SIZE AND FREQUENCY OF GAPS IN NEWLY SYNTHESIZED DNA OF XERODERMA PIGMENTOSUM HUMAN CELLS IRRADIATED WITH ULTRAVIOLET LIGHT

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ABSTRACT Native newly synthesized DNA from human cells (xeroderma pigmentosum type) irradiated with ultraviolet light releases short pieces of DNA (L-DNA) when incubated with the single-strand specific S_1 nuclease. This is not observed in the case of unirradiated cells. Previous experiments had shown that the L-DNA resulted from the action of S_1 nuclease upon gaps, i.e., single-stranded DNA discontinuities in larger pieces of double-stranded DNA. We verified that the duplex L-DNA, that arises from the inter-gap regions upon S_1 nuclease treatment, has a size which approximates the distance between two pyrimidine dimers on the same strand; this has been observed at different fluences of ultraviolet-light and indicates that the gap is related to or opposite the dimer. A method was devised to measure the size of the gaps. A Poisson distribution analysis of the percentage of the L-DNA produced as a function of S_1 nuclease concentration made this possible. 65% of the gaps corresponded to stretches of 1,250 nucleotides and 35% to stretches of 150 nucleotides. These parameters have been considered in the proposition of a model for DNA synthesis on a template containing pyrimidine dimers.

INTRODUCTION

Many physical and chemical agents alter DNA in such a way that it loses its codifying properties at the modified region. All these agents are mutagenic and/or carcinogenic and their mode of action in mammalian cells is still poorly understood. One such damage is pyrimidine dimer produced by UV light. In this case, the mutagenic and killing actions seem to be triggered during DNA replication on a template containing an unexcised pyrimidine dimer (Maher et al., 1979). As a consequence, a detailed understanding of the events of DNA replication on damaged templates, especially at the point where the replication complex encounters the pyrimidine dimer, has a direct bearing on the mechanisms of mutagenesis and lethality. In vitro experiments have shown that *Escherichia coli* DNA polymerase I and mammalian DNA polymerase α are blocked at the dimers during replication on a UV-irradiated single-stranded DNA (Moore and Strauss, 1979). The replication complex has also been reported to be blocked in vivo by dimers in the cases of mammalian mitochondria (Clayton et al., 1974) and HeLa cells (Edenberg, 1976). A different interpretation has been

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given in other cases, namely that the replication complex halts at the damaged site and resumes synthesis at a point beyond, thus leaving a gap opposite the damaged site (Lehmann, 1972). This controversy is attributable in part to experimental design. Approaches employing DNA sedimentation under alkaline conditions give results whose interpretation is ambiguous in the case of mammalian cells (Painter, 1974). Recently, we introduced an alternative technique in which the native newly synthesized DNA is treated with the single-strand specific nuclease S_1 from *Neurospora* (Meneghini, 1976) or *Aspergillus* (Cordeiro-Stone et al., 1979), and sedimented in neutral sucrose gradients. Under these conditions, the high molecular weight DNA (H-DNA) can be partially transformed into low molecular weight DNA (L-DNA). This transformation is observed only for newly-synthesized DNA from UV-irradiated cells (Meneghini, 1976; Cordeiro-Stone et al., 1979). Furthermore, the action of the S_1 nuclease was shown to be on both sides of the L-DNA, thus precluding the possibility that the L-DNA results from the action of the enzyme only on single-stranded regions of the fork (Cordeiro-Stone et al., 1979). In view of these results, we proposed that L-DNA corresponds to duplex DNA located in between gaps. We further hypothesized that these gaps are formed opposite the pyrimidine dimers (Cordeiro-Stone et al., 1979). In the present work, we report more precise measurements of the L-DNA and compare them to the interdimer distance to test the above hypotheses. A method was also devised to measure the size of the gaps. We have employed human fibroblasts derived from patients with the disease xeroderma pigmentosum and transformed by simian virus-40. Since these cells are almost completely defective in excision repair of dimers (Menck, C., and R. Meneghini, unpublished results), interpretations of our results based solely on this phenomenon can be ruled out.

METHODS

Cells

Xeroderma pigmentosum (XP) fibroblasts, transformed by simian virus-40 (XP12RO, complementation group A) were routinely grown in glass petri dishes in Dulbecco's modified Eagle's medium, pH 7.0, supplemented with 10% calf serum (Grand Island Biological Co, Grand Island, N.Y.), 472 U/ml penicillin, 94 μg/ml streptomycin, and 24 μg/ml ampicillin. The cells were kept in 5% CO_2 humidified atmosphere at 37°C.

