Slow DNA Rejoining in Ultraviolet-irradiated Human Diploid Fibroblasts Treated with the Mitogens Trypsin and Insulin

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ABSTRACT

Normally in mammalian cells the postincision steps of UV-induced excision repair are much more rapid than the recognition of damage and incision. This means that at any one time the level of repair-generated single-stranded DNA breaks is very low. Here we report that detectable levels of DNA breaks accumulate in quiescent human fibroblasts which are UV irradiated a few hours after replating in conditions that stimulate progress through the cell cycle. Most DNA breaks accumulate in cultures trypsinized and seeded in medium supplemented with insulin, and irradiated in early G1. Because trypsin and insulin have no effect on UV-induced incision rates, as measured by DNA break accumulation in the presence of DNA synthesis inhibitors, we argue that our ability to detect incomplete repair-sites is due to a significant reduction in the rate of gap sealing indicative of a shift in the steady state of excision repair. Provision of DNA precursors prevents the enhancing effect of trypsin and insulin on the accumulation of DNA breaks, implying that these agents affect DNA precursor metabolism.

Perturbation of the repair process, which leads to the accumulation of 1500–2000 DNA breaks/genome, is also associated with other effects including increased lethality, the appearance of double-strand breaks and the loss of NAD, the last effect presumably arising as a consequence of break-stimulated poly(ADPR) transferase activity. Addition of 3-aminobenzamide, an inhibitor of poly(ADPR) synthesis, completely blocks the decline in NAD levels, but does not change the rate of sealing of the accumulated DNA breaks. These results strongly suggest that ligation is largely, if not entirely, independent of ADP ribosylation in this system.

INTRODUCTION

The execution of UV-induced excision repair in eukaryotes involves an unknown number of coordinated enzymic events (1). Normally, once the recognition/incision steps have occurred at a site of damage, the completion of the repair event is rapid, ensuring that any breaks in the DNA are extremely short lived (2–4). This suggests that the rate-limiting step of the excision repair process involves recognition of a lesion and incision nearby—and, by inference, that a premium is placed on the rapid restoration of structural integrity to the DNA. When incision events are experimentally dissociated from the synthetic steps of repair by treating UV-irradiated cells with short pulses of DNA synthesis inhibitors, thereby preventing repair synthesis and ligation (2, 4), the biological consequences of the long-lived, repair-related single-strand DNA breaks become apparent (5, 6). These include the appearance of double-strand DNA breaks (7–9), an increase in the frequency of chromosomal aberrations (9–11), and enhanced cell killing (12).

By comparing the frequencies of DNA breaks accumulated in the presence versus the absence of DNA synthesis inhibitors we have earlier identified two heritable human conditions (46BR and Cockayne's syndrome) which display normal rates of UV-induced incision coupled with abnormally slow rates of one or more of the postincision events (13). The post-UV accumulation of single-strand DNA breaks in 46BR fibroblasts may be due to a defect in DNA strand ligation (13–15), while the similar phenotype in fibroblasts from individuals with Cockayne's syndrome may be due to perturbation of their deoxyribonucleotide pools (13).

In the present work we demonstrate that normal human fibroblasts which are released from density inhibition by trypsin detachment and then UV irradiated in early G1 will accumulate many single-strand DNA breaks in the absence of DNA synthesis inhibitors. Additional treatment with insulin increases the frequency of DNA breaks in these cells. We present evidence that the retardation of repair site completion is not due to altered NAD pools or poly(ADPR) synthetase activity which might affect DNA strand ligation. On the other hand, break accumulation is reduced by supplying deoxyribonucleosides exogenously. This ability to manipulate the steady-state level of repair induced DNA breaks has allowed us to estimate repair site completion times in different physiological conditions and to examine some of the biological consequences of long-lived breaks. In particular, we have examined the dependence of long-lived DNA break accumulation on methods of cell cycle synchronization, proteolytic detachment, and insulin treatment, seeking to establish whether protocols used to transform human or rodent cells in vitro (16–21) also impede the repair process.

MATERIALS AND METHODS

Cell Culture.

Normal embryonic human diploid lung fibroblasts (HEL, Gibco Biocult Ltd.) were cultured in MEM buffered with bicarbonate and supplemented with 10% FCS (Gibco Europe Ltd.); 46BR fibroblasts kindly provided by Dr. A. R. Lehmann (MRC Cell Mutation Unit, University of Sussex, Brighton) were taken from an immunodeficient 18-year-old female and were cultured in MEM supplemented with 15% FCS. The medium was changed or the cells subcultured at a split of 1:2 or 1:3 once a week.

Synchronization Procedure. Cells were synchronized in quiescent phase by growing the cultures to confluence for 8–12 days. Then the medium was changed once more and the cells were incubated further for 3 to 5 days to cause them to enter the G0/G1 resting stage. By this time less than 2% of the cells were in S-phase, as monitored by autoradiography (22). Cells were released from density inhibition either by serum stimulation or by detachment with pancreatic (Gibco Europe Ltd.), and replated at a concentration of 1–2 × 10⁶ cells per 35-mm dish in MEM plus 10% serum and plus or minus 0.5–1 unit/ml insulin. Synchronization by AAS was based on the procedure of Milo et al. (17): 2 × 10⁶ cells were seeded per 35-mm dish into Dulbecco's modified Eagle's medium minus arginine and glutamine with 10% dialyzed FCS. Twenty-four h later, the amino acid-deficient medium was replaced with 10% complete medium plus or minus 0.5 unit/ml of insulin. At 3, 12, and 24 h after feeding the cells were UV irradiated

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The abbreviations used are: ADPR, ADP ribosylation; ADPRT, ADP ribosyltransferase; AAR, 3-aminobenzamide; DSB, double-strand breaks; MEM, