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Detection of DNA Single-Strand Breaks Produced during the Repair of Damage by DNA-Protein Cross-Linking Agents

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ABSTRACT

In this investigation, the combination of 1-β-D-arabinofuranosylcytosine and hydroxyurea was used to inhibit the polymerase step of DNA excision repair. The DNA single-strand breaks (SSB), which accumulated in the presence of these agents, were measured by alkaline elution. With this approach, DNA SSB were detected in normal human fibroblasts after exposure to *trans*-platinum(II)diamminedichloride, formaldehyde, or potassium chromate. These agents all share the common feature that they induce DNA-protein cross-links in mammalian cells. In the case of *trans*-platinum(II)diamminedichloride or formaldehyde, the frequency of these SSB was markedly less in excision-deficient xeroderma pigmentosum cells. With chromate, a high level of SSB was induced in both normal and xeroderma pigmentosum cells; these results indicate that chromate damage to DNA is repaired by a mechanism different than the classical excision pathway since xeroderma pigmentosum cells responded normally. Several other agents were investigated with this approach, and no SSB were detected with nickel sulfate, 12-*O*-tetradecanoylphorbol-13-acetate or asbestos fibers in the presence or absence of the polymerase inhibitor. This approach was found to be a very sensitive method to detect DNA excision repair.

INTRODUCTION

Inhibitors of DNA synthesis have been shown recently to inhibit DNA excision repair in mammalian cells (3, 6, 13, 14, 21). In these reports, DNA SSB[2] were found to accumulate in cells exposed to UV radiation and treated with agents such as ara-C or HU. These SSB were apparently generated during excision repair, since Dunn and Regan (6) found that they were markedly decreased in excision-deficient XP cells. In the same report, treatment of normal human skin fibroblasts with 0.01 mM ara-C in the presence of HU resulted in the maximum accumulation of SSB after UV radiation; this correlated well with the number of incised lesions found during the same period of excision repair. These findings indicated that the incision step of excision repair continued in the presence of ara-C and HU and that these incision SSB accumulated due to an inhibition of the polymerase step of excision repair (6).

The alkaline elution technique can be used to measure very low levels of the DNA SSB (16). DNA SSB generated during excision repair have been detected in human cells with this technique (9) following UV radiation. This has also been observed in *Drosophila* cells (13); the level of SSB was increased in these cells with the addition of HU and 1 mM ara-C during a 30-min incubation after UV. The objective of this report was to determine if these inhibitors of the polymerase step of DNA repair could be used to detect excision repair of damage induced by certain chemical and physical agents in human cells. In particular, my interest has been in agents which induce DNA-protein cross-links in cells (8, 10, 11) such as *trans*-Pt. This agent has been shown to transform rodent cells in culture (11) and induce sister chromatid exchanges. Recently, I have found that DNA-protein cross-links, produced by *trans*-Pt, were significantly more persistent in XP cells compared to normal cells (7); there was also increased cytotoxicity in XP cells compared to normal cells (7). Formaldehyde has also been found to be more toxic to XP cells (17); UV-sensitive excision-deficient strains of bacteria (10) and yeast (2) were found to be more sensitive to formaldehyde than are the respective wild strains of bacteria or yeast. Recently, we have found that chromate salts induce high levels of DNA-protein cross-links in mammalian cells (12). Chromate salts have been found to be mutagenic (4) and carcinogenic (4); they induced DNA repair in human cells as measured by unscheduled DNA synthesis (20). By using the format developed by Dunn and Regan in human fibroblasts (6) and the alkaline elution technique, I have found that significant levels of DNA SSB accumulated in the presence of the DNA polymerase inhibitor (a combination of ara-C and HU) when cells were treated with *trans*-Pt, formaldehyde, or potassium chromate. The level of SSB was markedly less in excision-deficient XP cells treated in a similar manner with *trans*-Pt or formaldehyde. This approach was found to be an extremely sensitive method to detect the incision step of DNA repair in human cells.

MATERIALS AND METHODS

Cells. The human fibroblast strains CRL 1187, XP12BE (CRL 1223), and XP25RO (CRL 1261) were obtained from the American Type Culture Collection, Rockville, Md.; AG 1522 was obtained from the Human Genetic Mutant Cell Repository, Camden, N. J. The cells were used at passages 9 to 19 and grown as described previously (8). [2-^{14}C]Thymidine was added to growing cells for several days and then removed several days prior to the experiment. Exponentially growing L1210 mouse leukemia cells were grown and labeled with [^{3}H]thymidine as described previously (8).

