EQUAL SENSITIVITY OF THE TWO STRANDS OF ϕX174 REPLICATIVE DNA TO BREAKAGE BY IONIZING RADIATION

PHILLIP M. ACHEY *and* DEBORAH G. COURTNEY

From the Radiation Biology Laboratory and the Department of Microbiology, University of Florida, Gainesville, Florida 32601

ABSTRACT The supertwisted, double-stranded, replicative intermediate of ϕX174 DNA (RFI) has been used to determine whether one of the two strands of the double helix is uniquely sensitive to induction of single-strand breaks by ionizing radiation. This could result from a particularly sensitive base sequence or a transfer of energy to a specific location of the DNA molecule. The results indicate that both strands of the double helix are equally broken, even though their base compositions are significantly different. If there are "hot spots" in the strands, then they are present in equal amounts in each strand.

INTRODUCTION

This study was designed to answer whether one of the two strands of the DNA double helix is preferentially sensitive to single-strand break induction by ionizing radiation. Unlike ultraviolet radiation, ionizing radiation releases energy in a random fashion. This does not preclude the possibility that biological molecules may contain regions uniquely sensitive to ionizing radiation action. Since the *G* values for destruction of DNA bases have been shown to be different (Weiss, 1964), it is possible that unique base sequences in one of the strands could cause a high sensitivity of that strand to radiation damage. Another possibility is that energy deposited by ionizing radiation is transferred to a specific region of the DNA where it then interacts with the mollecule. Energy transfer along a DNA molecule has been suggested by Pearlstein (1968). A recent publication has indicated an extensive intramolecular migration of radiation-induced charge or excitation energy to the 5-bromodeoxyuridine (BUdR) sites in the DNA (Fielden et al., 1971). It should be noted that neither incorporation of BUdR nor a change in the gross base composition of DNA has any significant effect on the production of strand breaks (Szybalski, 1967); however, this does not distinguish whether one of the two strands of the double helix is more sensitive to strand breaks.

The supercoiled intermediate of ϕX174 DNA synthesis (RFI) has been used in these studies. After ionizing radiation, the RFI form is converted to an open form

(RFII) by one or more single-strand breaks in either strand. Upon melting those RFII molecules which contain only one break, a single-strand rod (broken) and ring (unbroken) are obtained. Equal sensitivity of the two strands would be demonstrated by an equal distribution of the strands in the ring and rod populations.

We first separated on alkaline sucrose gradients the single-stranded rings and rods resulting from those molecules which contained single-strand breaks after irradiation. The dose range for these experiments was 20–80 krad. This dose range was chosen so that only a small fraction of the molecules contained two or more single-strand breaks. The D_{37} for single-strand break production in RFI DNA is 200 krad under our conditions (Achey et al., 1971). The difference in base composition of the two strands of the RFI molecules results in a density difference between the two strands (Siegel and Hayashi, 1967). The heavy component is the ϕX174 phage strand ("+" strand), and the light component is the complementary strand ("−" strand). These strands can be separated on equilibrium alkaline CsCl gradients (Burton and Sinsheimer, 1965). The results show an equal proportioning of the + and − strands into the ring and rod populations. We conclude that the two strands of the double helix are equally sensitive to single-strand break induction.

MATERIALS AND METHODS

Bacterial and Viral Strains

The bacterial and viral strains were obtained from Dr. Fred Funk, then of the laboratory of Dr. R. L. Sinsheimer, California Institute of Technology, Pasadena, Calif. The bacterial strain used as a host for preparing RFI DNA was *Escherichia coli* HF 4704 (*hcr*$^-$, *thy*$^-$). The viral strain was ϕX174 am3, a lysis-defective mutant (Hutchison and Sinsheimer, 1966).

