

Cellular and Adenovirus dl312 DNA Metabolism in Cycling or Mitotic Human Cultures Exposed to Supralethal Gamma Radiation

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Abstract. Cellular repair of DNA damage due to lethal gamma irradiation was studied to reveal differences between strains and cell cycle stages that are otherwise difficult to detect. Cycling and metaphase-blocked cultures of normal fibroblasts and carcinoma cells were compared for repair of gamma sites (gamma radiation-induced nicks, breaks, and alkali-labile sites in DNA) at supralethal exposures ranging from 7 to 150 krad ^{137}Cs radiation and at postirradiation incubations of 20–180 min. Fibroblasts from normal human skin or lung repaired gamma sites efficiently when cycling but did not repair them when blocked at mitosis. Bladder (253J) or lung (A549) carcinoma cells, unlike normal fibroblasts, repaired gamma sites efficiently even when blocked at mitosis. HeLa cells degraded their DNA soon after exposure at all doses tested, regardless of mitotic arrest.

Whether the above differences in DNA repair between cell cycle stages and between strains result from differences in chromatin structure (*cis* effects) or from differences in the nuclear enzymatic environment

(*trans* effects) could be resolved by placing an inert, extrachromosomal DNA molecule in the cell nucleus. Specifically, *cis* effects should be confined to the host chromosomes and would not be detected in the inert probe whereas *trans* effects should be detected in host chromosomes and inert probe DNA alike. Indeed, we found a suitable DNA molecule in the adenovirus deletion mutant dl312, which does not proliferate in the absence of E1A complementation. Gamma sites in ^{32}P -labeled adenovirus dl312 DNA were repaired efficiently in all hosts, regardless of mitotic arrest. Failure of mitosis-arrested fibroblasts to repair gamma sites was therefore due to a *cis* effect of chromatin organization rather than to a *trans* effect such as repair enzyme insufficiency. In sharp contrast, chromosomes of mitotic carcinoma cells remained accessible to repair enzymes and nucleases alike. By means of these new tools, we should get a better understanding of higher-order chromatin management in normal and cancer cells.

WHEREAS many properties of cultured cells, including donor age (Liu et al., 1982), previous DNA damage to tissue of origin (Liu et al., 1985), viral transformation (Smith and Hanawalt, 1976), and cell lineage (Taichman and Setlow, 1979), appear not to influence DNA repair competence, local chromatin structure and the cell cycle stage do influence DNA repair in eukaryotic cultured cells. Ionizing radiation damage in active genes exceeds that in total cell DNA, which is mostly not in genes (Chiu et al., 1982). Transcribed, chromosomal genes are also repaired more efficiently than total cell DNA (Chiu and Oleinick, 1982; Bohr et al., 1985). In heavily irradiated, mitotic hamster fibroblasts, gamma radiation damages in DNA complementary to polyA⁺ RNA were repaired more rapidly than similar damages in nontranscribed DNA (Oleinick et al., 1984). However, the cell cycle itself influences chromosomal DNA repair; mitotic cells repair gamma radiation damages less efficiently than interphase cells (Oleinick et al., 1984).

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9L tumor cells proliferating in rat brains repair ionizing radiation damage more completely than postmitotic, normal brain cells (Wheeler and Wierowski, 1983). In hamster ovary cells blocked at mitosis by N_2O , the K_m for the removal of pyrimidine dimers was elevated and V_{max} was depressed relative to cycling cells, as if repair enzymes acted less effectively on mitotic chromatin (Collins et al., 1980).

Greater availability of "active" chromatin to solutes could possibly explain the above data. However, the sensitivity of chromatin to endogenous DNA repair is not distributed exactly like its sensitivity to digestion by exogenous DNase. For example, repair in extrachromosomal *Tetrahymena* ribosomal genes did not exceed that in total cell DNA (Chiu and Oleinick, 1980). Also, a globin gene not transcribed in fibroblasts underwent damage and repair just like a transcribed collagen gene (Nose and Nikaido, 1984). In contrast, transcribed genes are generally hypersensitive to exogenous DNase I when compared with the same genes when inactive or with other inactive DNA (Yaniv and Cereghini, 1987). Solvation alone cannot readily explain the observed differences. Instead, it may be that repair enzymes have evolved for selec-

tive maintenance of chromosomal genes over nongene DNA. Also, endogenous repair enzyme levels could vary with the cell cycle. Thus, chromatin structure is not the only possible explanation for decreased repair in mitotic cells (Collins et al., 1980). One way to distinguish chromatin condensation effects on repair (*cis* effects) from effects of fluctuations in enzyme activity (*trans* effects) would be to introduce into the nucleus an inert, nonchromosomal DNA molecule and then to monitor damage and repair in that molecule and in cell DNA. For this purpose, we selected a DNA virus with a deletion in an early function gene.

Adenovirus replicates in the cell nucleus. Cells of patients from some xeroderma pigmentosum complementation groups do not reactivate UV-damaged adenovirus as efficiently as normal cells (Day, 1974; Day et al., 1978). Since xeroderma pigmentosum cells fail to repair UV damage in their own DNA, virus DNA repair is thought to depend upon cellular enzymes. Similarly, cells of patients with ataxia telangiectasia repair ionizing radiation damage poorly and reactivate gamma-irradiated adenovirus poorly (Jeeves and Rainbow, 1986). In contrast, UV reactivation of adenovirus was normal in Cockayne syndrome cells (Hoar and Davis, 1979), as might have been expected since cells from these sun-sensitive patients repair their DNA normally.

The above data indicate that adenovirus can be used to study human DNA repair enzyme action on extrachromosomal DNA. However, in each case, the end point was plaque assay rather than actual DNA repair. We found no reports in which molecular repair of adenovirus DNA was measured. Also, the chromatin of cells undergoing vegetative adenovirus infection may be modified or degraded (White et al., 1984). Since vegetative growth is a requirement for plaque formation—but not for biochemical measurements of DNA damage and repair—it seemed logical to study repair in DNA of adenovirus host range mutants (i.e., mutants unable to grow on some normal adenovirus hosts) where the defect is in virus replication. Adenovirus dl312 (dl312) replicates like wild-type adenovirus in human embryonic kidney cells that carry the viral E1A region (e.g., 293 cells; Graham et al., 1977; Aiello et al., 1979). dl312 carries a 1-kbp deletion in the E1A early transcription unit (Jones and Shenk, 1979). In cells that cannot complement this viral defect, transcription of viral genes is delayed, and dl312 fails to proliferate (Jones and Shenk, 1979; Berk et al., 1979). dl312 DNA is 34 kbp long—large enough to measure some kinds of DNA damage at levels that are sublethal to infected cells.