UV Irradiation and Cell Labeling

The cells were plated in small petri dishes (12 or 20 cm^2) at ~¼ of the confluent density. Before irradiation, the cells were washed twice with prewarmed buffer solution (PBS) containing 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, and 1 mM $CaCl_2$, pH 7.0. Irradiation was performed with a low pressure germicidal lamp (254 nm, fluence rate 0.5 $J/m^2.s$) with the cells under a 2-ml layer of PBS. The cells were then incubated with fresh medium for 30–45 min and pulse-labeled with [^{3}H]thymidine (30–60 μCi/ml, 50 Ci/mmol, New England Nuclear, Boston, Mass.) for a further 30–45 min period.

Treatment with S_1 Nuclease

The DNA, extracted as previously described (Meneghini, 1976), was treated with S_1 nuclease purified from crude α-amylase according to Vogt (1973) up to and including the diethylaminoethyl (DEAE)-cellulose chromatography. The peak fractions exhibiting nuclease activity upon single-stranded DNA were pooled and concentrated by ultrafiltration (Millipore Corp., Bedford, Mass., immersible molecular separator kit; 10,000 = nmwl). Assay solutions contained [^{3}H]DNA (usually 2–4 μg) in 200 μl of 30

mM sodium acetate, pH 5.0, 300 mM NaCl, 1 mM $ZnSO_4$, 5% glycerol, and 0.1–60 U/ml of S_1 nuclease (1 U of nuclease activity is the amount of enzyme that solubilizes 1 μg of single-stranded DNA in 1 min at 45°C in 30 mM sodium acetate, pH 4.6, 50 mM NaCl, 1 mM $ZnSO_4$, and 5% glycerol (Vogt, 1973). The incubation was performed at 35°C for 30 min and the reaction was stopped by chilling the samples and adding 50 μl of 0.5 M Tris, 0.05 M EDTA, pH 7.5.

Centrifugation and Molecular Weight Determination

Neutral gradients were 5–20% sucrose (wt/vol) in 0.1 M NaCl, 10 mM EDTA, pH 8.0. Alkaline gradients were 5–20% sucrose (wt/vol) in 0.1 M NaCl, 0.1 M NaOH. In both cases the gradient volume was 4.0 ml, on the top of which samples (250 μl) were layered. Centrifugation was carried out in a Spinco SW 50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 32,000 rpm, 20°C, for 100 or 200 min (neutral gradients) or for 120 or 240 min (alkaline gradients). Fractions were pumped out from the bottom of the gradient and dripped onto strips of Whatman 17 paper (Whatman Inc., Clifton, N.J.), which were then washed once in cold 5% trichloroacetic acid, twice in ethanol and once in acetone. After drying, the paper strips were cut, placed in vials with 5.0 ml of scintillation liquid, and their radioactivity measured in a Beckman L-200 liquid scintillation spectrometer.

Molecular Weight Determination

Standards for molecular weight were DNA from phages T2, T4, and λ. In one case, fragments of λ DNA obtained by treatment with *Eco*RI restriction enzyme were used as markers. The molecular weight corresponding to each fraction was determined using the expression of Studier (1965). The number average molecular weight (M_n) was calculated from the expression $M_n = \Sigma r_i / \Sigma (r_i / M_i)$ and the weight average molecular weight (M_w) by the expression $M_w = \Sigma (r_i\ M_i) / \Sigma r_i$, where r_i and M_i are the amount of radioactivity and molecular weight of DNA, respectively, in fraction i. For M_n calculations, the top three fractions were ignored. These expressions are valid when the specific activity of all molecules considered is the same. This requirement is reasonably fulfilled in all cases where the expression was applied. In a few cases, the size distribution of the molecules approximated a random distribution, M_n being calculated from the expressions $M_n = 0.50\ M_w$ and/or $M_n = 0.60\ M_{1/2}$, where $M_{1/2}$ is the median molecular weight (Ehmann and Lett, 1973). The frequency of single strand breaks (ssb/10^8 daltons) produced was calculated from $10^8 (M_n(c)/M_n(t) - 1)/M_n(c)$, where $M_n\ (c)$ is the M_n of untreated (control) DNA and $M_n\ (t)$ is the M_n of treated DNA.