Preparation of RFI DNA

A slightly modified version of the procedure of Knippers et al. (1969) was followed in RFI preparation. A 600 ml culture of *E. coli* HF 4704 was grown to exponential phase and a titer of 2×10^8 in minimal salts medium which contains 0.5 g NaCl, 8.0 g KCl, 1.1 g NH_4Cl, 12.1 g Tris, 28 mg KH_2PO_4, 0.8 g sodium pyruvate, 2.7 g Casamino acids, 14 mg L-tryptophan, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.1 mmol $CaCl_2 \cdot 2H_2O$, 0.16 mmol Na_2SO_4, and 2.0 g glucose per liter, called TPGA medium. Mitomycin C (K & K Laboratories, Inc., Plainview, N. Y.) was added to a final concentration of 50 μg/ml, and aeration was stopped and the culture protected from light. This treatment stopped host cell DNA synthesis, but did not interfere with RFI synthesis (Lindquist and Sinsheimer, 1967). After 10 min, the cells were washed free of mitomycin C on a 150 mm diameter S & S type B6 membrane filter (Schleicher and Scheull, Inc., Keene, N. H.) with 3 vol prewarmed TPGA salts (TPGA medium without glucose). The cells were resuspended into 600 ml TPGA medium (37°C), and after 2 min ϕX174 am3 was added to a multiplicity of infection of 5. 30 s later, [*methyl*-^{3}H]thymine (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) was added to a 7.5 μCi/ml final concentration, then followed 30 s later with chloramphenicol at a final 30 μg/ml concentration. After 90 min of growth, the cells were pelleted and resuspended into 50 ml 0.05 M Tris, 0.001 M ethylenediaminetetraacetate (EDTA), pH 8.1 (TE buffer), frozen, thawed, and lysed with lysozyme (80 μg/ml). After treatment with Sarkosyl (0.4%) and pronase (40 μg/ml) for 2 h at 37°C,

the lysate was extracted twice in the cold with TE-saturated phenol. The phenol was dialyzed away against TE buffer, and the preparation was treated with RNase A (1 mg/ml concentration, heated 10 min at 100°C, Worthington Biochemical Corp., Freehold, N. J.) at a concentration of 50 μg/ml for 1 h at 37°C. Linear sucrose gradients consisted of 18 ml of 5% sucrose and 17 ml of 20% sucrose in 0.05 M Tris, 0.001 M EDTA, 0.001 M NaCl at pH 8.1. The gradients were prepared at room temperature and then allowed to stand in the cold for 18 h. A 4 ml volume of the preparation was layered onto each of six sucrose gradients. Centrifugation was performed in a Spinco SW 27 rotor (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) at 21,000 rpm for 20 h at 5°C. The gradients were collected from the top with an ISCO gradient fractionator (Instrumentation Specialties Co., Lincoln, Neb.) at 2½ ml/min collection rate into 0.9 ml fractions. 25 μl samples were removed from each fraction for radioassay. Those fractions containing RFI DNA were pooled and extensively dialyzed against 0.015 M NaCl. The DNA, at a concentration of 14 μg/ml (determined spectrophotometrically at 260 nm), was stored at −76°C.

Radioactive Assay of Gradients

Radioactive profiles of all gradients were obtained in the same manner. Fractions were collected on Whatman No. 4 paper disks, dried, and washed in cold 5% trichloroacetic acid and cold ethanol. After drying, the disks were counted in a toluene-base scintillator.

Irradiation

Irradiation with ^{60}Co gamma rays was as described previously (Achey et al., 1971). The dose rate was 7420 rad/min. All irradiations were at −196°C in air.

Neutral Sucrose Gradient Analysis

Conversion of RFI to RFII by irradiation was measured by layering 10 μl of the sample onto 5–20% linear sucrose gradients. The gradients contained 2.5 ml of 5% sucrose and 2.4 ml of 20% sucrose dissolved in 0.05 M Tris, 0.001 M EDTA, 0.001 M NaCl at pH 8.1. After centrifugation for 2.5 h in a Spinco SW 50.1 rotor at 36,000 rpm at 20°C, five-drop fractions were collected from the bottom onto paper disks and counted for radioactivity.