Gamma rays cause nicks, breaks, and alkali-labile sites as well as other types of damage in DNA (Mee and Adelstein, 1987; Teoule, 1987). For any lesion registered as a chain scission in alkali, we use the term gamma site. Denatured DNA containing gamma sites sediments more slowly in alkali than unirradiated DNA. Cellular repair enzymes remove the gamma sites, increasing the single-strand molecular weight of the DNA and its alkaline sedimentation velocity (Lett, 1981). We report here on dl312 as a nonreplicating, chromatin-like DNA molecule for molecular studies of DNA repair in human cells. We report also on differences in the repair of gamma sites between (a) cycling and mitotic cells;

(b) various normal fibroblast strains and carcinoma cell lines; and (c) host cell DNA and that of infecting dl312.

Materials and Methods

Human Cell and Adenovirus Culture

HeLa, 293 (Graham et al., 1977), 253J bladder carcinoma (Elliot et al., 1974), and A549 lung carcinoma (Fogh et al., 1977) cells (all from Joseph R. Nevins, Department of Biology, Duke University, Durham, NC) and secondary, normal skin fibroblasts (from this laboratory) or WI-38 lung fibroblasts (from Arthur K. Balin, Chester, PA) were cultured in DME (Gibco Laboratories, Grand Island, NY) containing 10% FBS. All strains tested negative for mycoplasma by fluorescence microscopy and uridine/uracil uptake. HeLa cells were also cultured in spinner flasks in Joklik medium (Gibco Laboratories) containing 5 or 10% FBS.

Plate stocks of dl312 were prepared by infection of 293 cells (Graham et al., 1977). ³²P-labeled dl312 was prepared by a method from the laboratory of J. R. Nevins as follows: dl312 at a multiplicity of infection (MOI) of 10⁵ particles/cell was added to 293 cell spinner cultures (~5 × 10⁵ cells/ml) in phosphate-free Joklik medium to which a trace of phosphate was added back to encourage growth. Cells were fed with 1 vol complete low-phosphate medium containing 20 μCi/ml H₃[³²P]O₄ (New England Nuclear, Boston, MA) 3 h after infection. 3–4 d after infection, cells were centrifuged, frozen and thawed three times in PBS, and sonicated for lysis. The virion-rich lysate was delipidated twice with freon (to which adenovirus is impervious) and then sedimented through a discontinuous CsCl gradient (*d* = 1.2–1.45 g/cm³) for 2 h at 20,000 rpm followed by a linear CsCl gradient (*d* = 1.2–1.45 g/cm³) for 12 h at 20,000 rpm. The band obtained from the last gradient was supplemented with a mixture of 4 vol 50% glycerol, 0.1% BSA, 0.25 M NaCl, and 0.1 M Tris (pH 7.4) and then stored at ~5 × 10¹¹ particles/ml (1 OD₂₆₀ = 10¹² particles/ml) at –20°C for up to 1 mo for use in experiments. In this manner, yields of 10¹³ particles (2–5 × 10¹¹ plaque-forming units; determined by plating on 293 cells) per liter cells at specific activity of ~10⁵ cpm/particle were obtained. When virus yield was low, purified virus was dialyzed against the glycerol solution (without BSA) to remove CsCl, which is toxic to cells.

Labeling of Cell DNA

To label their DNA uniformly, cells were grown for several doublings in medium containing 0.05 μCi/ml [*methyl*-³H]thymidine (7 Ci/mmol; NEC-027; New England Nuclear) or 0.01 μCi/ml [*methyl*-¹⁴C]thymidine (40 mCi/mmol; NET-568; New England Nuclear) and then incubated overnight in nonradioactive medium. The specific activity of [³H]DNA so obtained exceeded that of [¹⁴C]DNA by ~10-fold.

Infection with Virus

Cells were washed in serum-free medium (pH 6.8–7). Virus in the same medium was added at ~10⁹ particles/ml (MOI = 60–300 particles/cell) or a different amount as shown. The culture was shaken gently at 37°C for 1 h. Nonadsorbed virus was aspirated with the supernatant. HeLa spinner cultures in serum-free medium were infected at ~10⁶ cells/ml for 1 h, washed, and suspended in growth medium.

Mitotic Arrest

To cause mitotic arrest, cells were cultured in growth medium containing colcemid, which binds to the growing ends of nascent microtubules thus preventing cytokinesis (Andreu and Timascheff, 1986). Virus-infected (or uninfected) cells were incubated in nonradioactive, complete medium with or without colcemid (0.1–0.5 μg/ml) for 12–36 h, depending on the yield of mitoses (spinner cultures) or refractile (mitotic) cells (stationary cultures; i.e., cultures grown without agitation). Cells remained alive (excluded trypan blue) for at least 96 h of incubation in colcemid. Infection by dl312 had no effect on the yield of mitoses in any of our experiments.

Mitotic Selection

Three categories of cells were used in these experiments: control, mitotic, and nonmitotic. Control cells were not exposed to colcemid. Mitotic cells could be removed in two steps from stationary cultures to which colcemid had been added from the postlabeling chase onward. Loose, mitotic cells

1. *Abbreviations used in this paper:* dl312, adenovirus dl312; MOI, multiplicity of infection.

were shaken off into the medium and used if viability was >90%. Remaining mitotic cells were detached by trypsin (1–3 min on ice; monitored by inverted phase-contrast microscopy), aspirated, and combined with those cells released by shaking. Nonmitotic cells adhered to the flask and were detached by adding more trypsin at 37°C. Tryptic action was stopped by adding fresh medium (with or without colcemid). Cells were centrifuged and suspended in fresh medium. In experiments using spinner cultures, mitotic and nonmitotic cells were not separated. To determine the fraction of total cells possessing condensed chromosomes, or mitotic index, cells were washed and suspended in 0.5% KCl, fixed (20 min) in 3:1 CH₃OH/CH₃COOH, then washed in 3:1 CH₃OH/CH₃COOH, dropped on slides, and stained with Giemsa (Gibco Laboratories) at 1% in 0.02 M KH₂PO₄, pH 6.5. Mitotic index of the mitotic cell fraction was 0.8–0.95 for stationary cultures and 0.65–0.85 for spinner cultures. Mitotic indices of control and nonmitotic fractions were <0.05.

Gamma Irradiation

Cultures in growth medium (pH 6.8–7) were immersed in ice water and exposed to gamma rays from a ¹³⁷Cs source at 1.5 krad/min. Irradiated cells were then incubated at 0 or 37°C. DNA repair in cells held on ice would artifactually depress the apparent level of DNA damage. However, postirradiation incubation at 0°C had no effect on the sedimentation profile of DNA from irradiated cells. The yield of gamma sites from our ¹³⁷Cs source was approximately one per 8 kbp/60 krad in naked DNA (from initial slope analysis; Matsuo and Ross, 1987); one per 17 kb/60 krad in dl312 virions (by alkaline sedimentation profile); and one per 12 kb/60 krad in cell DNA or intracellular virus (using internal markers).