Irradiation. Cells were γ-irradiated with a ^{137}Cs source which delivered 130 rads/min. The cells were irradiated at 0° while suspended in medium. UV radiation (254 nm) was carried out as described previously (8).

Chemical Agents. K_2CrO_4, $NiSO_4 \cdot 6H_2O$, and formaldehyde (37%) were obtained from Fisher Scientific Co.; K_2CrO_4 and $NiSO_4 \cdot 6H_2O$ were dissolved in water immediately before the experiments. *trans*-Pt

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[2] The abbreviations used are: SSB, single-stranded break(s); ara-C, 1-β-D-arabinofuranosylcytosine; HU hydroxyurea; XP, xeroderma pigmentosum; *trans*-Pt, *trans*-platinum(II)diamminedichloride; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SDS, sodium dodecyl sulfate; RE, relative elution.

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was supplied by Mathe Bishop, Inc. (Malvern, Pa.) and prepared as described previously (11). ara-C was kindly supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute and HU by the Aldrich Chemical Co. (Milwaukee, Wis.). These agents were dissolved separately in phosphate-buffered saline and stored in liquid nitrogen. ara-C was used at a concentration of 0.01 mM and HU at 2 mM. Superoxide dismutase was obtained from P-L Biochemicals (Milwaukee, Wis.) and catalase from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). TPA was a gift from H. Nagasawa (18), which was originally supplied by Consolidated Midlands Corp. (New York, N. Y.); it was dissolved in acetone. TPA was also obtained from C. C. R., Inc. (Eden Prairie, Minn.) and dissolved in dimethyl sulfoxide. Union International Centre Cancer reference amosite asbestos fibers were provided by Dr. V. Timbell, Medical Research Council, Great Britain; the asbestos was washed with water and autoclaved before use. Cells were treated with these agents as described previously (10).

Alkaline Elution. The procedure used was developed by Kohn *et al.* (15) and has been reviewed recently (16). Briefly, the cells were filtered onto a 2-μm-pore size polycarbonate filter (Nuclepore, Pleasanton, Calif.) and lysed with 2% SDS-0.1 M glycine-0.02 M sodium EDTA (pH 9.6); 2 ml of the same solution containing 0.5 mg of proteinase K per ml were then pumped through the filter at 0.04 ml/min. This solution was followed by 0.02 M EDTA (acid form)-0.1% SDS tetrapropylammonium hydroxide added in the amount required to give a pH of 12.2. Eluted fractions were collected and assayed for radioactivity as described previously (16). The combination of the polycarbonate filters, proteinase K digestion, and SDS in the eluting solution minimized the cross-linking effect caused by agents which induce DNA-protein cross-links (8, 10, 16). In these experiments, no cross-linking effect was detected with any agent. In order to provide for an internal standard, ^{3}H-labeled L1210 cells which had received 300 rads at 0° were included in each assay, and the results were plotted as described previously (10).

Extensive evidence has been presented that the alkaline elution technique can detect DNA SSB produced by ionizing radiation and many chemical agents, as well as excision breaks produced after UV radiation (9, 10, 16). With this technique, DNA SSB can be detected following doses of radiation as low as 25 rads or approximately 1 DNA SSB per 10^{10} daltons (10, 16). The rate of elution has been found to be a first-order function of the DNA SSB frequency when no DNA cross-linking is present (10, 16). This has been quantitated in our experiments by the value of the RE which is derived by $\log R_0 - \log R$, where R represents the fraction of DNA retained on the filter at some reference time and R_0 is the fraction retained in an untreated sample (10). The reference time was when 27% of the ^{3}H internal standard DNA was retained on the filter, 12 hr of elution on a corrected time scale (10). The RE is proportional to the DNA SSB frequency (10), and an estimate of the SSB frequency can be obtained by comparing this value to the RE induced by 300 rads in L1210 cells, where the DNA SSB frequency is 8.1 SSB/10^{10} daltons, based on the SSB efficiency of 2.7×10^{-12}/rad/dalton found in L1210 cells (15). If the DNA SSB frequency was very high (RE >2), the SSB frequency was determined by taking an earlier intercept when 70% of the internal standard DNA was retained on the filter or after approximately 3 hr of elution.