RESULTS

The Frequency of Gaps

When XP12RO cells are irradiated with UV light and pulse-labeled with [^{3}H]thymidine, the newly synthesized native DNA becomes a substrate for the S_1 nuclease, a typical pattern of action of the enzyme being shown in Fig. 1. At three different fluences of UV light, roughly 50% of the original H-DNA was transformed into L-DNA by the S_1 nuclease treatment. This trend of about half the original DNA being refractory to the S_1 nuclease treatment has been observed in other experiments (not shown) with different fluences and has also been noticed with other cells. As previously shown (Cordeiro-Stone et al., 1979) in the case of unirradiated cells, the newly synthesized DNA is virtually unattacked by the S_1 nuclease (Fig. 1 *a*). Rough estimates of the M_n of the L-DNA indicate that its size tends to decrease as the fluence of UV light increases. To evaluate more precisely the size of the L-DNA and, as a consequence, the frequency of the gaps, experiments were carried out in which the DNAs were spun for a longer time (Fig. 2 *a–e*). Under these new conditions, the H-DNA reaches the bottom of the tube and the L-DNA forms a more distinct profile which can be better visualized if one plots the differential values (S_1 treated DNA minus untreated DNA) for each fraction (Fig. 2 *f–j*).

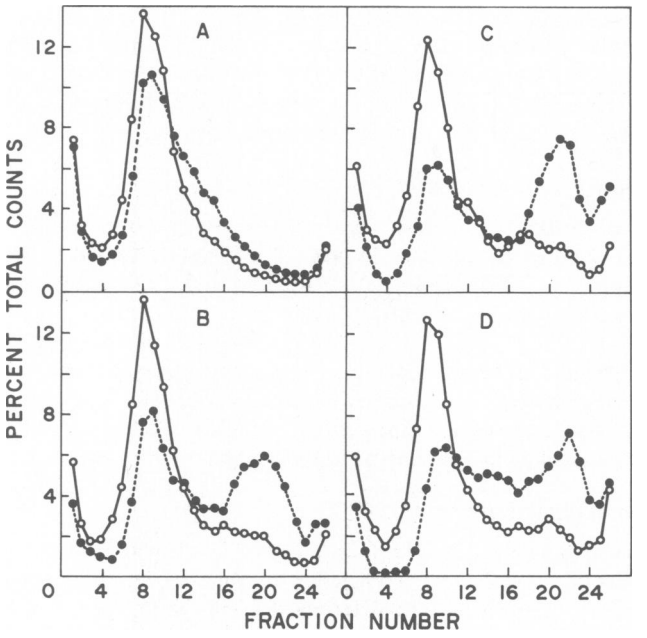

FIGURE 1 Formation of L-DNA upon treatment of newly synthesized DNA with S_1 nuclease. The cells were irradiated with 0 (*A*), 12.5 (*B*), 25.0 (*C*), and 50 (*D*) J/m^2, incubated for 30 min in fresh medium and pulse-labeled for a further 30 min with 60 μCi/ml of [^{3}H]thymidine. The DNA was extracted and incubated with (●) or without (○) 20 U/ml of S_1 nuclease for 30 min at 35°C and centrifuged in neutral sucrose gradients for 100 min. Sedimentation is from right to left. Fractions 4–16 correspond to H-DNA and fractions 17–24 to L-DNA.

The M_n values calculated both from the M_w values and the $M_{1/2}$ values for each distribution agreed reasonably well (Table I), indicating that the size distribution approximates a normal one. It should be emphasized that the L-DNA molecules are uniformly labeled (Cordeiro-Stone et al., 1979), their radioactivity thus being directly proportional to their mass. Fulfillment of these two requirements, namely the normal distribution of sizes and the direct proportionality between mass and radioactivity, makes it possible to calculate M_n values in the manner indicated in Table I. The averages of the values in columns A and B, presented in column C, were considered to be the best estimates of L-DNA size and, consequently, of the intergap distance. Comparing the values of columns C and D it can be seen that the intergap distances (C) are 37–72% of the value of the interdimer distances (D). It is not possible to preclude the possibility that these differences are due to systematic errors in molecular weight determinations, which in each case involve different sedimentation procedures, molecular weight standards and constant parameters. Regardless if the differences between interdimer distance and intergap distance have quantitative significance the results are strongly indicative of a causal relationship between these two parameters.

The Size of the Gaps

The S_1 nuclease assay was also employed to measure the size of the gaps. We noticed that the transformation of H-DNA into L-DNA was dependent on the S_1 nuclease concentration up to a certain value, as shown in Fig. 3. To calculate the percentage of L-DNA formed, the

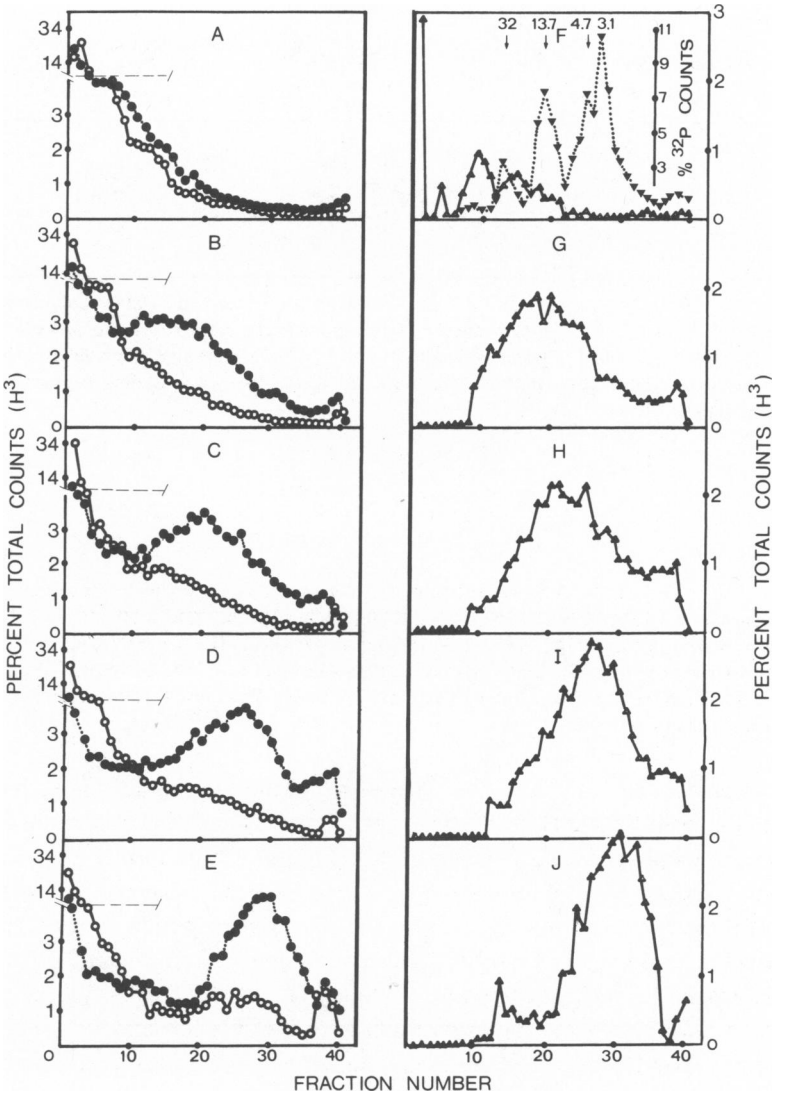

FIGURE 2 Size of L-DNA at different fluences of UV light. The cells were irradiated with 0 (*A*, *F*), 5.0 (*B*, *G*), 10 (*C*, *H*), 20 (*D*, *I*), and 40 (*E*, *J*) J/m^2, incubated for 45 min in fresh medium and pulse-labeled for a further 45 min with 30 μCi/ml of [^{3}H]thymidine. The DNA was extracted and incubated with (●) or without (○) (*A–E*) 20 U/ml of S_1 nuclease for 30 min at 35°C and centrifuged in neutral sucrose gradients for 200 min. In *F–J* the differential values were plotted for each fraction of *A–E*, respectively (S_1 treated DNA minus untreated DNA, Δ). *F* also shows the sedimentation profile of a mixture of untreated ^{32}P-λDNA and *Eco*RI fragments of ^{32}P-λDNA (▼), used as markers for molecular weight determination. The molecular weight values in millions of daltons is shown for each peak. The lightest peak is a mixture of the three smaller *Eco*RI fragments and its molecular weight represents a weight average of the molecular weight of these fragments.

TABLE I

COMPARISON BETWEEN INTERGAP DISTANCE AND INTERDIMER DISTANCE

Fluence	A M_n from $M_{1/2}$ $\times 10^{-6}$	B M_n from M_w $\times 10^{-6}$	C Average M_n $\times 10^{-6}$	D Interdimer distance (double stranded DNA) daltons $\times 10^{-6}$	E C/D
(J/m^2)					
5	7.4	7.1	7.3	20.0	0.37
10	4.3	4.7	4.5	10.0	0.45
20	2.5	3.2	2.9	5.0	0.58
40	1.3	2.3	1.8	2.5	0.72

Data in columns A and B were obtained from Fig. 2 (*F–J*) as described in Methods. Data in column C correspond to the average of columns A and B. The values of column D correspond to the average distance between two pyrimidine dimers in the same strand, measured in terms of double-stranded DNA. These values were determined in these cells (C. F. Menck, unpublished results) by using an UV endonuclease from *M. luteus* according to a methodology already described (Meneghini and Menck, 1978).

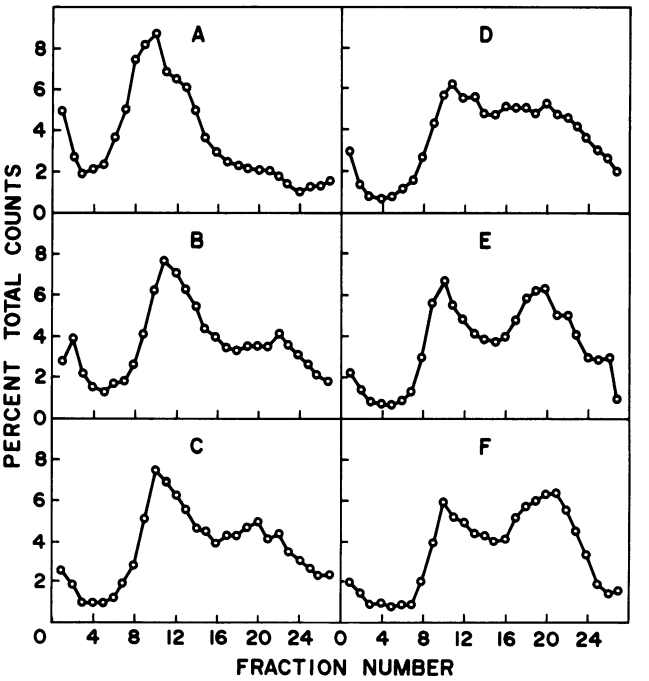

FIGURE 3 Effect of S_1 nuclease concentration on the formation of L-DNA. The cells were irradiated with 12.5 J/m^2, incubated in fresh medium for 30 min and pulse-labeled for a further 30 min with 30 μCi/ml of [^{3}H]thymidine. The DNA was extracted, incubated with 0 (*A*), 0.5 (*B*), 1.0 (*C*), 3.0 (*D*), 9 (*E*), and 52.5 (*F*) U/ml of S_1 nuclease for 30 min at 35°C, and centrifuged in neutral sucrose gradients for 100 min.

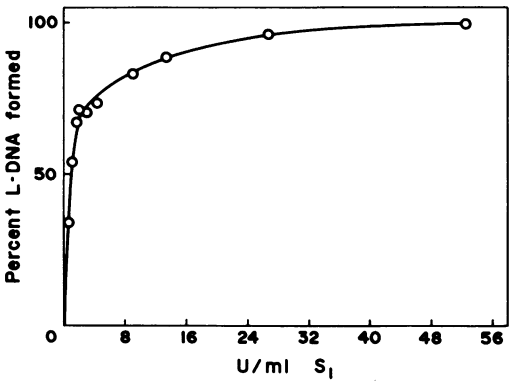

FIGURE 4

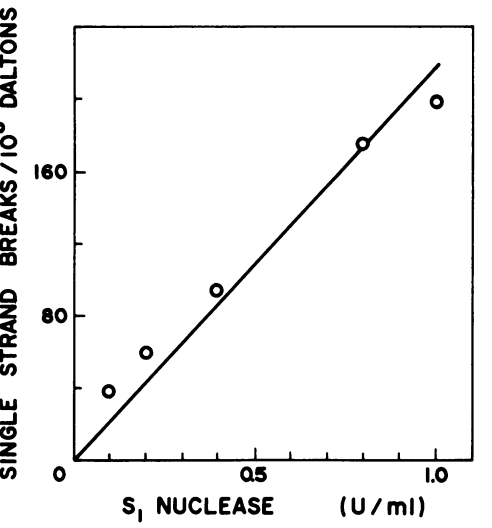

FIGURE 5

FIGURE 4 Kinetics of L-DNA formation as a function of S_1 nuclease concentration. The points were obtained from several experiments like the one of Fig. 3. The sums of percent of counts for H-DNA (fractions 4–16) and L-DNA (fractions 17–24) were scored in each case. In this procedure, the background for L-DNA (fractions 17–24, no enzyme added) was discounted. The resulting value, corresponding to incubation with large amounts of S_1 nuclease (52.5 U/ml), was taken as 100% of L-DNA (the maximum amount obtainable from H-DNA, see text), which was used to calculate the percentage of L-DNA at other enzyme concentrations. Each point represents the mean of two or more determinations.