Lysis and Sedimentation in Alkaline Sucrose Gradients

Linear, 5-ml, 5–23% alkaline sucrose gradients were prepared with 10 mM EDTA, 100 mM NaCl, and 50 mM KH₂PO₄, adjusted to pH 12.5 with NaOH. In most experiments, an 0.05-ml cushion containing 60% sucrose and 0.3 M NaOH was placed at the bottom of the tube. Then, 0.17 ml of 0.5 M NaOH and 0.05 ml of 0.2% triisopropylphenyl sulfonic acid (Eastman Kodak Co., Rochester, NY), 0.2% *p*-amino-salicylic acid (Sigma Chemical Co., St. Louis, MO), and 12% 2-butanol were carefully placed in layers atop each gradient. Identical results were obtained with a single layer composed of 0.1 M NaOH, 0.01 M NaEDTA, and 1% *N*-lauroyl sarcosinate (CIBA-GEIGY, Basel, Switzerland).

Cells were washed in divalent cation-free PBS (Gibco Laboratories) and suspended at 5×10^6 /ml in 0.85% saline. Volumes of 75 or 100 μ l of cells in saline were applied to the surface of each gradient. After a 30-min incubation at 20°C to denature DNA, gradients were centrifuged at 30,000 or 40,000 rpm to $w^2t = 6 \times 10^{10}$ (w = angular velocity in radians/s and t = time in seconds; cells exposed to 7–40 krad) or at 49,000 rpm to $w^2t = 1.5 \times 10^{11}$ (cells exposed to >40 krad). Fractions withdrawn from the top were supplemented with 5 vol (~1 ml) H₂O and shaken with 10 ml Hydrofluor (National Diagnostics, Inc., Sommerville, NJ) until they cleared. Radioactivity was measured by liquid scintillation counting at ambient temperature. Except for dl312-infected HeLa cells, duplicate gradients or samples were reproducible within 5% whenever tested; wide error bars found in summary figures result from differences in conditions between experiments. A script written in Symphony (Lotus Development Corporation, Cambridge, MA) subtracted background and then determined the median fractional distance sedimented. Data are shown as ratios of median molecular weights.²

2. A choice had to be made to compare DNA molecular weights. Ideally, the data should be summarized as lesions repaired. The number averaged molecular weight (\bar{M}_n) is inversely related to the average frequency of interruptions, regardless of their distribution (Charlesby, 1954). \bar{M}_n is extremely sensitive to label at the top of sucrose gradients, so it is sometimes calculated by ignoring fractions out of the main peak (Youngs and Smith, 1976). However, we had difficulty identifying the cutoff point in this analysis. Alternatively, the weight averaged molecular weight (\bar{M}_w) may be used to infer \bar{M}_n and break frequency for certain distributions (Charlesby, 1954); for random fragment length distributions, \bar{M}_n may be calculated exactly by omitting the top of the gradient and the three bottom fractions and then subtracting the molecular weight at the upper cutoff point (Lehmann, 1981). However, the fragment length distributions caused by cell lysis, by damage, and by repair in cellular DNA are neither random nor uniform, so such calculations are approximations. Moreover, some of our samples yielded dual peaks; in others, gradients were slightly over- or underrun, so that part of the sample remained in the lysis layer or on the cushion. Such phenomena

Results

Effect of Colcemid on Gamma Site Repair in Fibroblast DNA

We first tested for the effect of colcemid-induced mitotic arrest on the ability of normal human fibroblasts to remove gamma sites from their DNA. Both mitotic and nonmitotic fibroblasts (see Materials and Methods) were incubated either at 0 or 37°C before lysis and sedimentation in alkali. In Fig. 1 *b*, the DNA from ³H-labeled nonmitotic cells held at 37°C sedimented further into the gradient than did DNA from nonmitotic fibroblasts held at 0°C (Fig. 1 *a*). In sharp contrast, DNA from mitotic fibroblasts held at 37°C (Fig. 1 *d*) cosedimented with DNA from mitotic or nonmitotic cells incubated at 0°C after irradiation (Fig. 1, *a* and *c*, respectively). Thus, mitotic cells were unable to remove gamma sites from their DNA; nonmitotic, colcemid-treated cells repaired gamma sites. When similar numbers of ¹⁴C- and ³H-labeled cells were mixed just before sedimentation (Fig. 1, *e* and *f*), ¹⁴C-labeled cells behaved like similarly treated ³H-labeled cells. Importantly, ³H sedimentation profiles were identical with those for gradients run without ¹⁴C-labeled cells. There was no interaction of DNA species in the gradients, so sedimentation profiles reflect true molecular weight distributions.

Dose-Response Curves for HeLa Cells and Fibroblasts

Nonmitotic and mitotic fibroblasts from colcemid-treated cultures were compared with control fibroblasts for repair of gamma sites caused by various ionizing radiation doses (12–60 krad). In Fig. 2 *a*, the fractional increase in \bar{M} (median molecular weight) due to incubation at 37°C is plotted against gamma radiation. In this plot as in other scatter plots and bar graphs presented here, the ratio $\bar{M}_{37}/\bar{M}_0 = 1$ when there was no effect of postirradiation incubation on \bar{M} ; $\bar{M}_{37}/\bar{M}_0 > 1$ when there was repair at 37°C; and $\bar{M}_{37}/\bar{M}_0 < 1$

distort calculated \bar{M}_n values. To permit analysis of more data and to avoid the problem that an average frequency of nonrandom events has questionable meaning, we sought another molecular weight measurement.

The median, \bar{M} , is the molecular weight where half the DNA strands in a sample are longer and half are shorter. Unlike \bar{M}_n and \bar{M}_w , \bar{M} is not weighted to fragment size or number and so cannot be used to calculate average break frequency. Because it is not weighted, the numerical magnitude of effects on \bar{M} is often smaller than that of effects on the weighted averages. However, \bar{M} is insensitive to background and to distribution shape; its physical significance can be readily grasped and it is readily picked out of a distribution by the human eye (Matsuo and Ross, 1987). Also, unlike the weighted averages, its accuracy is relatively impervious to nonlinearity caused by lysis layer or cushion. The median fractional distance,

$$\bar{d} = \frac{P + F}{T}$$

where P is the number of the fraction preceding that containing >50% of total elapsed counts, T is the total number of fractions in the gradient, and

$$F = \frac{tc/2 - pc}{ac - pc}$$

where tc is the total counts in the gradient, pc is the elapsed counts to fraction P , and ac is the elapsed counts to the fraction after P . We calculated ratios of median molecular weights from the relation $\bar{M}_1/\bar{M}_2 = (\bar{d}_1/\bar{d}_2)^a$, where the subscripts 1 and 2 represent the two gradients being compared (usually the 0 and 37°C samples) and the exponent a is a constant (Abelson and Thomas, 1966). Using markers, we found $a = 2.6$.

