the acid-insoluble radioactivity was determined as before.

Treatment of DNA with RNase. A solution of pancreatic RNase (1 mg/ml) in TNE buffer (TN buffer containing 0.01 M EDTA) was held in boiling water for 5 min. The activity of this stock enzyme solution was checked routinely, using [^{3}H]uridine-labeled RNA from 3T3 mouse embryo cells. Native and heat-denatured DNA samples were incubated with the enzyme (final concentration, 25 μg/ml) in TNE at 37 C. Then acid-insoluble radioactivity of treated and untreated samples was determined as for alkali-treated DNA.

Shearing of DNA. ϕW-14 DNA was sheared by blending in a Virtis microblender. The size of the sheared fragments was determined by velocity sedimentation in neutral sucrose gradients.

Isotopes. [*methyl*-^{3}H]thymidine, [2-^{14}C]uracil, [G-^{3}H]ornithine, and ^{32}P as $H_3{}^{32}PO_4$ were from New England Nuclear Corp.

RESULTS

Velocity sedimentation of ϕW-14 DNA in neutral sucrose gradients. ϕW-14 DNA sedimented as a single, sharp band in neutral sucrose density gradients (Fig. 1A). Its molecular weight was calculated (3) to be $92.4 \pm 5.7 \times 10^6$ (10 determinations) relative to a value of 110×10^6 for T4 DNA.

Velocity sedimentation in alkaline sucrose gradients. In alkaline sucrose gradients, ϕW-14 DNA showed a markedly heterogeneous band pattern (Fig. 1B). The fastest-sedimenting component was calculated (19) to be of molecular weight $42.9 \pm 4.0 \times 10^6$ (seven determinations). The slowest-sedimenting components were calculated to be in the molecular weight range of 10^6. Although some intact single strands were probably present, their relative amount and the overall band pattern varied from experiment to experiment, even with the same preparation of DNA. The amount of intact single strands present was always considerably less than 50% of the material recovered from the gradient (Fig. 1B). Recoveries were usually greater than 90%.

Velocity sedimentation of heat-denatured DNA in neutral sucrose gradients. Heat-denatured DNA was analyzed to see whether there were single-strand interruptions present before exposure to alkali. ϕW-14 DNA is unusual in that the modified base it contains is positively charged at neutral pH. Heat-denatured ϕW-14 DNA adsorbed very strongly to the polyallomer tubes, even after treatment of the tubes with bovine serum albumin and polyvinylpyrrolidone. The T4 reference DNA also adsorbed to the tubes in the presence of heat-denatured ϕW-14 DNA. In alkaline gradients, the putrescinyl groups were uncharged, and these effects were not seen.

This difficulty was overcome by: (i) filling the centrifuge tubes with freshly boiled ϕW-14 DNA (5 μg/ml), leaving them at room temperature for 30 min, draining them dry, and storing them at 4 C; (ii) denaturing the labeled DNA in 50% formamide at 80 C for 10 min; and (iii) using sucrose gradients containing 50% formamide. In this way, it was shown that ϕW-14 DNA did not contain single-strand interruptions before exposure to alkali (Fig. 1C). ϕW-14 DNA that had been sheared to approximately sixteenths by blending, or which had been