FIGURE 5 Effect of S_1 nuclease concentration on the production of ssb in single-stranded DNA. Cells were labeled for 24 h with 1 μCi/ml of [^{3}H]thymidine, their DNA was extracted and denatured by adding 1 M NaOH to a final 0.1 M concentration. After 10 min at 25°C the pH was lowered to 7.4 by adding 1 M HCl. This DNA (4.4 μg/ml) was incubated for 30 min at 35°C with the indicated concentrations of S_1 nuclease and centrifuged in alkaline sucrose gradients for 240 min at 32,000 rpm. The frequency of ssb was calculated from M_n values as described in Methods and plotted vs. S_1 nuclease concentration.

gradients were divided into two parts, one corresponding to H-DNA (fractions 4–16) and another to the L-DNA (fractions 17–24). The sums of percent of counts in fractions 17–24 were determined in each case and normalized relative to the corresponding sum obtained at an S_1 nuclease concentration of 52.5 U/ml, assumed to be 100%, i.e., the enzyme concentration at which the maximum amount of L-DNA was formed. This assumption is important for the data treatment which will be developed below and its justification resides in the fact that the resulting curves (Fig. 4) attain a plateau at this enzyme concentration.

To calculate the size of the gap it is assumed the ssb produced by the enzyme are randomly distributed among the single-strand regions. For this calculation we must define the following parameters: N, concentration of ssb produced by the nuclease; D, concentration of single-stranded DNA; G, concentration of gaps. Thus, D/G is the size of the gap and N/G the average number of ssb produced per gap. The distribution of ssb over the entire population of gaps will, under this assumption, follow a Poisson distribution. All classes of this distribution except class zero will give rise to L-DNA, and the fraction corresponding to this class will be

given by $u = e^{-N/G}$. The ordinate in Fig. 4 corresponds, accordingly, to $1 - e^{-N/G}$. If it is further assumed that the number of ssb per gap is proportional to the enzyme concentration, E, and to the size of the gap, $N/G = kED/G$, then the expression above becomes $u = e^{-kED/G}$, or $\log u = -0.4343\ k\ E\ D/G$, where k is a constant which represents the frequency of ssb per unit of S_1 nuclease (ssb $\times$ dalton^{-1} $\times$ US_1^{-1}). This latter assumption was confirmed by the experiment of Fig. 5, in which denatured DNA was treated with different nuclease S_1 concentrations. From the changes in Mn the frequency of ssb, N/D, is calculated and this gives a straight line when plotted vs. E (Fig. 5). Thus k can be determined from the slope of the curve and equals 1.93×10^{-6} ssb $\times$ dalton^{-1} $\times$ US_1^{-1}. The average value of k in three distinct experiments like the one shown in Fig. 5 was 1.75×10^{-6} ssb $\times$ dalton^{-1} $\times$ US_1^{-1}. Another important consideration is that k does not depend on the concentration of single-stranded DNA, at least up to a concentration of 68.4 μg/ml. This was verified in the experiment of Table II, which shows that over the range from 2.0 to 68.4 μg/ml DNA, the ssb frequency at a given S_1 concentration is independent of the denatured DNA concentration. One is therefore working in a concentration range in which N is first order relative to D (N/D is constant at a given E). Hence, it is reasonable to expect that the value of k calculated at relatively high D will be the same at low D, as is the case of gaps belonging to double-stranded DNA.

Several experiments like the one of Fig. 3 were carried out and the mean values of $\log u$ were plotted as a function of E. According to the above description, straight lines obeying the equation $\log u = -0.4343\ kED/G$ should be obtained. Since k is known, the value of D/G can be determined from the slopes. The result (Fig. 6) is surprising in that two distinct slopes are observed. One of these corresponds to 65% of the single stranded regions, i.e., those that are rapidly attacked by the S_1 nuclease. The remaining 35% corresponds to single-strand regions of 4.5×10^4 daltons or ~150 nucleotides. The slope of the curve of slower kinetics should be subtracted from the one of fast kinetics since the slope of this latter is contributed by the two different families of single-stranded regions. In doing so the value of 1,250 nucleotides is obtained for the single-stranded regions rapidly attacked by the S_1 nuclease. The u values shown in Fig. 6 come from five different experiments and in each of them it was possible to detect the two different slopes.