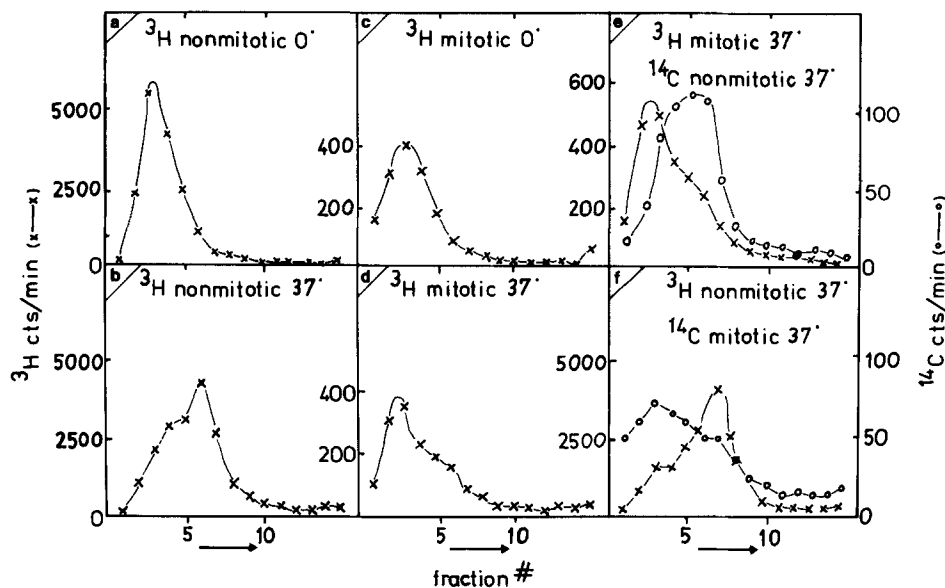


Figure 1. Effects of mitotic block by colcemid on the repair of gamma sites in the DNA of normal human fibroblasts. DNA of cells blocked in mitosis at the time of irradiation retained a lower single-strand molecular weight after incubation for repair than did DNA in nonmitotic cells from the same culture. Normal human lung fibroblasts (WI38; midway through their proliferative lifespan) were labeled with [³H]- or [¹⁴C]thymidine, held overnight in fresh, non-radioactive medium, supplemented with 0.1 μg/ml colcemid, and incubated 4 h before mitotic selection. The mitotic cells were ~10% of the total. The mitotic and nonmitotic cells were exposed in ice water for 15 min to (22.5 krad) ¹³⁷Cs gamma rays, held at 0 or 37°C for 180 min, mixed to the appropriate levels of radioactivity, and then lysed and sedimented in alkali as described in Materials and Methods.

when there was degradation at 37°C. In Fig. 2 *a*, both nonmitotic and control cells repaired their gamma sites ($\bar{M}_{37}/\bar{M}_0 = 3-7$); mitotic fibroblasts did so much less efficiently ($\bar{M}_{37}/\bar{M}_0 = 1-2$). Data for each condition, assembled from various experiments with normal fibroblasts from various foreskin biopsies, normal adult skin, or fetal lung, do not follow a simple curve. Two-tailed *t* tests showed no significant

difference between the matched data for control and nonmitotic cells. However, data for control and nonmitotic cells both differed significantly from the data for mitotic cells ($p < 0.05$ and $p < 0.001$, respectively). Effects in Fig. 1 and Fig. 2 *a* were therefore due to mitotic arrest per se rather than to nonspecific effects of the colcemid.

Control and mitotic HeLa cells substantially degraded their DNA when incubated at 37°C after all gamma exposures tested (7.5–60 krad; Fig. 2 *b*). Similar results were obtained with spinner and stationary cultures, when the medium was changed before exposure and the cells were exposed at room temperature, and in double-label experiments using ¹⁴C- and ³H-labeled cells. Mycoplasma contamination of the cells was excluded as an explanation by both fluorescence microscopy and uridine/uracil incorporation tests. Moreover, HeLa cells labeled in medium containing 200 μg/ml gentamycin degraded their DNA in similar experiments. The disruption of HeLa cell DNA metabolism by supralethal gamma irradiation appeared to be inherent to the cell line.

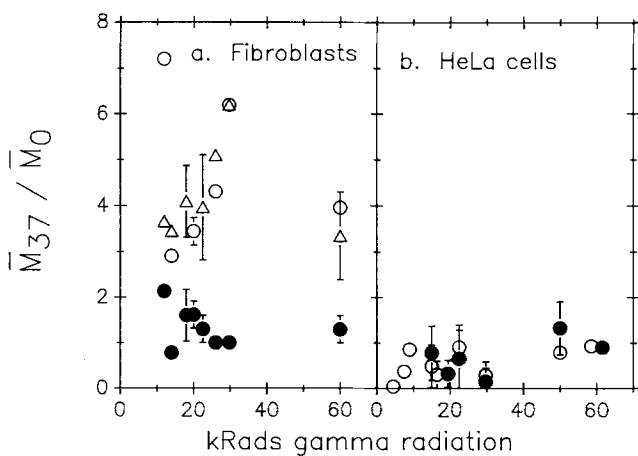


Figure 2. The effect of postirradiation incubation temperature on median molecular weight of DNA from (a) skin or lung fibroblasts and (b) HeLa cells is plotted against radiation dose. Control, mitotic, and nonmitotic cells exposed to radiation were held in growth medium at 0 or 37°C for 120–150 min (each set of paired samples was incubated for the same interval). Cells were lysed and sedimented in alkali. The abscissa represents the dose of gamma radiation and the ordinate represents the ratio of median molecular weight for cells held at 37°C for repair to that for cells held on ice. See text for further explanation. This scatter plot shows averaged data from 28 distinct experiments. (Open circles) Control cells; (triangles) nonmitotic cells; (solid circles) mitotic cells.

Time Course of Gamma Site Repair

The difference in Fig. 2 between fibroblasts and HeLa cells might reflect processes occurring in all cells exposed to supralethal radiation but which may occur at different rates in different strains. If so, HeLa cells might have repaired their DNA early after irradiation, just like the fibroblasts, and then subsequently degraded their DNA. Thus, if the fibroblasts were tested at longer postirradiation incubations, they might degrade their DNA like HeLa cells did in Fig. 2. Alternatively, the patterns in Fig. 2 could indicate relatively stable, dynamic equilibria of DNA repair and degradation in these different cell types. To distinguish between these two possibilities, we performed experiments in which postirradiation incubation was varied.

When mitotic and nonmitotic fibroblasts were exposed to 60 krad gamma radiation and incubated at 0 or 37°C for

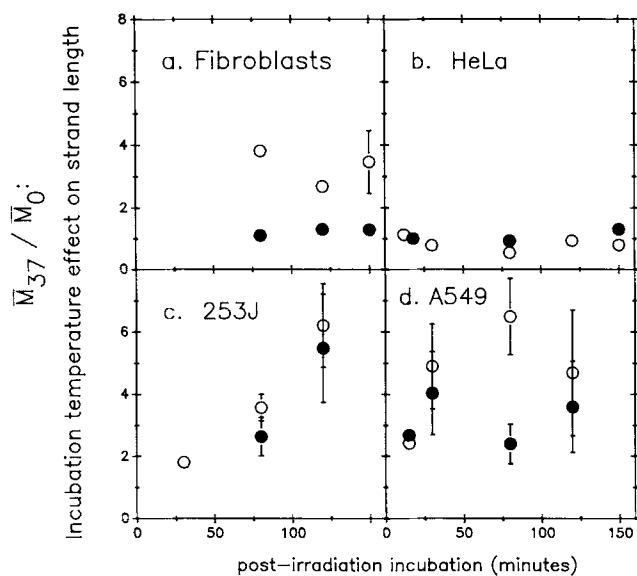


Figure 3. Time course of gamma site repair of cell DNA. Cells exposed to 60 krad gamma radiation were incubated for the intervals shown on the abscissa at 0 or 37°C. Median molecular weight ratios were determined as in Fig. 2. Matched data for (open circles) no mitotic and (solid circles) mitotic cells are shown for several experiments each with (a) fibroblasts; (b) HeLa cells; (c) 253J bladder carcinoma cells; (d) A549 lung carcinoma cells.