RESULTS

Human fibroblasts were treated with the *trans*-Pt for 2 hr at 37° and analyzed by alkaline elution as shown in Chart 1. The cross-linking effect was removed with proteinase digestion of the cell lysates as described in "Materials and Methods." No residual DNA cross-links were detected when measured by a reduction in the γ-ray sensitivity (10) with alkaline elution (data not shown). In Chart 1, *trans*-Pt alone induced a negligible increase in elution. When the cells were treated with this agent

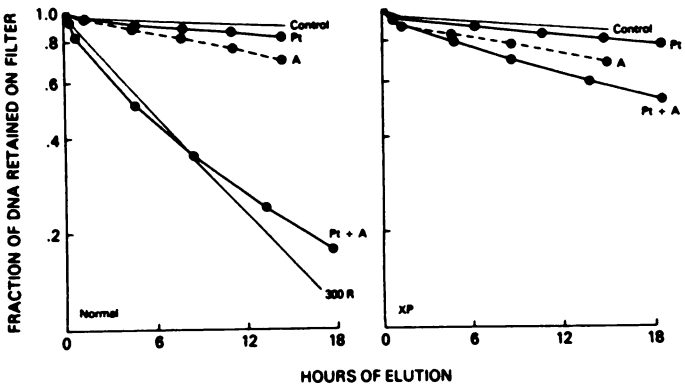

Chart 1. Effect of *trans*-Pt (*Pt*) and polymerase inhibitor (*A*) (ara-C plus HU) on alkaline elution. Cells were exposed to 50 μM *trans*-Pt with or without the polymerase inhibitor for 4 hr at 37°, and they were then rinsed and analyzed by alkaline elution. *Left*, normal human fibroblasts, AG 1522; *right*, XP, fibroblasts, XP12BE. Untreated cells (*Control*) or cells exposed to γ-radiation (300 rads) are included for comparative purposes. *Pt*, *trans*-Pt alone; *A*, polymerase inhibitor alone; *Pt + A*, *trans*-Pt and repair polymerase inhibitor; *R*, rads.

and the polymerase inhibitor ara-C and HU, a marked increase in elution was seen in normal cells. This increase in elution was comparable to that induced by 300 rads of γ-rays alone. In excision-deficient XP cells, no marked increase in elution was seen; the effect was only slightly greater than that seen by treatment with the polymerase inhibitor alone. A consistent small increase in elution was seen in all cells treated with the polymerase inhibitor; it did not appear to significantly vary with cell type. A reasonable interpretation of these results is that lesions induced in DNA by *trans*-Pt were excised in normal cells but not in XP cells; in the presence of the polymerase inhibitor, the transient SSB generated during this repair accumulated in normal cells. This result was similar to that seen with UV radiation (Chart 2), where XP cells are known to be deficient in excision repair. In normal cells with this dose of UV radiation, transient DNA SSB are seen during excision repair but by 1 hr approach control levels (9); in the presence of the polymerase inhibitor, they apparently accumulated to a high level as seen in Chart 2. In XP cells, this effect was not seen.

When cells were exposed to chromate salts, DNA-protein cross-links and transient DNA SSB were detected by alkaline elution (12). Cells were treated with potassium chromate and the polymerase inhibitor to see if an effect similar to that of *trans*-Pt or UV could be observed. In Chart 3, a high level of DNA SSB was again detected in normal cells treated in this manner; surprisingly, the result in XP cells was the same as in normal cells. The results with potassium chromate are summarized in Table 1. With chromate alone, only after the highest dose (50 μM) was an appreciable level of DNA SSB observed. With the polymerase inhibitor, DNA SSB were detected with all concentrations of chromate. An attempt was made in Table 1 to estimate the frequency of these presumed excision SSB by subtracting the background level induced by the polymerase inhibitor alone and by the chromate alone; this frequency appeared to be roughly proportional to the concentration of chromate used. The result with XP cells was similar to that seen with normal cells at all concentrations of chromate. If these DNA SSB seen with chromate and the polymerase inhibitor were the result of an accumulation of SSB generated during excision repair, then the repair mechanism must be different

