

Single strand breakage and repair in eukaryotic DNA as assayed by  $S_1$  nuclease

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ABSTRACT

A sensitive new approach for measuring the repair of single strand breaks in DNA induced by low doses of gamma irradiation was tested in cultured fibroblasts from Chinese hamster lung, human afflicted with ataxia telangiectasia or Fanconi's anemia and in normal cells of early and late passages. The assay is based on the increasing rate of strand separation of DNA duplexes in alkali for molecules with increasing numbers of single strand scissions. DNA strand separation is shown to follow the relation,  $\ln F = -(1/M_n \cdot \text{const}) \cdot t^\beta$  where F is the proportion of double-stranded DNA, detected as  $S_1$  nuclease resistant, after alkaline denaturation time, t.  $M_n$  is the number-average molecular weight of DNA between single strand breaks.  $\beta < 1$  is an empirically determined constant. The results suggest an increase in the number-average molecular weight between breaks,  $M_n$ , with increasing times for repair. The final level attained corresponds to the  $M_n$  of control DNA in unirradiated cells. As few as one break introduced into  $10^9$  daltons of single-stranded control cell DNA can be detected. The kinetics, requirements and sensitivities of this assay are described.

INTRODUCTION

The major technique for measuring the repair of single strand breaks induced in mammalian cell DNA has been velocity sedimentation through alkaline sucrose gradients<sup>1</sup>. This procedure avoided mechanical shear of high molecular weight DNA's by lysing the cells directly on the top of the gradient<sup>2,3</sup>. Unfortunately, the rate of sedimentation appeared to be a function of both the lysis time and the rotor speed<sup>4,5</sup>. Also anomalous sedimentation behavior resulted from incomplete strand separation of the DNA<sup>6</sup> and from the formation of DNA networks by interacting single-stranded molecules<sup>1</sup>.

Studier<sup>7</sup> has shown that the sedimentation rate (S) of completely single-stranded DNA in alkali was a function of its molecular weight (M) such that  $S = a \cdot M^{0.4}$ , where a is a constant.

However, Davison<sup>8</sup> demonstrated that the time for complete separation of two DNA strands was a function of the square of their molecular weights. Accordingly, if the time of strand separation could be measured, then molecular weight measurements could be more accurately determined. Unfortunately, for very large DNA's such as those from eukaryotic cells, this is not practical.

A theory has recently been advanced for estimating the rate of DNA strand separation in alkali<sup>9,10</sup>. This is based on the assumption that DNA strand breaks serve as origins of rotation during strand separation. The amount of double-stranded DNA remaining after a given period of denaturation is proportional to the number-average molecular weight,  $M_n$ , of DNA between single strand breaks. For a given amount of DNA,  $M_n$  is inversely related to the initial number of single strand scissions. Thus, such a prediction is useful in designing methods for the detection of single strand breaks in DNA. Recently, two procedures based on this principle have been developed using either hydroxylapatite column chromatography<sup>10</sup> or alkaline elution through a polyvinylchloride filter<sup>11,12</sup>.

This paper presents a simple technique based on alkaline induced strand separation of DNA and applies it to the repair of gamma ray induced damage in DNA's from several eukaryotic cell strains. Alkali is used to denature whole cell DNA in situ for a fixed time followed by neutralization. The fraction of double-stranded DNA remaining is then assayed by its resistance to the single strand specific nuclease  $S_1$  from Aspergillus oryzae. It is shown that the initial number of induced breaks is proportional to the dose of irradiation and that for very low doses, repair of these lesions in cultured cells from five different sources is essentially complete by 120 minutes.

### THEORY

When DNA is denatured in alkali above the transition pH 11.4<sup>7</sup> the rate of unwinding is inversely proportional to the number-average molecular weight of the DNA and to the viscosity of the solvent<sup>8,13</sup>. This suggests that the rate limiting factor in strand separation is the viscous drag of solvent molecules on the rotating DNA strands.

If DNA containing random single strand scissions is subjected to alkali above pH 11.4, denaturation will begin independently at each break. The initial rate of unwinding will be proportional to the number of breaks. As strand separation proceeds, the smaller molecular weight fragments will be rapidly converted to single-stranded DNA while the larger fragments continue to unwind. The viscous drag on the rotating single-stranded molecules is the rate limiting factor so as the rotating portions become longer the rate of unwinding slows down. Thus strand separation takes place more rapidly in the beginning than towards the end of denaturation.

Theoretical assumptions involve the lack of external factors which might influence the rate of unwinding of DNA. The cells do not lyse in the presence of the high salt alkaline solution but rather slowly lose their characteristic morphology. Therefore to assume that the DNA is released from the cell free of complexed proteins and lipids may not be accurate<sup>14</sup>. However, it appears from our work that these factors do not alter the kinetics of strand separation.

According to the model if random breaks are introduced into DNA molecules then strand separation can be described by the relation,

$$\ln F = (-1/M_n \times \text{const}) \cdot t^\beta$$

where F is the fraction of double-stranded DNA, as assayed by resistance to S<sub>1</sub> nuclease, after denaturation time, t. M<sub>n</sub> is the number-average molecular weight between breaks and β < 1 is a constant. The model suggests that if the constant β can be evaluated then a plot of log F against t<sup>β</sup> should yield a straight line. β is determined by computing the slope of the regression line in a log-ln F verses log t plot. β was found to have a slight dependence on dose leveling off at about 600 rads. The average β was computed to be 0.508 ± 0.093. A rigorous mathematical derivation of the relation  $\ln F = -(1/M_n \times \text{const}) \cdot t^\beta$  appears in Rydberg<sup>10</sup>.