80–150 min, the fibroblasts behaved essentially as in the previous experiments conducted at lower radiation dosages: \bar{M} for the colcemid-treated nonmitotic cells (open circles) tripled at 37°C and \bar{M} for the mitotic cells (solid circles) was unaffected by incubation temperature (Fig. 3 a). Even when fibroblasts were exposed to 20 krad gamma radiation and incubated at 37°C overnight, net DNA repair was detected (not shown). Cycling fibroblasts evidently repaired supralethal DNA damage, and DNA integrity was maintained after relatively long postirradiation incubations, even though the cells were technically dead.

In the time course tests of HeLa cells exposed to 7.5 krad, there was an early indication of increased molecular weight followed at 20 min by a rapid, irreversible degradation of the

cell DNA (not shown). After exposure to 60 krad, the early increase in \bar{M} was negligible (Fig. 3 b), and colcemid had little sparing effect on degradation of HeLa cell DNA. Like fibroblasts, irradiated HeLa cells continued to exclude trypan blue and exhibited normal staining with Giemsa; there was no gross microscopic evidence for catastrophic autolysis.

We also tested the 253J bladder carcinoma (Fig. 3 c) and A549 lung carcinoma (Fig. 3 d) cell lines. Each of these lines repaired gamma sites efficiently, even when blocked in mitosis by colcemid (Fig. 3, c and d). The marginal (~20%) decrease in gamma site repair due to mitotic block in these cells contrasts with the virtual elimination of gamma site repair in fibroblasts (Fig. 3 a).

The qualitative differences between repair in the various cultured cell types and also the inhibition by mitotic arrest of gamma site repair in fibroblasts were reproducible at various doses and incubations and between experiments and thus are intrinsic properties of the cells. The observed effects could be explained by chromosome structure (*cis* effects) or by variables like active gamma site repair enzyme or cofactor levels (*trans* effects). We therefore evaluated nonreplicating dl312 as a nonchromatin probe of the processes we had uncovered.

Suitability of Adenovirus dl312 as a Nonchromatin Probe for DNA Repair

Our criteria for assessing virus attachment and nuclear translocation of ³²P-labeled dl312 DNA are shown in Table I. dl312 was variably infective. Once in the cell, its DNA reached the nucleus efficiently in all hosts since both ultraviolet endonuclease-sensitive sites and psoralen interstrand cross-links were quantitatively removed from DNA of infecting dl312 exposed to these agents *in vitro* before infection. Efficient entry of adenovirus 2 into the nucleus upon attachment to KB cells was shown previously (Sergeant et al., 1979). Intranuclear dl312 was a reasonably stable, nonreplicating, nonchromosomal DNA molecule. The virus genome remained intact since the restriction pattern of viral DNA was detected for at least 2 wk in quiescent, infected cells. The virus DNA appeared not to undergo extensive methylation. Cell cloning efficiency was undiminished by dl312 infection. Yet, colonies grown from cells infected with 50–1,000 particles yielded no evidence for viral sequences, even when the infecting virus

Table I. Determination of Suitability of Adenovirus dl312 for Our Use

Criterion	Results
Virus attachment to cells	Between 30 and 50% of ³² P-labeled dl312 attached to 253J, A549, HeLa, or 293 cells, but only 4% of added virus attached to fibroblasts.
Translocation to nucleus	>70% of the virus attaching to cells reached the nucleus intact as judged by (a) repair of gamma sites in virus DNA, (b) removal of UV endonuclease-sensitive sites, and (c) loss of reversible bihelicity from virus DNA cross-linked <i>in vitro</i> before infection.
Intranuclear stability	Label from dl312 associated with washed cells remained constant for at least 1 wk (estimated 90% accuracy; geiger counter readings). Normal virus restriction pattern detected at 2 wk after infection (quiescent cells). Repair of ³² P-dl312 DNA to normal size at 5 d after infection.
Illegitimate recombination into host chromosomes	Nuclear DNA purified from colonies grown from infected cultures (MOI = 50–1,000 particles/cell) of fibroblasts or 253J, A549, or HeLa cells were negative for dl312 sequences or restriction pattern whether or not the virus had been exposed before infection to one or three LD ₅₀ (250–800 J/m ²) 254-nm UV radiation.
Effect on cell viability	Plating efficiency of A549 and 253J cells and fibroblasts was unaltered by infection with dl312. Plating efficiency was determined by twofold serial dilution of infected cells in 96-well plates, scoring dilution at which growth occurred.

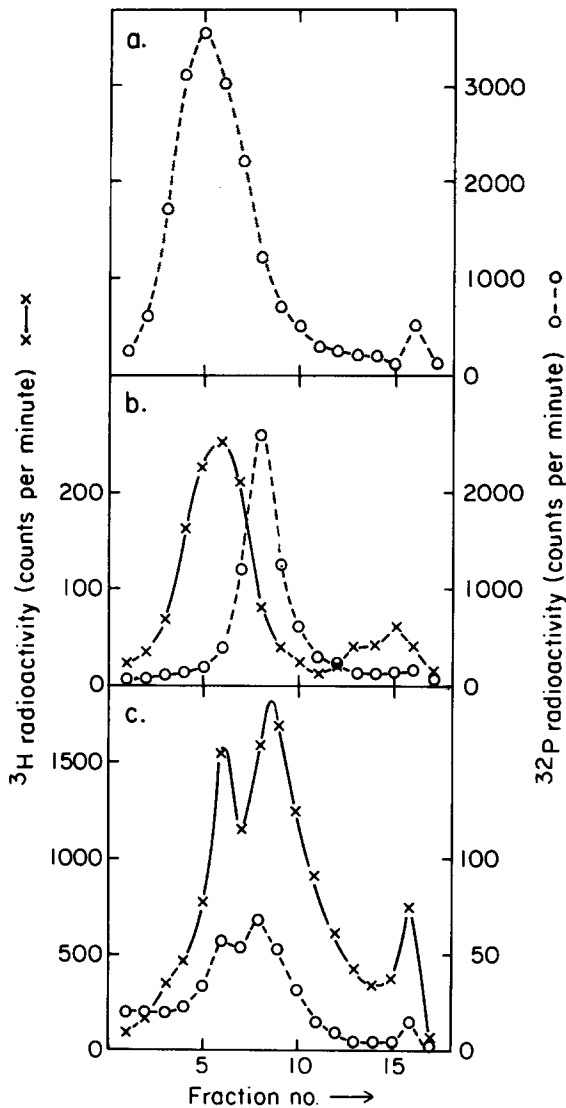


Figure 4. Tests for the suitability of dl312 as a nonchromatin probe for DNA repair. Sedimentation profiles of (a) virus irradiated with 90 krad; (b) unirradiated virus mixed with colcemid-treated, irradiated fibroblasts (90 krad) incubated at 37°C for repair; (c) infected, control fibroblasts irradiated with 90 krad and incubated at 37°C (MOI = 10,000 particles/cell; 40 min postirradiation incubation).