TABLE II
FREQUENCY OF SSB PRODUCED BY A CONSTANT S_1 NUCLEASE CONCENTRATION ON DENATURED DNA AT DIFFERENT CONCENTRATIONS

DNA concentration	ssb/10^8 (N/D)
(μg/ml)	
2.0	42
18.6	54
35.2	54
68.4	60

The experimental procedure was the same as the one described for the experiment depicted in Fig. 5, except that DNA concentration varied as indicated and S_1 nuclease was kept constant at 0.11 U/ml.

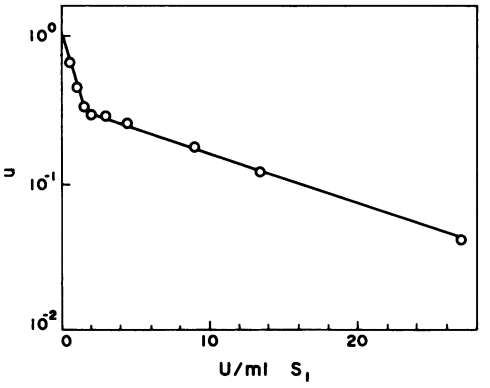

FIGURE 6

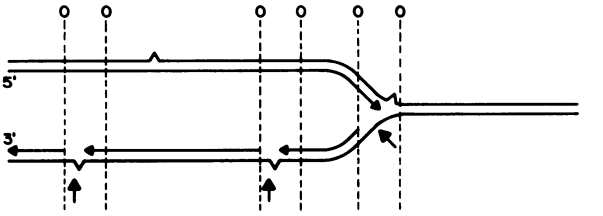

FIGURE 7

FIGURE 6 Determination of the size of the single-stranded regions. Values of u are plotted on a log scale and represent the mean of two or more determinations; u represents the fraction of the total L-DNA that can be obtained, but that still remains as H-DNA at the indicated enzyme concentration (see text for details). The values of u and their respective standard deviations (from lower to higher enzyme concentrations) are 0.66 ± 0.18 (5); 0.46 ± 0.08 (2); 0.33 ± 0.07 (5); 0.29 ± 0.16 (2); 0.30 ± 0.06 (2); 0.27 ± 0.07 (5); 0.17 ± 0.03 (2); 0.12 ± 0.07 (5); 0.04 ± 0.00 (2). The figures in parentheses represent the number of experiments.

FIGURE 7 Model of the effect of pyrimidine dimers on DNA synthesis. It is assumed that when the dimer is in the template for the leading (5'–3') strand it will constitute a temporary block to the fork movement. When the dimer is in the template for the lagging (3'–5') strand the fork ignores the dimer and progresses up to the next origin of an Okasaki fragment (O). The fragment initiates and elongates in a direction contrary to that of the fork movement up to the dimer when further elongation is temporarily blocked and a stable gap is formed. Only the relevant Okasaki fragment origins are shown (O). The arrows indicate regions that are attacked by S_1 nuclease.

DISCUSSION

Recently, we showed that native pulse-labeled DNA from human cells irradiated with UV light gives rise to pieces of L-DNA upon treatment with S_1 nuclease (Meneghini, 1976; Cordeiro-Stone et al., 1979). This is not observed in the case of unirradiated cells. Due to some properties of this L-DNA we postulated that they originated from regions in between gaps, which were points of attack by the S_1 nuclease. Possible sites of S_1 nuclease action other than gaps which might be considered are pyrimidine dimers (Shishido and Ando, 1974) and putative base mismatches, induced by replication with poor fidelity on a dimer-containing sequence (Shenk et al., 1975). Under the conditions employed here no action of S_1 on pyrimidine dimer-containing DNA could be observed (Meneghini, 1976; Cordeiro-Stone et al., 1979). The possibility that the enzyme is recognizing mismatches seems unlikely due to the following: (*a*) The condition under which Shenk et al. (1975) detected mismatches in simian virus-40 DNA was much more severe than the one employed here; they used pH 4.4 instead of pH 5.0 and the enzyme concentration was one order of magnitude above the highest concentrations that were used in this work. These two conditions can be compared by the fact that Shenk et al. (1975) detected action of the enzyme opposite nicks whereas we could not observe any double strand breaks produced by the S_1 nuclease on UV-irradiated DNA,

pretreated with *Micrococcus luteus* UV endonuclease (Schumacher, R. I. and R. Meneghini, unpublished results). (*b*) The kinetics of disappearance of the sites sensitive to S_1 nuclease in a pulse-and-chase experiment parallels the kinetics of DNA elongation observed by alkaline sucrose sedimentation analysis (Schumacher, R. I. and R. Meneghini, unpublished results), suggesting that these sites are real discontinuities in the DNA, rather than mismatches.