## MATERIALS AND METHODS

### Cell Culture Conditions

Cultured V79 Chinese hamster lung fibroblasts (CHL) and diploid human fibroblasts were used throughout this study. The CHL cells were grown as monolayers in milk dilution bottles in Ham's

F-10 medium supplemented with 10% fetal calf serum and 1X antibiotic/antimycotic mixture. The diploid human fibroblasts were similarly grown in MEM's supplemented with 15% fetal calf serum and 1X antibiotic/antimycotic mixture. Conditions for the maintenance of these cells have been described elsewhere<sup>15</sup>.

### Radioactive Labeling of Cells

Cells were trypsinized and added to medium containing 0.1 $\mu$ Ci/ml [<sup>3</sup>H-methyl] thymidine (Nuclear Dynamics 25 Ci/mMole, 1 mCi/ml) to a final concentration between 4 x 10<sup>4</sup> to 4 x 10<sup>5</sup> cells/ml. 2.5 ml of this mixture was then distributed into plastic culture flasks (Falcon plastics, 25 cm<sup>2</sup> surface area). The cells were allowed to attach, followed by incubation at 37° and 5% CO<sub>2</sub> in a humidified chamber for two generation times (18 hours for CHL cells and 48 hours for human fibroblasts). Labeling of cultures took place in the exponential growth phase with a final level of incorporation of 0.21 dpm/cell. Assuming 0.2 to 0.7 rad per <sup>3</sup>H disintegration<sup>16,17</sup> for CHL cells, the average nucleus received a maximum dose of 2.5 to 8.4 rads/hr.

### Cell Irradiation

Irradiation was performed with a <sup>137</sup>Cs gamma-source (Model M-38) giving a dose rate of 2 Krad/min as determined by ferrous sulphate dosimetry. One hour before irradiation the radioactive medium was replaced with fresh unlabeled medium. After this equilibration period, the cells were cooled to 4°C then irradiated. The culture flasks were maintained on ice (4°C) at all times prior to alkali treatment except for the brief irradiation time and the time allowed for repair incubation at 37°C.

### Cell Repair Conditions

Unirradiated control cells and irradiated 0 repair time cells were denatured immediately after irradiation. Cells which were allowed to undergo repair were transferred from ice to a humidified chamber with 5% CO<sub>2</sub> and incubated at 37°C for specified times. Repair was terminated by returning the cultures to ice.

### Alkaline Unwinding

The culture medium was removed followed by a rinse with chilled Puck's balanced saline (PBS). The PBS was aspirated off. Cultures were returned to ice until all flasks in the group were

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handled. Next, to each flask (in a group) was added 2.5 ml of an alkaline solution containing 0.03 N NaOH and 0.9M NaCl at pH 12.0. This alkaline solution was first added to each flask in an inverted position. Then the flask was immediately turned over so that the denaturing solution flowed quickly and evenly over the cell monolayer. DNA strand separation was allowed to take place in the dark for specified time<sup>5</sup>.

#### Neutralization

Alkaline denaturation was terminated by the rapid injection of an equal volume of either 0.076 M acetic acid (final pH 4.3) or 0.038 M acetic acid (final pH 4.9). The neutralized solution was mixed and transferred to a 6 ml plastic culture tube. Aggregates were broken up by sonication (Branson sonifier W185, microtip, setting 4, 10 sec), followed by addition of a 5% solution of recrystallized sodium dodecyl sulfate (SDS) to give a final concentration of 0.1%. The tubes were stored on ice until all flasks were treated and ready for further processing.

#### S<sub>1</sub> Nuclease Digestion

Before digestion, the samples were heated to 55°C for 15 minutes to solubilize any remaining aggregates and to strip the DNA of protein. Due to the high ionic strength of the solution, this treatment does not cause further DNA denaturation nor is significant renaturation expected since this corresponds to a  $Cot = 9.7 \times 10^{-3}$  (moles secs · liter<sup>-1</sup>). After heat treatment, heat denatured calf thymus DNA was added to a final concentration of 5 µg/ml<sup>18</sup>. Addition of zinc acetate (5 µmoles/ml) just prior to digestion maximally activated the nuclease. The solution was evenly distributed to two tubes. To one tube was added 0.05 I.U./ml of the single strand nuclease S<sub>1</sub> (Calbiochem). [One international unit (=I.U.) is defined as that amount of enzyme which converts 1 µmole of single-stranded DNA to mononucleotides per min at 30°C]. Both tubes were maintained for 1-2 hrs at 40°C. The reaction was terminated by addition of an equal volume of chilled 14% trichloroacetic acid. Acid insoluble DNA was recovered by retention on 0.45 µ Millipore filters and quantitated by scintillation counting in toluene flour.

#### Kinetics Experiments

Many of the kinetic experiments were carried out using heat

denatured  $^{32}\text{P}$  labeled DNA from Hemophilus influenzae (sp. act.  $9 \times 10^4$  cpm/ $\mu\text{g}$ ). High voltage electrophoresis was run on DE 81 paper at pH 3.5 (pyridine-acetate buffer), 1.65 KV (30 volts/cm) for 2.5 hours.

RESULTS

I. Characterization of  $S_1$  Nuclease digestion conditions

The kinetics of the  $S_1$  nuclease digestion of single-stranded DNA at  $40^\circ\text{C}$  in 0.45 M NaCl and 0.1% SDS was investigated. These are the conditions of digestion for the dose response and repair kinetics experiments. The results in Figure 1 show that the reaction is complete by 30 minutes. Addition of more enzyme at 15 minutes has no significant effect on the final extent of the reaction. Native, double-stranded  $T_7$  DNA is insensitive to  $S_1$  nuclease under that same conditions.