was UV irradiated to stimulate illegitimate recombination. This might be expected from the association of adenovirus recombination with its replication (Young et al., 1984). Apparently, dl312 DNA was a stable, inert episome in 253J cells, 549 cells, or fibroblasts. We tested by reconstitution for the ability of our sucrose gradient system to resolve repair of gamma sites in dl312 DNA. DNA from irradiated virus particles (Fig. 4 a) sedimented to about the same position as DNA from colcemid-treated fibroblasts that were irradiated and held at 37°C (Fig. 4 b). DNA from unirradiated virus mixed with cell DNA in the lysis layer sedimented several fractions further (Fig. 4 b) than irradiated virus (Fig. 4 a). This shows that our system is capable of resolving excess repair in dl312 DNA over that in cell DNA. In various experiments with cell DNA using unirradiated dl312 as a marker, there was no effect of cellular DNA repair on the dl312 DNA sedimentation profile (not shown). DNA from infected, irradiated cells that were incubated without colcemid sedimented to an intermediate position, indicating that gamma sites were repaired similarly in both the cell and virus DNA (Fig. 4 c).

There were exceptions to the generally favorable behavior of dl312 in suitability tests. First, dl312 attached much less well to fibroblasts than to carcinoma cells. Second, although replication of the viral DNA was delayed, it was eventually seen in HeLa cells at MOI >200 particles/cell. This result would be expected from the known partial permissiveness of HeLa cells for dl312 replication (Nevins, 1986). We also tested for virus effects on repair in the DNA of infected HeLa cells. Cells labeled in their DNA with ³H or with ¹⁴C were infected with 600 unlabeled dl312 particles per cell and then tested with or without mitotic arrest for the repair of gamma sites. The data (Table II, left) show that HeLa cells infected with dl312 degraded their DNA in response to gamma radiation to a lesser degree than uninfected cells, irrespective of mitotic arrest. In contrast, there was no difference in gamma site repair between dl312-infected and uninfected A549 cells (Table II, right).

Gamma Site Formation and Repair in Adenovirus dl312-infected Cells

In the experiments of Fig. 1 and Fig. 2 a, mitotic fibroblasts failed to remove gamma sites although nonmitotic cells from

Table II. Effect of Adenovirus dl312 Infection and of Colcemid on Repair of Gamma Sites in Cultured, Human Carcinoma Cells*

Colcemid dl312	HeLa cells				A549 lung carcinoma cells			
	-	+	-	+	-	+	-	+
	0.49	0.09	2.53	3.75	7.00	3.91	7.76	5.79
	0.82	0.18	1.78	0.10	2.61	1.90	3.35	6.17
	0.33	1.43	5.10	3.66	4.83		5.58	3.34
	0.27	0.41	1.40	0.69	6.43		5.36	6.11
		0.14						
		0.32						
Average	0.48	0.43	2.70	2.05	5.22	2.91	5.51	5.35
Standard deviation	0.21	0.46	1.44	1.67	1.70	1.00	1.56	1.17

Labeled cells were infected with unlabeled virus (MOI = 600) and incubated with or without colcemid for mitotic block. Cells were exposed to 22.5 krad gamma radiation and held at 0 or at 37°C for 120 min and then tested for gamma sites. A two-tailed *t* test for independent samples was used to compare infected samples with their controls. Comparisons were not significant except control vs. +dl312 ($p < .05$) and +colcemid vs. +dl312+colcemid ($p < .05$) for the HeLa cells. * Data for gamma site repair are shown as fractional increase in median molecular weight due to 37°C incubation.

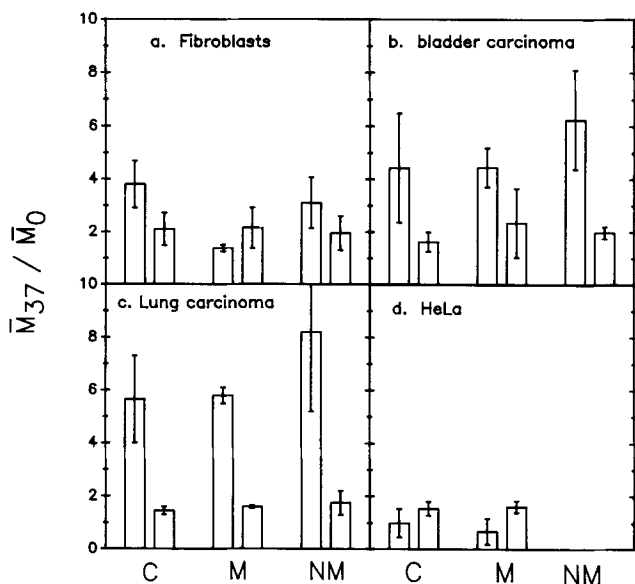


Figure 5. Effect of postirradiation incubation temperature on the repair of gamma sites in cell DNA (left-hand column of each pair) and infecting dl312 DNA (right-hand column of each pair) from irradiated control (C), mitotic (M), and nonmitotic (NM) cells, as shown on abscissa. Infected cultures were treated with colcemid or left as controls, exposed to 90 krad gamma radiation, and incubated 90 min for repair. Molecular weight increase due to postirradiation incubation at 37°C was determined from sedimentation profiles of the cell and viral DNAs.

the same cultures repaired such damages. If mitotic fibroblasts are repair enzyme deficient, dl312 DNA should also be repaired less well in the mitotic cells. If the mitotic chromosomes are a poor repair enzyme substrate, dl312 should be repaired as well as or better than cell DNA in mitotic cells.