It became interesting to know the size of this L-DNA as well as the size of the gaps to test a working hypothesis model (Fig. 7). The main features of this model (Cordeiro-Stone et al., 1979) are that gaps opposite dimers are formed only in the lagging strand where discontinuous synthesis takes place, and that they are formed as a direct consequence of the Okazaki mode of replication. This idea was suggested from the fact that only 50% of the H-DNA give rise to L-DNA. This proposition has not been proved though and other explanations are conceivable for the DNA resistant to the S_1 nuclease. The physical position of the gaps was tested by comparing the size of the L-DNA and the interdimer distance at different fluences. The results indicate that if the gap is opposite any lesion this should be a pyrimidine dimer, since there is no other lesion produced by UV irradiation with such frequency. The size of L-DNA averaged 50% of the interdimer distance and this difference, if not due to some systematic error in the *Mn* determination of the two parameters, might be ascribed to the fact that, according to the model (Fig. 7), at least the L-DNA coming from the nearest region to the fork is half the interdimer distance. This particular L-DNA is delimited by replication blocks imposed by contiguous pyrimidine dimers belonging to opposite templates. The result of Table I could be explained by assuming that this type of L-DNA predominates at low fluences.

Several authors have presented models according to which gaps were not formed in the daughter strands synthesized from lesion-containing templates (Edenberg, 1976; Higgins et al., 1976; Fujiwara and Tatsumi, 1976; Park and Cleaver, 1979). Our previous results (Meneghini, 1976; Cordeiro-Stone et al. 1979) and the present ones are hard to explain if the formation of gaps is not assumed. As to the localization of these gaps the present results indicate that they may be opposite dimers. However, a direct search of dimers opposite gaps by UV endonucleases has proven unsuccessful (Meneghini and Hanawalt, 1976; Clarkson and Hewitt, 1976).

To determine the gap size the approach taken was to consider the production of L-DNA as depending on the S_1 nuclease concentration according to a Poisson distribution. One point not mentioned as yet is that if L-DNA comes from intergap regions then two breaks should have been introduced, one in each gap, for L-DNA to be released. However, according to the theoretical approach used, a single break in one gap was considered sufficient to generate L-DNA. A theoretical analysis was also carried out for the situation in which two breaks, one in each gap, were necessary for the production of L-DNA, yielding a curved line for log u vs. concentration of enzyme, like a curve for cell killing produced by multiple hits. Instead, the results obtained (Fig. 6) show that a single hit is sufficient to produce the effect although two different size targets are present. There is a reasonable way to reconcile these facts by considering that a single break in one of the gaps (any of the arrows in Fig. 7) will produce a new molecule of lower molecular weight and this will be shifted to the range of the gradient where molecules are scored as L-DNA. Additional breaks at unattacked gaps will further shorten the size of these molecules but will not shift them from the range of the gradient they attained after the first break. In accordance with this view it was seen that the distribution size

of L-DNA (fraction 16–24, Fig. 3) tended to shift from an original polydisperse profile to a final sharp peak.

The results of these experiments showed two distinct populations of single-stranded regions, 65% corresponding to stretches of 1,250 nucleotides and 35% to stretches of 150 nucleotides. According to the model proposed (Fig. 7) the size of the gaps should average half the Okazaki fragment. This has not been measured in the cells employed here but in other eukaryotes the size of the fragments seem to fall in the range of 100–200 nucleotides (Edenberg and Huberman, 1975). Therefore only one-third of the gaps have a size which fits the above prediction. The other two-thirds have a size that is more in agreement with the values determined by others (Lehmann, 1972; Buhl and Setlow, 1972) using a totally distinct methodology. This does not rule out a possible correlation between the gap and the Okazaki fragment if, for instance, these cells have different families of Okazaki fragments, differing in size in the same way as the gaps do. An alternative explanation for the data is that S_1 nuclease becomes inhibited as it approaches the replication fork. This could explain the minimal sensitivity of pulse-labeled DNA in nonirradiated cells to S_1 nuclease, under the conditions employed here. The single-strand region at the replication fork would behave as a smaller "target" for the S_1 nuclease, giving rise to the double slope observed in Fig. 6.

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