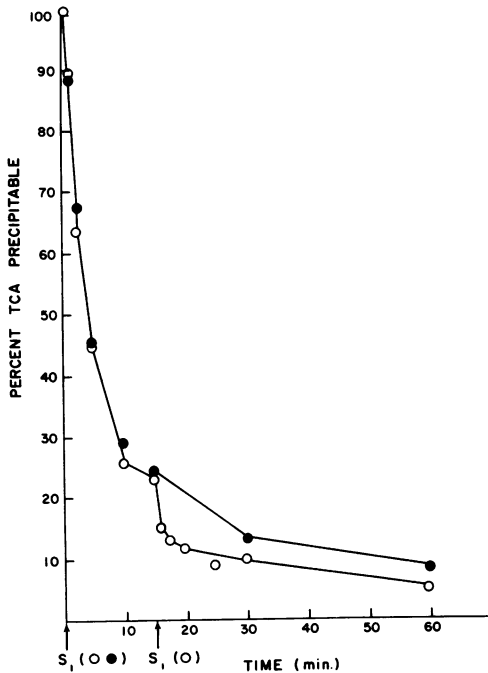


Figure 1. Kinetics of  $S_1$  nuclease activity. To a tube containing 1 ml of 0.03 N NaOH of 0.9 M NaCl, pH 12.0 was added  $5 \times 10^5$  CHL cells and  $3 \times 10^4$  cpm  $^{32}\text{P}$  DNA. Denaturation was continued for 1 hour followed by neutralization with an equal volume of 0.076 M HOAc. The sample was sonicated followed by the addition of 0.1% SDS, 5  $\mu\text{moles/ml}$  Zn (Ac) $_2$  and 10  $\mu\text{g/ml}$  single-stranded calf thymus DNA. The tube was split into two 1 ml fractions and 0.05 I.U./ml  $S_1$  was added to each (●). Aliquots of 100 $\lambda$  were taken at indicated times between 0-60 minutes for determination of TCA precipitable counts. At time 15 minutes, one sample (o) received a second 0.05 I.U./ml of enzyme.

The sensitivity of  $S_1$  nuclease to SDS and NaCl is presented in Figure 2. It is evident that at the enzyme/substrate ratio (1.6 I.U. enzyme/ $\mu\text{mole}$  DNA nucleotide) used, the nuclease is insensitive to concentrations of SDS up to 0.2%. Maximal activity is maintained up to 0.4M NaCl with less than a 5% loss in the final extent of digestion in 0.1 M salt.

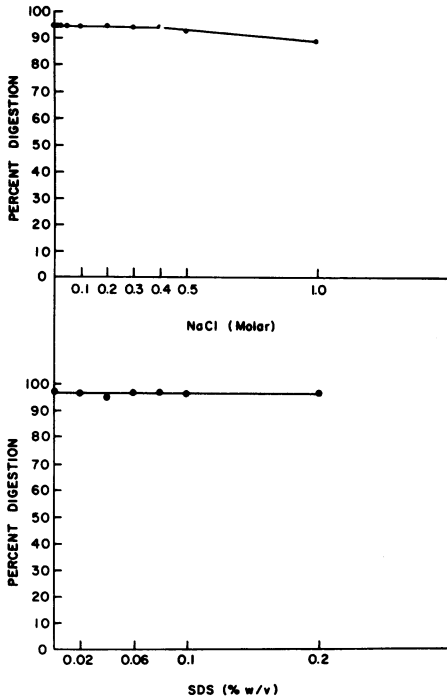


Figure 2. Activity of  $S_1$  nuclease in SDS and NaCl. To tubes containing 1 ml 25mM NaOAc, pH 4.5, at different ionic strengths or SDS concentrations were added 10  $\mu$ g heat denatured calf thymus DNA,  $3 \times 10^4$  cpm heat denatured  $^{32}$ P DNA 5  $\mu$ moles  $Zn(Ac)_2$  and 0.05 I.U.  $S_1$  nuclease. The reaction was terminated after 1 hour at 40°C by the addition of an equal volume of cold 14% TCA. After 1 hour on ice, the precipitate was collected on Millipore filters. The extent of the digestion was computed as  $[1 - (\text{TCA ppt cpm} / 3 \times 10^4 \text{ cpm})] \times 100\%$ .

In a second kinetics experiment, the extensiveness of the digestion was determined by high voltage electrophoresis of the products. In addition to quantitation of the TCA precipitable counts, the total reaction product from each time point was applied directly onto DEAE paper and analyzed by electrophoresis. From Figure 3, it can be seen that by 360 minutes of digestion the four deoxyribonucleoside-5'-monophosphates and inorganic phosphate are the major hydrolysis products. The larger slow moving oligonucleotides, as indicated by the arrow in the figure are precipitable by TCA.

The reproducibility of the  $S_1$  digestion was considered further. Triplicate flasks containing CHL cells were denatured for 30 minutes and neutralized as in the repair kinetics experiments. An aliquot from each was pooled and the combined sample was distributed into 4 tubes. To three of these tubes was added  $S_1$  with the fourth being saved for determination of the initial level of radioactivity. The computed fraction of double-stranded DNA remaining after  $S_1$  digestion was  $57.11 \pm 1.24$  for the pooled samples.

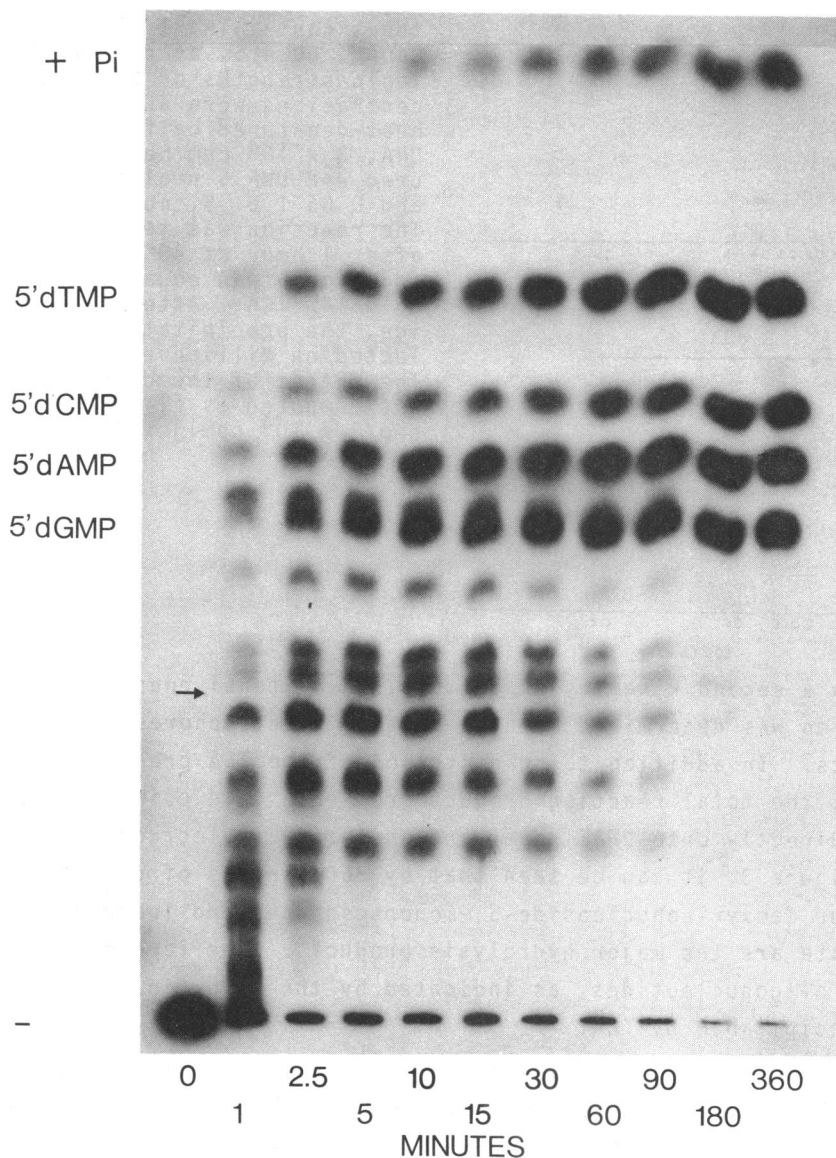


Figure 3. Autoradiography of Electrophoretogram of  $S_1$  nuclease Digestion Product from DNA. This experiment was similar to the previous kinetic study except aliquots were spotted directly onto DEAE paper and electrophoresed for 2.5 hours, 30 volts/cm at pH 3.5. The four non-radioactive deoxyribonucleoside - 5'-monophosphates were run as standards and detected by monitoring with a UV lamp. The digestion products were identified by autoradiography. All the oligonucleotides running behind the arrow are precipitable by TCA.



Table 1 shows the effect of increasing cell concentration on the extent of  $S_1$  digestion of  $^{32}\text{P}$  labeled single-stranded DNA. The enzyme/ $^{32}\text{P}$  DNA ratio remained constant for each experiment while the amount of cell lysate was changed. It is apparent that over a two order of magnitude range, the cell concentration has no effect on the digestion of this exogenously added DNA.

TABLE I. Extent of Nuclease Digestion at Various Cell Concentrations.

Equivalent Cell Content cells/ml	Extent of Digestion %
$7.8 \times 10^3$	94.1
$3.9 \times 10^4$	94.1
$7.8 \times 10^4$	95.4
$3.9 \times 10^5$	94.9
$7.8 \times 10^5$	93.9

Cell lysate was prepared from a stock of unlabeled cells denatured for 1 hour in alkali followed by neutralization, sonication, and the addition of SDS as outlined under materials and methods. The reaction mix contained 0.45 M NaCl, 0.1% SDS in NaOAc pH 4.5,  $3 \times 10^4$  cpm single-stranded  $^{32}\text{P}$  DNA, 10  $\mu\text{g/ml}$  heat denatured calf thymus DNA, 5  $\mu\text{moles/ml}$  Zn (Ac) $_2$ , 0.05 I.U.  $S_1$  and various amounts of cell lysate. As before, the extent of digestion was measured after 1 hour at 40°C.

## II. Dose Response

The increase in the number of single strand breaks in DNA to increased level of radiation was measured by the dose response relation which is presented as the inset to Figure 4. As indicated by this graph, the number-average molecular weight,  $M_n$ , of the DNA between breaks is a decreasing linear function with increasing dose.

The DNA strand separation kinetic curves as a function of radiation dose is also shown in Figure 4. The least squares regression of  $\log F$  against  $t^\beta$  was computed and graphed as shown. The correlation coefficients were used to estimate goodness of fit; they characteristically ranged from -0.94 to -0.96. The results show that the rate of DNA unwinding in alkali increases with increasing dose. The dose response relation was derived from the strand separation kinetics by plotting the slope,  $1/M_n \times \text{const}$ , against dose. By plotting the fraction of double-stranded

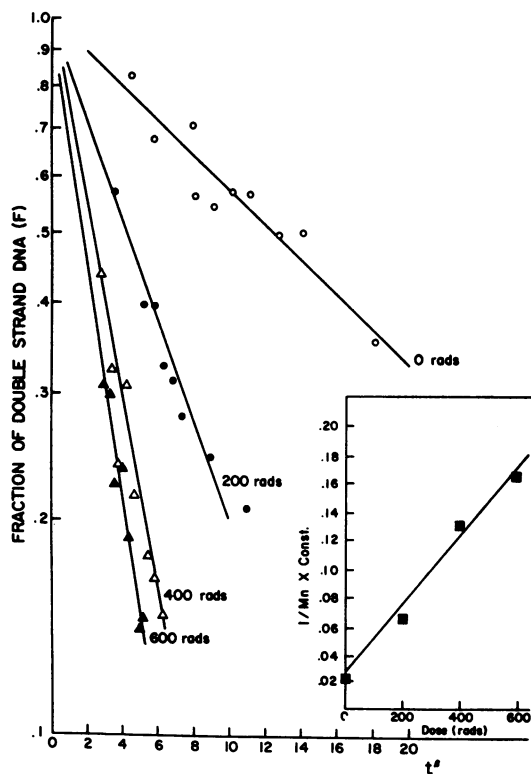


Figure 4. Dosage Response. Chinese hamster lung fibroblasts were seeded at  $5 \times 10^4$  cells/flask in four groups of ten flasks each. Radioactive labeling was allowed to take place for 18 hours followed by medium change. Each group of flasks were given different amounts of gamma irradiation (0.200,400,600 rads) and then treated with alkali as outlined in materials and methods. Each flask in a given group was allowed to denature for a different length of time (10,15,20,25,30,35,40,50,60 or 90 minutes) before neutralization. Following digestion the fraction of double-stranded DNA, F, as assayed by resistance to  $S_1$  nuclease was computed.

$$F = \frac{S_1 \text{ resistant acid insoluble cpm}}{\text{Total acid insoluble cpm}}$$

The line represents the best fit computed by the method of least squares. The inset is a plot of the slopes,  $1/M_n \times \text{const}$ , of the strand separation kinetic curves versus dose.

DNA remaining at a constant  $t^\beta$  versus dose, it can be calculated that the  $D_{37}$  is 520 rads. The apparent target size of the DNA molecule can be estimated from the relation:  $1/M_n = 2.3 \times 10^{-12}$  rad, where  $M_n$  is the number average molecular weight between breaks<sup>23</sup>. By this calculation, the DNA target size in CHL cells is approximately  $8.4 \times 10^8$  daltons.

### III. Repair Curve

Repair of gamma ray induced breaks in DNA results in an increase in the number-average molecular weight ( $M_n$ ) of DNA between breaks; therefore,  $M_n$  should increase with the time of repair incubation. For a constant alkali treatment time of  $t^\beta$ , the fraction of double-stranded DNA, F, should also vary as a direct function of repair time.

Figure 5 shows the kinetics of repair for CHL cells given 400 rads of gamma irradiation followed by incubation at either 37°C or 4°C. DNA repaired for each incubation time following 30

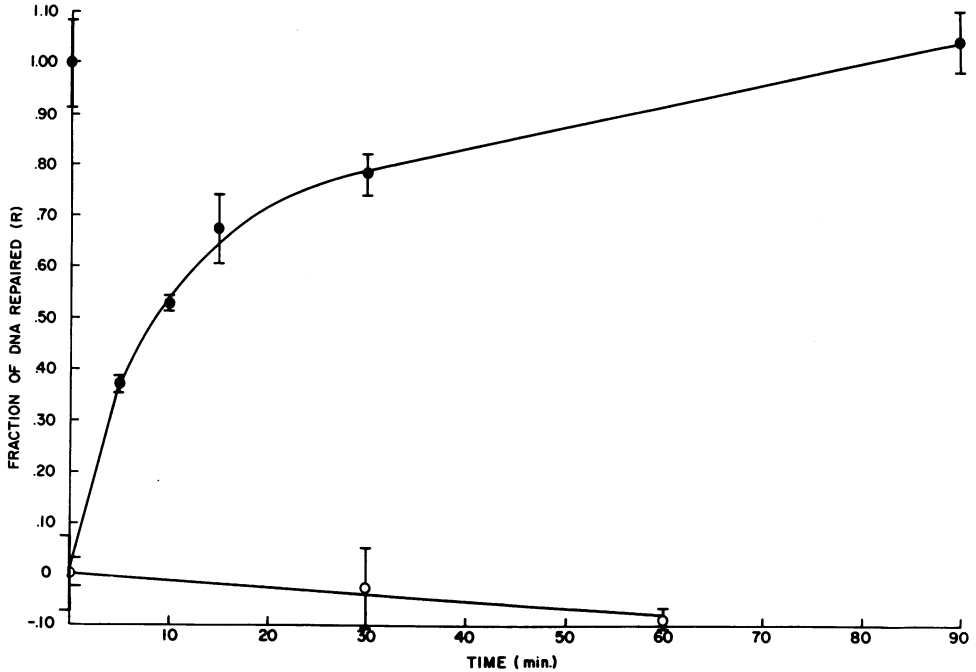


Figure 5. Repair Curve. In the first experiment (●) seven groups of three culture flasks were prepared as in Figure 4. Each group of three, except the control group, was given 400 rads of gamma radiation followed by repair incubation at 37°C for 0, 5, 10, 15, 30, or 90 minutes. All 21 flasks were denatured for 30 minutes and then neutralized for nuclease digestion. After digestion by  $S_1$  the percent double-stranded DNA for each repair time was determined and the percent repair (R) was computed. Each point was repeated in triplicate and the bars represent the range of standard deviations. In the second experiment (○) three groups of three culture flasks were irradiated and held in ice for 0, 30, or 60 minutes prior to denaturation and digestion. A fourth group was not irradiated and served as a control.

minutes of alkaline denaturation is shown in the graph. From this graph, it can be seen that for cultures incubated at 37°C, the percent double-stranded DNA returns to that of the unirradiated control by 120 minutes of repair. The time required for one half of the alkali labile sites to be repaired is about ten minutes. For the cells held at 4°C, the fraction of double-stranded DNA actually decreased with time.

From the slope of the  $\log(1-\bar{R}_c)$  versus time of repair regression it is possible to determine the rate constant for repair. The rate constant reflects the increase in  $M_n$  with in-

creasing time of repair. In cultures allowed to repair at 37°C the corrected rate constant for repair is 0.022 min<sup>-1</sup> while for cells kept on ice, the post irradiated DNA appears to accumulate new alkali labile sites at the rate of 1.47 x 10<sup>-3</sup> units/min.

The results of the screening procedure for possible DNA repair deficiency in four human fibroblast cultures are summarized in Table II.

TABLE II. Cultures Screened for Repair Deficiency

	Control	0 min	5 min	10 min	15 min	30 min	90 min
CHL	80.28	28.87	47.93	56.09	63.53	69.31	83.53
EHF	88.38	39.21	44.83	54.01	63.78	77.95	79.24
LHF	74.94	54.70	54.66	60.61	64.39	69.12	73.40
AT	77.87	35.17	42.60	50.58	55.66	71.64	78.81
FA	81.94	40.42	45.90	49.68	58.60	69.40	85.66
$\bar{F}$	80.68	39.67	47.18	54.19	61.19	71.48	80.13
S.D.	5.05	9.53	4.60	4.42	3.86	3.76	4.74
$\bar{R}$	100.00	0.00	18.31	35.41	52.48	77.57	98.65
$\bar{R}_C$	100.00	0.00	18.32	35.46	52.60	77.91	99.96

Cultures were treated as presented in the legend to Figure 5. The following abbreviations are used: Chinese Hamster lung fibroblasts (CHL), Early passage human fibroblasts (EHF), Late passage human fibroblasts (LHF), Ataxia telangiectasia (AT) and Fanconi's anemia (FA). In the upper portion the percent double-stranded DNA for each repair time is presented. In the lower table these values are averaged ( $\bar{F}$ ) and are accompanied by their associated standard deviations (S.D.), normalized percentage repair ( $\bar{R}$ ) and percentage repair corrected for increased development of alkali labile sites ( $\bar{R}_C$ ). The following abbreviations are used: Chinese hamster lung fibroblasts (CHL), Early passage human fibroblasts (EHF), Late passage human fibroblasts (LHF), Ataxia telangiectasia (AT) and Fanconi's anemia (FA).

The data show that within the accepted experimental error no difference in repair capacity, as measured by the recovery of S<sub>1</sub> nuclease resistance, can be distinguished among the various cell strains. In Figure 6 the regression of log (1- $\bar{R}_C$ ) verses time of repair gives a first order rate constant of 0.022 min<sup>-1</sup> with a  $t_{1/2}$  = 14.14 minutes. The correlation coefficient is -0.999.

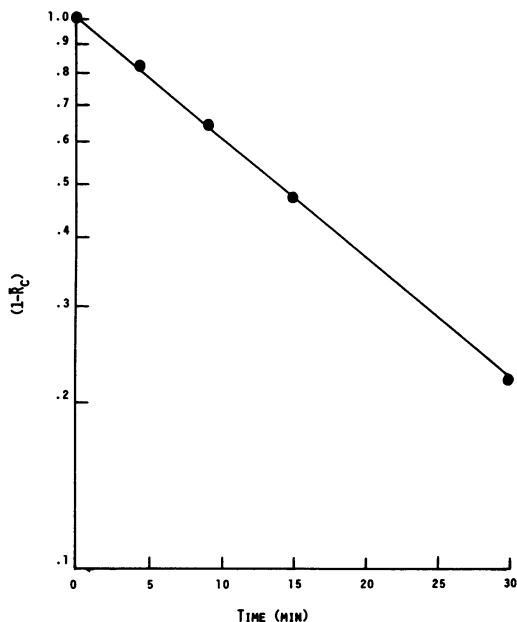


Figure 6. The least squares regression of  $\log(1-R_c)$  versus time of repair gives a first order rate constant of  $0.022 \text{ min}^{-1}$  with a  $t_{1/2}$  of repair equal to 14.14 minutes. The correlation coefficient is  $r = -0.999$ . Values obtained from Table II.

## DISCUSSION

In this study, the new approach to monitoring single strand scission in DNA upon gamma irradiation originally suggested by Ahnström and Erixon<sup>9</sup> has been extended. This approach relies on the increasing rate of DNA unwinding in alkali as a function of decreasing number-average molecular weight induced by strand breakage.

The present method of assaying the fraction of double-stranded DNA resistant to  $S_1$  nuclease after alkaline denaturation has proven to be a sensitive technique for measuring radiation induced single strand breaks in DNA (Fig. 4). One consequence of the sensitivity of this method is the ability to monitor the kinetics of repair (Fig. 5) after administering small doses of gamma irradiation to eukaryotic cells having high molecular weight DNA.

Normal capacity for repair of alkali labile sites induced by gamma radiation was demonstrated in cultured cells from different genetic backgrounds. Both ataxia telangiectasia and Fanconi's anemia are thought to be deficient in some form of re-

pair of damage induced by ionizing radiation. However, this result confirms and extends earlier observations with ataxia telangiectasia<sup>19,20</sup> and Fanconi's anemia (as cited in 21) that there is no diminished capacity to rejoin single-stranded DNA breaks. By comparing the capacity of CHL, EFH and LHF to repair alkali labile regions, it is evident that there is no significant difference in the ability of an immortal cell line (CHL) and a cell strain (EHF, LFH) with limited life span to repair gamma ray induced single-stranded DNA breaks. There is also no evidence to support a reduced capacity for repair in aging fibroblasts. Since LHF cultures were in a late stage of senescence (65d) with less than 10% cycling cells the observation that repair was complete was inferred from the behavior of a small proportion of the total population. It should be noted that LHF is the largest contributor to the observed standard deviations. The low level of incorporation of radioactive label (<0.021 dpm/cell) by LHF may limit our ability to accurately determine the proportion of double-stranded DNA associated with alkaline induced unwinding.

In order to be sure that  $S_1$  nuclease digestion of single-stranded DNA was compatible with the conditions used in alkaline denaturation, much of this report detailed conditions which would assure the reliability of the nuclease probe at moderate salt and detergent concentrations. The single strand specific  $S_1$  nuclease from Aspergillus oryzae has been shown earlier to be active over a range of ionic strengths and retain 55% of its maximal activity in 0.4 M NaCl<sup>18</sup>. The results of this study show 100% maximal activity at 0.4 M NaCl. This can be explained by the difference in the enzyme to substrate ratios used in the two studies, i.e.  $10^{-4}$  vs. 1.6 I.U./ $\mu$ mole DNA in the previous and the present study, respectively. Thus, higher enzyme to substrate ratios are required for digestion to reach completion, especially at high salt concentrations.

Other studies of  $S_1$  have shown that this enzyme at  $4 \times 10^{-5}$  I.U./ $\mu$ mole DNA was unaffected by SDS up to 0.04%, but its activity declined to 5% maximal in the presence of 1.25% SDS<sup>22</sup>. However, in the same study the rate of digestion was found to be constant for at least forty minutes even at 0.6%. These data suggest that  $S_1$  enzyme is not inactivated by the detergent but rather its ac-

tivity is reduced. Results from the present study shows insensitivity of  $S_1$  to SDS up to 0.2%. In fact, in an experiment not described,  $S_1$  nuclease was preincubated in 0.45 M NaCl, 0.1% SDS, 30 mM NaOAc, pH 4.5 before labeled single-stranded DNA was added. For at least one hour of preincubation,  $S_1$  retained 100% of its ability to digest the single-stranded DNA. This is probably due to an excessive enzyme input.

The time kinetics of digestion (Fig. 1) suggest that the reaction is complete by 30 minutes with a  $t_{1/2}$  of 5 minutes. This is in complete agreement with Sutton's studies. Since the addition of more nuclease after 15 minutes of reaction has little effect on the final level of digestion, the condition of 0.45 M NaCl and 0.1% SDS in 30 mM sodium acetate pH 4.5 is compatible with this enzyme.

The lack of effect of increasing cell concentration up to  $7.8 \times 10^5$  cells/ml on the rate and extent of digestion suggests that the cell debris, protein and lipid found in the lysate do not interfere with the enzyme's activity (see Table I). It also has not protective effect on the DNA since the final level of digestion remains constant.

By electrophoretically analyzing the formation of digestion products, this study shows that the ultimate products from DNA are the 5'-deoxyribonucleoside monophosphates and free Pi. However, these are derived from larger fragments formed early during the reaction. This evidence supports the designation that the action of  $S_1$  is endonucleolytic<sup>18</sup>. Even though oligonucleotides exist after 90 minutes of digestion, no further decrease in DNA precipitable by TCA can be detected. Thus, treatment with  $S_1$  nuclease for 2 hours is sufficient to convert all the single-stranded DNA to acid soluble material for accurate determinations of the fraction of double-stranded DNA, F.

The use of  $S_1$  nuclease is preferred over hydroxylapatite column chromatography as used in earlier studies for its simplicity and reproducibility. Hydroxylapatite chromatography is insensitive to partial single-strandedness; therefore sonication is required in order to shear single-stranded fragments from double-stranded DNA. In a study not shown, the proportion of double-stranded DNA as assayed by hydroxylapatite was found to be a

function of the sonication time and that at the highest input (Branson W-185, microtip, setting #4, 30 sec) substantial single-stranded fragments remain attached to double-stranded DNA. The greater the sonic input, the greater the shear forces; consequently, the better the estimation of the proportion of double-stranded DNA. However, the observed value of the double-stranded fraction,  $F$ , is always an over estimate. With the  $S_1$  assay, sonication does not affect the final results but is included to break up cell lysate aggregates introduced during neutralization. Furthermore, in the hydroxylapatite assay the total recovery of input DNA is assumed but not always realized. In the  $S_1$  assay all input DNA can be accounted for as either acid soluble or acid insoluble counts. The method of direct alkaline elution through polyvinylchloride filters has not been compared<sup>10</sup>.

There is clearly linearity between the number-average molecular weight and the dose of gamma irradiation given (see Fig. 4). From this dose response curve it is possible to determine the number of single strand breaks introduced into any given unit of DNA. The relation is:

$$\text{number of single strand breaks} = \frac{M_n(\text{initial})}{M_n(\text{final})} - 1$$

For a dose of 400 rads we obtain,

$$\text{breaks/unit ssDNA} = \frac{M_n^0/\text{const}}{M_n400/\text{const}} - 1 = 4.8, \text{ where}$$

one unit ssDNA is the mass of single-stranded DNA (ssDNA) between breaks in unirradiated control cell DNA. Thus 4.8 additional single strand breaks are introduced into the single-stranded unit mass of the denaturing DNA duplex found in unirradiated CHL cells. The number of single strand breaks induced in DNA by ionizing radiation has been estimated to be  $2.3 \times 10^{-12}$  breaks/dalton/rad<sup>23</sup>. Assuming that an average CHL cell contains  $6.7 \times 10^{12}$  daltons DNA<sup>16</sup> then, after 400 rads, one expects  $6.15 \times 10^3$  breaks/CHL cell. If there are 4.8 breaks/unit ssDNA one can calculate that there are 1280 unit ssDNA's/cell, each with a mass of  $5.2 \times 10^9$  daltons. Therefore, this assay can detect about 1 break in  $10^9$  daltons single-stranded DNA. This value agrees well with the estimated target size of  $8.4 \times 10^8$  daltons.

It should be noted that radioactive decay from tritium label incorporated into the DNA may cause strand breakage thus affecting the calculated target size. In this study attempts were made to



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minimize this effect by allowing cells to receive no more than 5 rads/hr of radiation from tritium decay which is nominal compared to the dose of 400 rads administered to the cells prior to study. Indeed, the calculated target size of  $8 \times 10^8$  daltons agrees well with the estimated sensitivity of detection of one break per  $10^9$  daltons. Since this latter calculation has taken into consideration the initial Mn, it should eliminate bias due to decay of incorporated label during the experiment. However, for very low dose experiments (50-100 rads), the use of  $^{14}\text{C}$  labeled precursors rather than  $^3\text{H}$  is advised due to their low contribution to isotope decay effect.

In the application of this method to analyze a cells' ability to repair single-stranded DNA breaks at  $37^\circ\text{C}$  the results (Fig. 5) show that it could measure a return of DNA size to that of control level. It appears that all fibroblasts tested undergo repair and complete the rejoining process of single strand breaks in DNA within 120 minutes after irradiation. DNA was repaired with a rate constant of  $0.022 \text{ min}^{-1}$ , corrected for the increase in the number of alkali labile sites with time at  $4^\circ\text{C}$  (see below).

As presented in Figure 5, this assay could distinguish repair from absence of repair with a sensitivity great enough to detect an actual increase in the number of alkali labile sites with time after irradiation at  $4^\circ\text{C}$ . This phenomenon of increased alkali labile sites has been observed in isolated DNA irradiated with much higher doses<sup>24</sup>. This was shown to result from an increase in the number of actual single strand breaks plus release of bases from the sugar moieties producing alkali labile sites. The number of true strand breaks reached a maximum after two hours while base loss continued for longer times. Since 400 rads of irradiation produces about  $6.15 \times 10^3$  alkali sensitive sites/CHL cell and the rate of appearance of new lesions is  $1.47 \times 10^{-3}$  units/min at  $4^\circ\text{C}$ , it is estimated in this study that an additional 9 sites/min/cell are generated. This estimate is a minimum because most of these reactions are temperature dependent. Thus at  $37^\circ\text{C}$  new breaks are being produced while repair takes place. It should also be noted that the sites of base loss would not exist as spontaneous breaks in vivo but in alkali would be assayed as observed breaks. This suggests that the com-

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puted yield of strand breaks is not accurate. Rather, at best, it may be a close estimate of the number of alkali labile sites induced in DNA by 400 rads of gamma irradiation at a given time after irradiation. The actual production of assayable lesions is a dynamic process, increasing at the rate of 9 sites/min/cell at 4°C for at least one hour after irradiation. A second consequence of this degradative event is an under estimation of the rate of repair. Unfortunately the magnitude of the correction factor at 37°C cannot be determined unless the process of repair and degradation can be separated.

It should be noted that in this study repair was assayed with cell exposed to a low level of irradiation ( $\leq 400$  rads). In earlier studies where repair was measured by sedimentation through alkaline sucrose, analysis characteristically required kilorad levels of gamma rays in order to induce enough damage in DNA that could be reflected as a shift in the sedimentation profile (e.g. 19). Since such doses are 100% lethal, they are not within the biologically relevant range. At 400 rads of irradiation, many cells retain reproductive integrity (survival data not shown) even though their DNA is damaged. Thus this technique can accurately and quantitatively detect repair of single strand breaks in DNA in cells exposed to relatively low doses of ionizing radiation.

The source of the deviations within each triplicate point taken in the repair kinetics experiments (Fig. 5) was investigated. It was necessary to know whether the deviations reflected real differences in rates of strand separation of DNA from flask to flask or were an artifact introduced by the  $S_1$  nuclease incubation. In the results presented it was shown that the  $S_1$  nuclease digestion contributed only a 2% deviation in the final extent of digestion for pooled samples; therefore, some process prior to  $S_1$  digestion must contribute a larger error. Since the digestion takes place in an acidic solution, depurination might contribute to variation by partial destruction of the double-stranded portion of the DNA. Sedimentation coefficients were determined for single strand  $T_7$  DNA<sup>7</sup> treated, as double-stranded DNA, with 0.45 M NaCl, 30 mM NaOAc pH 4.5 at 65°C for 15 minutes. This value was compared to that of untreated  $T_7$  DNA to determine

the number of alkali labile sites introduced into the DNA by the acidic condition. Less than 0.4 breaks per  $10^6$  daltons DNA resulted from such a treatment. Thus the incubation mixture does not seriously degrade the proportion of DNA which remains double-stranded.

Variations within replicates in the proportion of double-stranded DNA remaining after treatment, may be a consequence of the geometry of denaturation. The kinetics of unwinding are different depending on whether the cells are denatured as a suspension or attached to a surface. Cells treated in monolayer show a slower rate of strand separation which may be a consequence of the high local viscosity resulting from the release of DNA from the surface. Inspection of such a monolayer during denaturation reveals what appears to be a gel overlaying the surface. Variations in the viscosity of this gel could drastically affect the rate of unwinding. Unfortunately, repair should only be measured in cells which have been attached to a growth surface for at least one generation time; otherwise, it is difficult to interpret the results.

Due to the simplicity and sensitivity of this technique, its applicability should be wide ranging. It should be possible to monitor changes in the number-average molecular weight of DNA in many systems in which denaturation of DNA can be effectuated by alkali or other means. The single strand specific nuclease,  $S_1$ , is a hardy enzyme and should quite easily adapt to many other reaction conditions. By adopting a similar technique, it should be possible to follow many processes involving breakage and rejoining of DNA such as excision of UV-induced dimers and recombination. This study shows that repair processes can be monitored in eukaryotic cells given nonlethal doses of gamma radiation. Thus this technique should be useful in the elucidation of molecular mechanisms for several mutations affecting DNA repair processes.

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