Fibroblasts and various carcinoma cell lines were infected with ^{32}P -dl312 and incubated with or without colcemid in their growth medium. Mitotic, nonmitotic, and control cells were prepared and exposed to 90 krad gamma radiation, which was optimal to detect gamma sites in the viral DNA. Data from this kind of experiment are summarized in Fig. 5. With the sole exception of HeLa cells, the controls for all cell strains tested repaired their DNA after exposure to gamma radiation. Mitotic arrest uniformly inhibited repair in fibroblast DNA when compared with the controls. In some experiments, nonmitotic fibroblast DNA repair was also diminished. Quite unlike fibroblasts, the 253J and A549 carcinoma cell lines behaved as in previous experiments: control, mitotic, and nonmitotic cells all exhibited the same good level of repair in cell DNA. The HeLa cells degraded their DNA as in previous experiments. dl312 DNA in the infected cells was repaired to about the same extent in all cases ($\bar{M}_{37}/\bar{M}_0 = 1.7\text{--}2.2$). Thus, dl312 DNA returned toward (and apparently achieved) its normal molecular weight upon incubation for repair; cellular DNA increased to a much larger molecular weight but was not completely repaired. There was little influence of host type or of cell cycle on the extent of repair (or of damage) in dl312 DNA. Remarkably, in the HeLa cells, dl312 DNA was repaired while the cell DNA was being degraded.

We performed statistical analysis on the above data. The

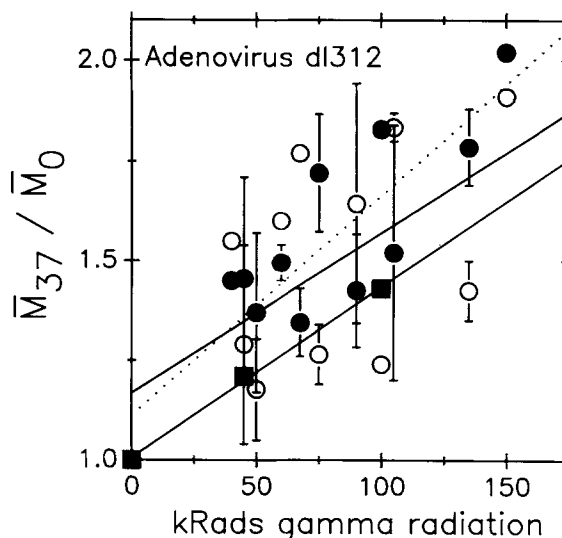


Figure 6. Molecular weight decrease due to gamma irradiation of dl312 (plotted as $M_{\text{control}}/M_{\text{irradiated}}$) (filled boxes and lower solid regression line) or increase due to repair of virus DNA irradiated in control (open circles and upper solid regression line) or mitotic (filled circles and dotted regression line) HeLa cells. Infected cells or virus in DME were exposed to gamma radiation as shown in the figure. Cells were held at 0 or 37°C for 90–120 min for repair.

repair of gamma sites in adenovirus DNA was unaffected by mitotic arrest in any host tested ($p > 0.1$). Similarly, there was no difference between conditions for repair of cell DNA in 253J, A549, or HeLa cells ($p > 0.1$), although HeLa cell controls differed significantly from all other controls in the repair of cell DNA ($p < 0.05$). The control fibroblasts did not differ from nonmitotic fibroblasts in repair of their DNA. However, both control and nonmitotic fibroblasts differed significantly from mitotic fibroblasts ($p < 0.005$ and $p < 0.01$, respectively). Thus, mitotic block did not affect repair of dl312 DNA; it prevented the repair of cell DNA in fibroblasts, but not in the other cell types.

We estimated the extent of gamma site repair in dl312 DNA by comparing data from many experiments with HeLa cells (Fig. 6). The regression lines for the mitotic and control cells do not differ significantly by t test for paired samples ($p > 0.05$). Scatter, due to pooling data from many different experiments, is eliminated in this analysis, which is exquisitely sensitive to systematic differences. The data for dl312 irradiated in vitro fall below the repair data because of the somewhat lower levels of gamma sites produced in the virion than in infected cells. Apparently, most or all gamma sites in the virus DNA were repaired by a 2-h incubation after irradiation in both control and mitotic HeLa cells. The same applies to other cell types tested (Fig. 5).

Discussion

Generality, Significance, and Selection of the Data Presented

Responses to lethal injury shown in these studies differed from those to sublethal damage (<1 krad ^{137}Cs radiation). For example, HeLa cells repair sublethal DNA damage

(e.g., Collins et al., 1981; Collins and Johnson, 1979) and are no more sensitive to DNA damage than other cell lines and strains we used. Yet, unlike other cells studied here they degraded their DNA after supralethal gamma irradiation. Our data reflect the dynamics of many cellular processes acting on the damaged DNA. Yet, in the >50 experiments for which data are shown here, a generally consistent picture was seen for each cell type and incubation condition. Each repair phenotype was seen in experiments with gamma radiation doses ranging from 5 to 100 krad (Fig. 2) and postirradiation incubations ranging from 20 min to 2.5 h (Fig. 3). Comparison of data between experiments as we did here tests reproducibility more rigorously than comparisons within any experiment. Molecular weight ratios were not selected for fit, so the data are not fitted by simple curves. The nevertheless striking reproducibility of the effects shows that differences in the dynamic repair equilibrium between cell types and conditions can be studied meaningfully in the manner shown here. We next discuss the three main comparisons we made of gamma site metabolism: mitotic vs. nonmitotic cells, fibroblasts vs. carcinoma cells, and cell vs. dl312 DNA.

Effect of Mitotic Arrest on Repair of Gamma Sites

Removal of gamma sites was inhibited strongly by mitotic arrest in fibroblasts (Figs. 1-3). Colcemid itself has no effect on the molecular weight of cell DNA (Ross et al., 1983). Nonmitotic, colcemid-treated fibroblasts repaired their DNA normally (Fig. 2), as did nonmitotic 253J and A549 cells (Figs. 2 and 3). This shows that colcemid itself did not inhibit repair. Psoralen cross-link incision occurs much more slowly than gamma site repair and is also inhibited in mitosis-arrested fibroblasts. Mitotic inhibition of psoralen cross-link incision was reversed on release from colcemid (Ross et al., 1983). This shows that the colcemid effect was reversible, like mitotic arrest. The 12 independently isolated fibroblast strains we tested originated from various tissues (adult skin, fetal lung, or neonatal foreskin) and differed widely in cultural lifespan and possibly other parameters. Nevertheless, all 12 failed to repair gamma sites when blocked at mitosis (Figs. 1-3). Thus, failure to repair gamma sites at mitosis was not a strain-specific phenomenon but instead was a property of normal fibroblasts. Either gamma sites were made inaccessible to repair enzymes by chromosome condensation at mitosis or else repair enzymes were absent or inactive in mitotic fibroblasts.

Fibroblasts vs. Carcinoma Cells

Mitotic fibroblasts failed to repair supralethal gamma sites that cycling fibroblasts repaired. In sharp contrast, no carcinoma cell line tested was influenced by mitotic arrest in the rate (Fig. 3) or extent (Fig. 2) of supralethal gamma site repair. The most karyotypically abnormal cells we tested were HeLa cells. HeLa cells not only failed to repair gamma sites, they permitted their DNA to be degraded (Figs. 2, 3, and 5). A549 and 253J cells, which were introduced into cell culture more recently than HeLa cells and are more karyotypically normal, repaired their DNA at mitosis. Karyotypically normal cultured fibroblasts failed to do so. If these differences are due to the intracellular environment (a *trans* effect), any DNA molecule in the cells for which the repair of gamma

sites depended upon cellular enzymes should be similarly affected. Alternatively, if the differences in gamma site metabolism inhere to the structure and management of the cellular chromatin itself (a *cis* effect), nonchromosomal DNA within the cell should be unaffected. dl312 was used to discern these possibilities.