Alkaline Sucrose Gradient Analysis

The broken (rods) and unbroken (rings) single strands of RFII DNA were separated on linear alkaline sucrose gradients, consisting of 2.4 ml of 5% sucrose and 2.3 ml of 20% sucrose dissolved in 0.2 M Na_3PO_4, and 0.001 M EDTA adjusted to pH 12.7 with NaOH. There was a 0.25 ml pad of 60% sucrose at the bottom to prevent the denatured RFI molecules (53S) from pelleting. A mixture of 140 μl irradiated RFI suspension, 10 μl unlabeled *E. coli* carrier DNA (60 μg/ml), 5 μl ^{14}C-labeled φX174 viral DNA (unbroken single strand rings), and 10 μl 0.5 N NaOH was allowed to stand in the cold for 5 min. This mixture was then layered on the alkaline sucrose gradient. The ^{14}C-labeled viral DNA served as a marker to identify the position of single-strand rings on the gradient. The gradients were centrifuged 21 h at 33,000 rpm and at 5°C in an SW 50.1 rotor. Alternate three-drop and one-drop fractions were collected into tubes and onto disks, respectively. Each tube contained 0.1 ml 0.1 M Tris, 0.02 M EDTA, 0.05 M sodium pyrophosphate, pH 8.1 (TEP buffer). The liquid fractions which contained single-strand rings and rods were determined by radioassay of the disks. The fractions containing rings were pooled together as were the fractions containing rods.

Equilibrium Alkaline CsCl Gradients

The amount of + and − strand DNA in the ring and rod populations was measured by alkaline CsCl equilibrium density gradients. To the pooled rings and rods were added 10 μl *E. coli* carrier DNA (60 μg/ml), 5 μl ^{14}C-labeled φX174 viral DNA and enough TEP buffer to adjust the volume to 0.6 ml. The pH was adjusted to 12.7 by adding 15 μl 6 N NaOH. The viral DNA was a marker to determine where the viral (+ strand) DNA banded. This was added to 3.5 ml alkaline CsCl (pH 12.7) in a polyallomer centrifuge tube so that the initial density was between 1.765 and 1.775 g/cm^3. The gradients were centrifuged to equilibrium for 65 h in a 50 Ti rotor at 33,000 rpm and 20°C. Four drop fractions were collected onto disks and counted (Billen and Hewitt, 1966).

RESULTS

Neutral Sucrose Gradient Analysis

Supertwisted RFI molecules are converted to open double-stranded RFII molecules by the introduction of one or more single-strand breaks (Taylor and Ginoza, 1967; Jansz et al., 1968). These two molecular species have sedimentation values of 21S (RFI) and 16S (RFII) on neutral sucrose gradients (Burton and Sinsheimer, 1965). Neutral sucrose gradient profiles of unirradiated and irradiated RFI samples are plotted in Fig. 1. The survival of RFI DNA from single-strand breaks at several doses is shown in Table I. Because of experimental variability, the survival values obtained at the various doses do not coincide exactly with the exponentially shaped dose-survival curve expected for this in vitro system as previously reported (Taylor and Ginoza, 1967; Achey et al., 1971). More important to these experiments, however, is that the fraction of molecules with two or more single-strand breaks be low since it is of interest to determine whether the first break is introduced randomly into the strands of the double helix. This criterion is met as demonstrated by the experiments of Table I. By Poisson statistics, the percentage of all the molecules with two or more breaks is 2 and 8% after doses of 20 and 80 krad, respectively.

Alkaline Sucrose Gradient Analysis

Separation of the unbroken (ring) and broken (rod) strands of the RFII molecules was performed on alkaline sucrose gradients. The alkaline sucrose profile of RFI DNA exposed to 20 krad is plotted in Fig. 2. The fast-moving component contains the denatured RFI molecules and has a 53S value (Burton and Sinsheimer, 1965). The sedimentation values for the rings and rods on alkaline sucrose gradients are 16S and 14S respectively (Schekman and Ray, 1971). A marker of ^{14}C-labeled viral single-strand DNA identified the banding position of the rings. As noted in a previous paper (Achey et al., 1971), there is an increase in the number of single-strand breaks when DNA is strand analyzed on alkaline sucrose gradients. The amount of alkaline-induced breaks was low in these experiments as shown in Table I. This is consistent with the previous results which showed that under primarily direct radiation action conditions (−196°C), there is little alkaline lability.