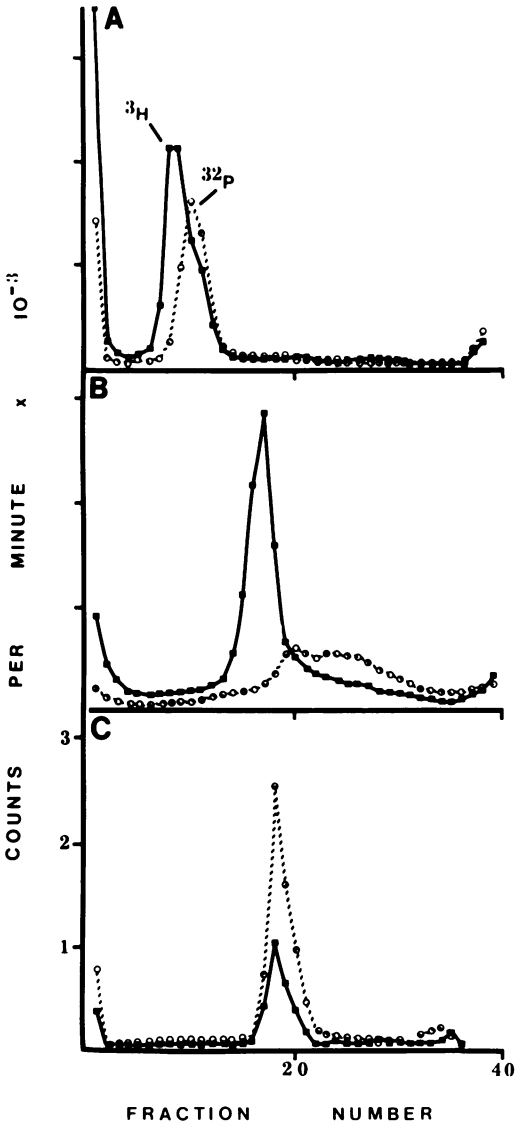

FIG. 1. *Sucrose gradient sedimentation of φW-14 and T4 DNA. φW-14 DNA was extracted from phage labeled with $^{32}PO_4$ (○), and T4 DNA was extracted from phage labeled with [^3H]thymidine (■). (A) Neutral gradient, native DNA. (B) Alkaline gradient. (C) Neutral gradient containing 50% formamide, DNA denatured by heating in 50% formamide.*

treated with 0.2 N NaOH at room temperature for 30 min and then neutralized, separated cleanly from intact T4 DNA when they were mixed, denatured, and centrifuged under these conditions. Therefore, the T4 and φW-14 DNAs were not forming a complex in the formamide gradients.

Hydroxyapatite chromatography verified that the conditions used did indeed denature φW-14 DNA (Fig. 2). Native and denatured T4 DNA were eluted by 0.220 and 0.114 M phosphate, respectively (averages of three determinations). Native and denatured φW-14 DNA were eluted by 0.180 and 0.111 M phosphate, respectively (averages of three determinations). There was also a 20% increase in the absorbance of φW-14 DNA at 260 nm after denaturation under these conditions.

Effect of alkali on φW-14 DNA. φW-14 DNA was labeled in the phosphodiester backbone with ^{32}P, in the pyrimidines with [^{14}C]uracil, and in the putrescinyl groups alone with [^3H]ornithine. The release of acid-soluble counts was measured after treatment of the preparations with alkali at 37 C.

The most striking effect of alkali was the solubilization of up to 25% of the radioactivity

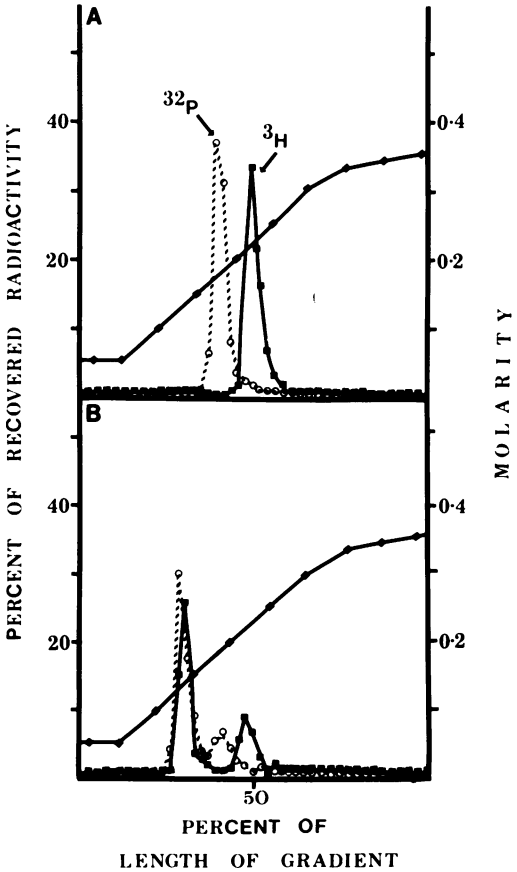

FIG. 2. *Hydroxyapatite chromatography of φW-14 and T4 DNA. φW-14 DNA was extracted from phage labeled with $^{32}PO_4$ (○), and T4 DNA was extracted from phage labeled with [^3H]-thymidine (■). (A) Native DNA. (B) Thermally denatured DNA. The conditions for chromatography are described in the text. (◆) Molarity of phosphate.*

of [^{3}H]ornithine-labeled DNA by treatment at room temperature. About 6% of the radioactivity of [^{14}C]uracil-labeled DNA and about 3% of that of ^{32}P-labeled DNA were also solubilized by alkali (Table 1). There was some variation in the amounts of radioactivity solubilized from experiment to experiment. Treatment of the DNA at 100 C did not increase the amounts of radioactivity solubilized by alkali.

Effect of RNase on ϕW-14 DNA. The radioactivity solubilized by alkali might have come, at least in part, from ribonucleotides in the DNA. However, pancreatic RNase solubilized only about 1% of the radioactivity of native and heat-denatured DNA labeled with ^{32}P, [^{3}H]ornithine, or [^{14}C]uracil (Table 2).

DISCUSSION

The molecular weight of ϕW-14 DNA is at least 92 $\times$ 10^6. This is a minimum estimate because the unusually low buoyant density of the DNA may affect its sedimentation rate in sucrose gradients.