Gamma Site Repair in Cell vs. Adenovirus dl312 DNA

We found the adenovirus E1A deletion mutant dl312 to be a relatively inert, stable episome and was therefore used to study DNA metabolism in infected, gamma-irradiated, human cells. Repair of gamma sites in dl312 DNA was similar between cell types and, within each cell type, between cell cycle conditions (Fig. 5). Importantly, repair in dl312 DNA exceeded that in the DNA of HeLa cells or of mitotic fibroblasts (Figs. 5 and 6). Since the virus was an internal control for the cell DNA in our experiments (i.e., was present in the same enzymatic environment as the cell DNA), this shows that mitotic fibroblast cell DNA was less accessible to cellular repair enzymes than was DNA of infecting dl312. It may be concluded that the effect of mitotic arrest on repair of gamma sites in fibroblasts (Figs. 1-3 and 5) is a *cis* effect of chromatin structure rather than a *trans* effect of enzyme activity. Similarly, comparing between cell types, whereas mitotic fibroblasts fail to repair gamma sites in their DNA, carcinoma cell lines tested repaired these sites nearly as efficiently as the same cells when cycling. Carcinoma cell DNA remained accessible to enzymatic action at mitosis whereas fibroblast DNA did not. We can conclude that chromatin structure differences between the cells control the effect of mitosis on DNA repair.

Clearly, the conditions required to make these distinctions stress the cell. dl312 DNA (34 kbp) is too small to measure sublethal gamma sites by sucrose gradient sedimentation. The greater than or equal to twofold increase in dl312 median molecular weight due to gamma site repair (Figs. 4 and 5) is virtually complete repair (Fig. 6). When cellular DNA is repaired to a much larger average molecular weight than that of intact dl312, dl312 cannot be used to evaluate whether differences in repair efficiency result from *cis* or *trans* effects. Instead, such conditions reveal the maximum expected repair in dl312 DNA. In some experiments like those of Fig. 5 *a* (not shown), the level of repair (\bar{M}_{37}/\bar{M}_0) was >10 for control fibroblasts, >3 for dl312, and depressed in the nonmitotic fibroblasts as well as mitotic fibroblasts. We think this was an artifact (although it was seen in two of six experiments run), and we have eliminated those data from the analysis and Fig. 5. Inclusion of these data did not affect the main result: mitotic fibroblasts repaired their own DNA less well than that of infecting dl312. We attribute the less uniformly good quality of data in such experiments to the relatively small size of adenovirus and consequently large amounts of damage required for these tests.

Fibroblasts contain fewer reducing equivalents than carcinoma cells (Engin, 1976) and so experience more hydroxyl radical damage. Thus, the level or quality of initial damage may have varied somewhat between strains at any dose of ionizing radiation. However, the relative independence of our data upon gamma dose (Fig. 2) or incubation time (Fig. 3) and the strikingly consistent behavior of dl312 repair in the experiments shown here (Figs. 5 and 6) diminish the possible role of initial damage effects.

Despite the above caveats, the striking difference in the effect of mitotic arrest on gamma site repair between cultured fibroblasts and carcinoma cells of the 253J or A549 lines is most simply explained by a *cis* effect, namely chromosome structure. The consistently efficient repair in dl312 makes it very difficult to explain differences in repair of cell DNA based on *trans* effects. Fibroblasts, but not carcinoma cells, were inhibited at mitosis in the repair of supralethal gamma sites either (a) because of differences in embryological origin (fibroblasts are mesenchymal while carcinoma cells are epithelial) or (b) because the ability to block repair at mitosis is a property of normal cell chromatin that is lost during carcinogenesis. Although several studies (e.g., Sellins and Cohen, 1987) indicate that normal, mature, peripheral leukocytes degrade their DNA after exposure to low levels of gamma radiation, there is no evidence for such unusual behavior in other types of cells. To distinguish the above possibilities, we have tested a human fibrosarcoma cell line. These cells behaved just like the A549 and 253J cells and unlike any normal fibroblast strain tested (Ross, P. M., and T. Inadomi, manuscript submitted for publication). Moreover, psoralen intercalation was influenced in a manner reminiscent of mitotic effects on DNA repair. As a whole, the data support the idea that we have detected an hereditary defect in DNA metabolism (i.e., loss of mitotic inhibition of DNA repair) that is specific to cancer cells. In either case, we have shown that carcinoma cells differ from fibroblasts in cell chromatin organization, a previously unrecognized phenomenon. Each cell type's characteristic stability to perturbation of higher-order chromatin structure is likely manifest, if more difficult to detect directly, at sublethal exposures. If so, it may be pivotal as cause or result of carcinogenesis.

HeLa Cell DNA Degradation

A cellular nuclease degraded HeLa cell DNA containing gamma sites (Figs. 2, 3, and 5). Like the HeLa cells, and unlike the fibroblasts in our experiments, mature blood leukocytes also degrade their DNA after exposure to ionizing radiation (Sellins and Cohen, 1987). The reasons for this degradation are not known. Partial repair of dl312-infected HeLa cell DNA in some experiments (Table II) probably results from variable synthesis of the viral E1B proteins, which retard cell DNA degradation during vegetative adenovirus infections apparently by preventing the transit of cytoplasmic nucleases into the nucleus (White et al., 1984). The nuclease activity in uninfected HeLa cells must be distinct from that of late, lytic adenovirus infections because the latter activity degrades viral DNA as well as cell DNA. Whereas most adenovirus in our experiments contained several single-strand sites, few adenovirus DNA molecules contained double-strand breaks. The much larger cellular DNA, by contrast, sustained many double-strand breaks, producing large numbers of chromatin ends. These may be a good substrate for a cellular exonuclease. In cell types other than HeLa, cell DNA was spared either because the nuclease was not induced or because the cellular chromatin structure was more ordered than in HeLa cells.

Summary

Cultured fibroblasts of various origins failed to repair gamma sites produced by 12–100 krad ¹³⁷Cs radiation when

blocked at mitosis by colcemid. Cells that were not blocked in mitosis repaired the gamma sites. Mitotic inhibition of gamma site repair was absent in two carcinoma cell lines, 253J and A549. HeLa cells degraded their DNA when gamma sites were present, regardless of mitotic arrest. In all cell types and under all conditions, gamma sites in dl312 DNA were repaired efficiently. This shows that the effects of cell origin on gamma site repair in cell DNA were mainly *cis* effects of chromatin structure rather than *trans* effects of enzymatic environment. The data also show that infecting dl312 DNA was suitable for the study of DNA repair and suggest that the hereditary instability of carcinoma cells can be detected and perhaps better understood through this kind of experiment.

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