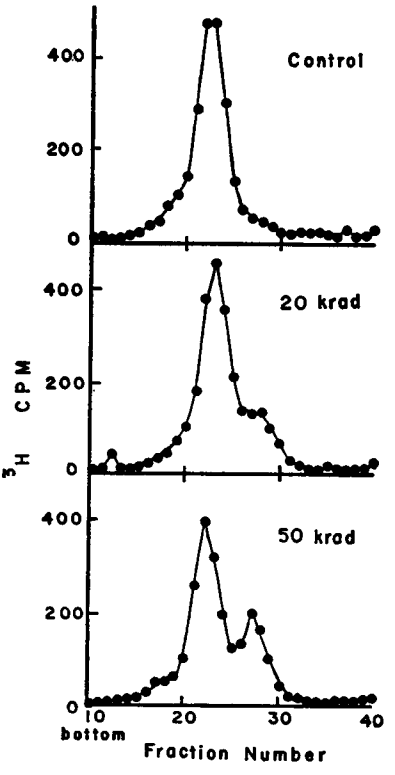

FIGURE 1 Neutral sucrose gradient profiles of RFI DNA for control and irradiated samples.

TABLE I

SURVIVAL OF RFI FROM STRAND BREAKS WHEN ANALYZED ON NEUTRAL AND ALKALINE SUCROSE GRADIENTS

Dose	Fraction surviving on neutral sucrose	Fraction surviving on alkaline sucrose
krad		
20	0.81	0.76
30	0.66	0.65
50	0.71	0.51
80	0.61	0.56

Alkaline CsCl Density Gradient Analysis

The two strands of the ϕX174 replicative form molecules have significantly different base compositions, causing a density difference between the complementary strands in alkaline CsCl density gradients. The viral strand (+ strand) density is 1.765 g/cm^3 and the complementary strand (− strand) density is 1.756 g/cm^3. The purified rings

and rods from the alkaline sucrose gradients are banded on alkaline CsCl gradients in order to measure the ratio of + strands and − strands in the broken and unbroken molecular populations. Density gradient profiles of the rods and rings obtained from the gradient of Fig. 2 are plotted in Fig. 3. The ^{14}C-labeled marker DNA is viral DNA which has the + strand density. In both profiles, there is more ^{3}H

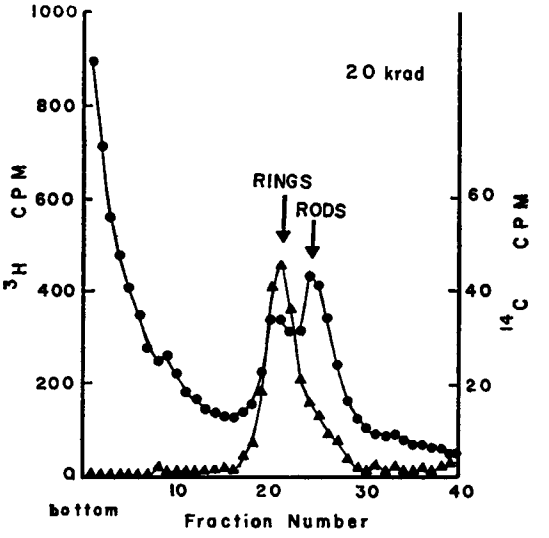

FIGURE 2 Alkaline sucrose gradient profile of RFI DNA after 20 krad dose. ^{14}C-labeled ϕX174 viral DNA is the marker to identify banding position of rings. ^{3}H (●) and ^{14}C (▲) counts per minute are plotted against fraction number.