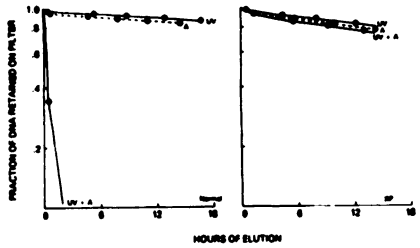

Chart 2. Effect of polymerase inhibitor on accumulation of DNA SSB after UV radiation as determined by alkaline elution. Cells were irradiated with 2 J/sq m and incubated with (*UV + A*) or without (*UV*) the polymerase inhibitor (*A*) for 1 hr at 37°. Samples were then analyzed and plotted as in Chart 1.

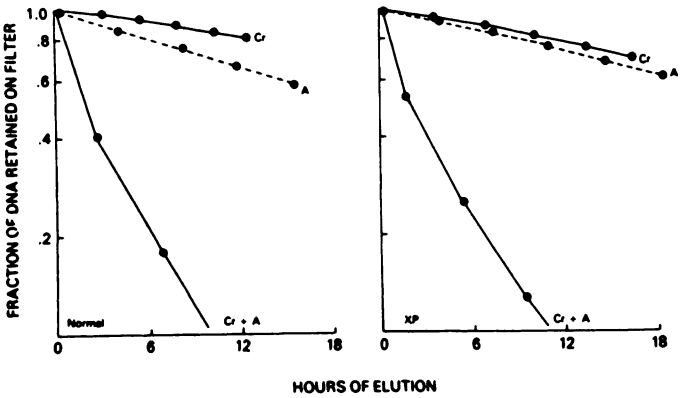

Chart 3. Effect of K_2CrO_4 and polymerase inhibitor on alkaline elution. Normal and XP cells were exposed to 5 μM K_2CrO_4 with (*Cr + A*) or without (*Cr*) the polymerase inhibitor (*A*) for 4 hr at 37° and analyzed as in Chart 1.

Table 1

Effect of K_2CrO_4 and polymerase inhibitor on alkaline elution

Cells were treated with varying concentrations of K_2CrO_4, with or without the polymerase inhibitor, for 4 hr as in Chart 3. Normal cell types are referred to as N1 (AG 1522) and N2 (CRL 1187), and XP cells are refersed to as XP1 (XP12BE). The increase in elution of DNA with treatment was measured by the value of the RE (see "Materials and Methods"). In Column 3, the effect of K_2CrO_4 alone is shown from representative experiments. In Column 4, the effect of K_2CrO_4 plus the polymerase inhibitor is shown. In Column 5, the net effect of the polymerase inhibitor on the accumulation of presumed excision DNA SSB is estimated by subtracting the RE induced by the polymerase inhibitor alone and the RE induced by K_2CrO_4 alone from the RE induced by both together (Column 4). An estimate of the frequency of DNA SSB represented in Column 5 by the RE is given in Column 6 (see "Materials and Methods").

K_2CrO_4 (μM)	Cell type	K_2CrO_4 alone (RE)[a]	K_2CrO_4 + A (RE)	Net A (RE)	SSB/10^{10} daltons
0	N1		0.15		
	N2		0.14		
	XP1		0.07		
2	N1	0.00	0.49	0.34	5.2
	XP1	0.00	0.54	0.47	7.2
5	N1	0.06	1.10	0.89	14.0
	N2	0.07	0.80	0.59	9.1
	XP1	0.04	0.98	0.88	13.0
50	N1	0.34	>2.00	>2.00	70
	XP1	0.30	>2.00	>2.00	70

[a] RE, relative elution; A, polymerase inhibitor.

than the usual UV excision pathway, since XP cells responded normally.

This approach was also used with a third DNA-protein cross-linking agent, formaldehyde. In Table 2, these results are summarized with several normal and XP cell lines. With formaldehyde alone, no appreciable level of SSB was seen; in the

Table 2

Effect of polymerase inhibitor on accumulation of DNA SSB induced by various agents

Cells were treated with various agents and the polymerase inhibitor as in Table 1. The cell types were as in Table 1 with the addition of XP25RO (XP2). Cells were treated in serum-free medium for 1 hr with formaldehyde and then incubated for an additional 3 hr in complete medium. In the case of TPA, the first sample was treated in serum-free medium for the entire 4 hr; the second sample was treated in medium containing heat-inactivated serum. The source of the TPA is given in parentheses. The cells were exposed to asbestos in medium containing heat-inactivated serum. Catalase was used at 1000 units/ml and superoxide dismutase at 15 units/ml. The increase in elution produced by exposing the cells to γ-radiation at 0° is included for comparative purposes.

Agent	Cell type	Agent alone (RE)	Agent + A (RE)	Net A (RE)	SSB/10^{10} daltons
UV (0.04 J/sq m)	N1	0.00	0.30	0.18	2.8
trans-Pt (50 μM)	N1	0.02	0.53	0.43	6.5
trans-Pt (50 μM)	XP1	0.02	0.15	0.03	0.5
Formaldehyde (800 μM)	N1	0.00	0.85	0.67	10.2
Formaldehyde (800 μM)	N2	0.03	0.89	0.71	10.9
Formaldehyde (800 μM)	XP1	0.01	0.14	−0.05	0
Formaldehyde (800 μM)	XP2	0.01	0.05	0.03	0.5
$NiSO_4 \cdot 6H_2O$ (250 μg/ml)	N1	0.00	0.01	0.00	0
TPA (C.C.R., Inc.) (2 μg/ml)	N1	0.00	0.21	0.03	0.5
TPA (Consolidated Midland) (5 μg/ml)	N1	0.02	0.14	−0.01	0
Asbestos (100 μg/ml)	N1	0.02	0.14	0.00	0
Control	N1		0.12 ± 0.02		
Catalase/superoxide dismutase	N1	0.00	0.12		
300 rads	N1	0.53			

[a] A, polymerase inhibitor; RE, relative elution.

presence of the polymerase inhibitor, a significant level of SSB accumulated in normal cells but not in XP cells. The effect with *trans*-Pt is also included in this table for comparative purposes. As seen in Table 2, this can be an extremely sensitive approach to detect DNA SSB generated during excision repair. For example, an effect was seen with as little as 0.04 J of UV radiation per sq m. Several other agents were investigated in this manner. With the metal carcinogen Ni^{+2}, no DNA SSB were detected either directly or with the polymerase inhibitor. No DNA SSB were detected with nickel sulfate with concentrations of 0.01 to 2 mg/ml (data not shown). Also, no effect was seen with TPA obtained from 2 sources. There is evidence that sister chromatid exchanges are induced by TPA in cells in medium with heat-inactivated serum (18), but no significant effect was seen by alkaline elution. Also, no effect was seen in medium without serum. No effect was observed with asbestos fibers. It should be noted that, if large amounts (>1 mg) of asbestos are put directly on the filter prior to alkaline elution, some degradation of DNA from untreated cells is observed (data not shown). In Table 2, a smaller amount of asbestos was used, and the cells were extensively washed on the filter to remove free asbestos fibers. When these precautions were taken, treatment of fibroblasts with asbestos had no effect on alkaline elution.

As seen in Table 2, treatment of cells with the polymerase inhibitor ara-C plus HU had a small effect on alkaline elution comparable to that produced by 70 rads of γ-rays, 1.8 SSB/10^{10} daltons. The cells at this time were confluent, and the radioactive thymidine had been removed several days earlier. The hypothesis was tested that a small level of excision repair may be due to continual spontaneous damage in the DNA such

as by peroxides or free radicals. However, treatment of the cells with catalase and superoxide dismutase had no effect on the frequency of SSB induced by the polymerase inhibitor.

DISCUSSION

Dunn and Regan (6) have presented evidence that the DNA SSB, which were seen after UV radiation in the presence of ara-C and HU, were generated during excision repair. Their result was confirmed in this investigation. However, with alkaline elution, one DNA SSB can be detected per 10^{10} daltons (10, 16), while no less than 50 SSB per 10^{10} daltons are detectable by the usual alkaline sucrose sedimentation method (15). With this increased sensitivity, excision SSB were detected after 0.04 J/sq m. DNA SSB were easily detected in normal cells after treatment with either *trans*-Pt or formaldehyde and the polymerase inhibitor. These were not markedly toxic doses because colony survival in rodent cells was greater than 10% of control with this concentration of *trans*-Pt (11); with formaldehyde, a similar dose resulted in 50% colony survival in normal human fibroblasts (5). Apparently, these DNA SSB are generated by the UV-type excision pathway, since a negligible effect was seen in XP cells. The excision SSB seen with these agents were much less than those seen with equitoxic doses of UV radiation (5). This probably accounts for the observation that no appreciable level of SSB was detected without the polymerase inhibitor with *trans*-Pt or formaldehyde, the polymerase inhibitor being necessary to accumulate a sufficient number of excision SSB to detect even by alkaline elution.

An argument could be raised that these SSB seen with the polymerase inhibitor and DNA-damaging agents might be due to some direct effect on the DNA. For example, the DNA from necrotic cells will occasionally appear fragmented by alkaline elution (16). As stated previously, the toxicity of the various DNA-damaging agents used was low. The cells were confluent and would not be expected to be very sensitive to S-phase-specific agents such as ara-C or HU. The most obvious objection to this argument is that, with several agents, no appreciable level of SSB was seen in excision-deficient XP cells. It should also be added that Dunn and Regan (6) found that, when the polymerase inhibitor was removed, the DNA SSB which had accumulated after UV radiation were rapidly rejoined.

Although this investigation indicates that *trans*-Pt and formaldehyde induce DNA repair, it does not address which lesion(s) is recognized by the excision repair mechanism. Since DNA-protein cross-links are removed in normal but not in XP cells (7), the implication is that this lesion is repaired by the excision mechanism with the resultant SSB in the presence of the polymerase inhibitor. In the case of formaldehyde, numerous lesions can be induced in DNA and nucleoprotein (1). While excision-deficient yeast (2) and bacteria (19) are more sensitive to formaldehyde by colony survival, DNA-protein cross-links were removed equally well in normal and in excision-deficient strains of yeast (17).

In the case of potassium chromate, a similar effect was seen in normal and XP cells. This would indicate that lesions induced by this agent in DNA are repaired by a mechanism different from the UV type. It is unlikely that chromate, ara-C, and HU could have such an effect on the cellular DNA that DNA SSB would be induced directly. The cells were confluent at the time of treatment, and the radioactive label had been removed several days earlier. The lower concentrations of chromate used have been shown to be nontoxic to human fibroblasts by colony survival (20). As mentioned earlier, chromate salts have already been shown to induce DNA repair as detected by unscheduled DNA synthesis (20). Chromate induced a surprisingly high frequency of excision SSB; on a toxicity basis (20), the level induced was at least the same order of magnitude as would be expected from an equitoxic dose of UV radiation (6). The lesion(s) recognized by this repair mechanism is uncertain. The level of excision SSB is probably significantly greater than the frequency of DNA-protein cross-links induced by the same concentration of chromate (12). Chromate salts are rapidly reduced to the trivalent form in the cell (reviewed in Ref. 12). The trivalent form of chromium readily forms stable coordination complexes with a very slow rate of ligand exchange (4). This might be expected to induce damage by binding to the lone pair of electrons of nitrogen and other nucleophilic sites in DNA or chromatin.

In the case of the tumor promoter TPA, no effect was seen in this investigation. The same lot of TPA was used as in the study where sister chromatid exchanges were detected (18). Sister chromatid exchanges are usually observed in cells after treatment with a DNA-damaging agent, but no perturbation of the cellular DNA was observed by alkaline elution. An argument could be made that the length of exposure was inadequate to rule out such an effect, but in the case of TPA, qualitative morphological changes were seen within 1 hr with these high concentrations of TPA (data not shown). This argument could also be raised with the asbestos results, but asbestos fibers have been shown to penetrate cells in culture within 1 to 2 hr.[3]

Finally, ara-C and HU alone were found to induce a small significant level of DNA SSB (see Table 2). This increased with the time of exposure (data not shown). The possibility exists that HU or ara-C may directly induce a low level of DNA SSB in nongrowing cells, but this would be inconsistent with their known mechanisms of action (reviewed in Ref. 6). It was hypothesized that a low level of "spontaneous" DNA damage was continually being repaired; however, the addition of catalase and superoxide dismutase had no effect on this SSB frequency. The elution profiles were relatively linear, which indicates that these SSB were probably randomly distributed (16). The cause for these DNA SSB is uncertain, but it is tempting to speculate that even nondividing DNA may be continually undergoing some sort of "text-editing" process where DNA SSB are generated.

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