The lack of correlation between the amounts of radioactivity solubilized by alkali and by RNase argues against the presence of ribonucleotides making ϕW-14 DNA alkali labile. In T5 (17) and herpes simplex type 1 (1, 6) DNAs, there is a correlation between the amounts of radioactivity solubilized by alkali and by RNase. Furthermore, the putThy content of ϕW-14 DNA is about 12% (10). Alkali solubilizes about 20% of the radioactivity of [^{3}H]ornithine-labeled DNA, 6% of that of [^{14}C]uracil-labeled DNA, and 2% of that of ^{32}P-labeled DNA. It is significant that 20% of the putThy residues is equivalent to some 2 to 3% of the total bases and some 5% of the pyrimidines in ϕW-14 DNA. (It is possible, of course, that putThy residues interfere with the cleavage of

TABLE 1. *Effect of alkali on ϕW-14 DNA*[a]

DNA sample[b]	Label in DNA								
	^{32}P			[^{14}C]uracil			[^{3}H]ornithine		
	Counts/min[c]		TCA soluble	Counts/min		TCA soluble	Counts/min		TCA soluble
	Before	After	(%)	Before	After	(%)	Before	After	(%)
1	1,730	1,680	3	1,100	1,000	9	1,580	1,400	11
2	3,270	3,220	2	2,750	2,680	3	1,810	1,440	20
3	4,370	4,220	3	3,010	2,820	6	1,610	1,210	25

[a] Reaction mixtures contained DNA in TNE made 0.2 N in NaOH in a total volume of 0.25 ml. After 4 h at room temperature, triplicate 25-μl samples from each tube were spotted on Whatman 3MM discs and the trichloroacetic acid (TCA)-insoluble radioactivity was determined as described in the text. The control mixtures contained DNA in 0.25 ml of TNE.

[b] These are different preparations of DNA, each containing ^{32}P, ^{14}C, or ^{3}H.

[c] Average of three samples.

TABLE 2. *Effect of pancreatic RNase on ϕW-14 DNA*[a]

DNA sample[b]	Label in DNA								
	^{32}P			[^{14}C]uracil			[^{3}H]ornithine		
	Counts/min[c]		TCA soluble	Counts/min		TCA soluble	Counts/min		TCA soluble
	Before	After	(%)	Before	After	(%)	Before	After	(%)
1									
Native	1,500	1,540	(0)						
Denatured	1,490	1,450	3						
2									
Native	3,170	3,130	1	2,700	2,700	0	2,180	2,150	1
Denatured	3,050	3,060	0	2,700	2,590	4	2,160	2,140	1
3									
Native	4,390	4,400	0	3,060	3,060	0	1,660	1,670	0
Denatured	4,190	4,070	3	2,850	2,860	0	1,540	1,520	1

[a] Native and thermally denatured DNA samples in TNE buffer were incubated with pancreatic RNase (final concentration, 25 μg/ml) for 2 h at 37 C. Then triplicate 25-μl samples from each tube were spotted on Whatman 3MM squares and the trichloroacetic acid (TCA)-insoluble radioactivity was determined as described in the text.

[b] These are different preparations of DNA, each containing ^{32}P, ^{14}C, or ^{3}H.

[c] Average of three samples.

adjacent ribonucleotide sequences by RNase.)

Rather, it seems that the presence of putThy makes φW-14 DNA alkali labile, since alkali removes preferentially radioactivity associated with the putThy groups. The putrescinyl moiety of putThy may be labile under other conditions. Mass spectrometry of the penta(trimethylsilyl) derivative of putThy yields, as a major product, an ion of m/e 269, which is formed by fragmentation of the molecule between the thymine methyl and the secondary amine nitrogen (J. A. McCloskey, personal communication). Preliminary evidence suggests that UV irradiation of φW-14 DNA may remove some or parts of some of the putThy groups (R. A. J. Warren, unpublished observations).

The way in which alkali-labile putThy groups also make the phosphodiester backbone alkali labile remains to be determined. It is significant that SP15 DNA, in which almost half the thymine methyls are substituted with dihydroxypentyl groups, is also extremely alkali labile. Exposure of SP15 DNA to 0.3 N NaOH at 37 C gradually reduces it to fragments of molecular weight about 10^5 (13).

The decreased hypodensity of φW-14 DNA in alkaline CsCl gradients (10) agrees with the loss of some or parts of some of the putThy groups.

It is not surprising that native φW-14 DNA elutes before T4 DNA during chromatography on hydroxyapatite. φW-14 DNA, with its smaller net negative charge, would bind less strongly than T4 DNA to the gel. On the other hand, denatured φW-14 DNA may have a more ordered structure than denatured T4 DNA, so that these forms of the DNAs are eluted together.

ACKNOWLEDGMENTS

This research was supported by grant A-3686 from the National Research Council of Canada.

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