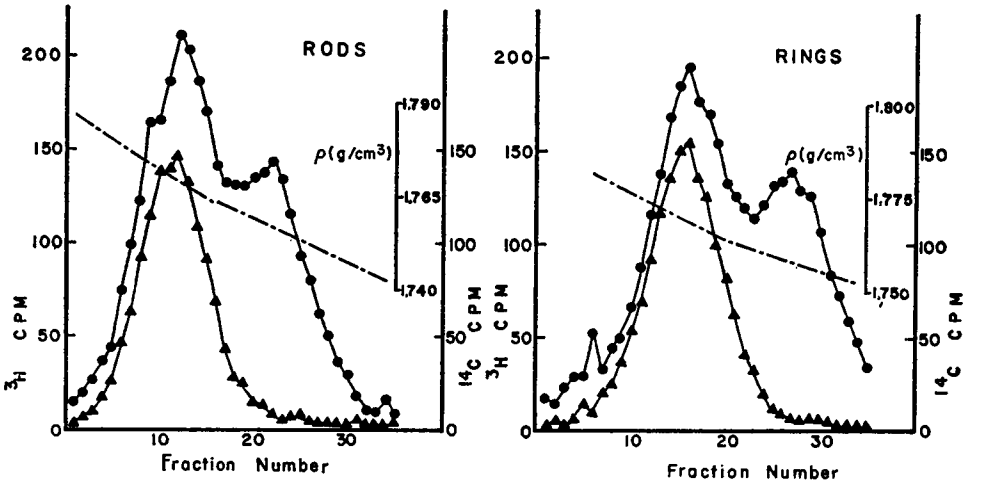

FIGURE 3 Alkaline CsCl density gradients of rings and rods obtained from the gradient of Fig. 2. ^{14}C-labeled ϕX174 viral DNA is the marker to identify position of + strands. ^{3}H (●) and ^{14}C (▲) counts per minute are plotted against fraction number. Buoyant density in grams per cubic centimeter, — — — —.

TABLE II

DISTRIBUTION OF + AND − STRANDS IN THE RING AND ROD POPULATIONS

Dose	Rings		Rods	
	% Label with + density	% Label with − density	% Label with + density	% Label with − density
krad				
20	61	39	63	37
30	52	48	57	43
40	59	41	58	42
50	58	42	59	41
80	53	47	60	40

label banding at the + strand density than at the − strand density. However, the mole percent content of thymine is 32 in the + strand and 24 in the − strand (Siegel and Hayashi, 1967). Since the [^{3}H]thymine was used to label the RFI DNA, the specific activities of the two strands are different. Therefore, equal chemical amounts of the two strands are contained in a population which has 57% of the radioactivity in the + strand and 43% in the − strand. The results of alkaline CsCl density gradients of DNA exposed to various doses of radiation are presented in Table II. There is no apparent selective strand sensitivity to single-strand break induction. The average percent of ^{3}H label with + strand density in the ring and rod populations is 57 and 59%, respectively. Correspondingly, 43% of the ^{3}H label from the fractions pooled as rings banded at the density position of − strand material, as did 41% of the ^{3}H label from the fractions pooled as rods. These values are predicted when equal amounts of the two strands are rings and rods, which would result when either strand is equally likely to receive the first break.

DISCUSSION

A distinguishing feature of ionizing radiation is transfer of energy in discrete packets. The spatial distribution of these interactions follows Poisson statistics (Dessauer, 1922; Dertinger and Jung, 1970), which predicts that ionizing radiation releases its energy in the target material in a random way. Nothing is said, however, about the response of the target. We have studied the response of DNA after in vitro irradiation to determine if one of the two strands of the double helix is uniquely sensitive to damage expressed as strand breaks. The data of Table II show that there is no salient difference in the sensitivtiy of the two strands of the RFI molecule to strand break induction by ionizing radiation. These results do not exclude the possibility of sensitive regions within the strands. If these regions are present, then there are equal numbers of these regions in the two strands.

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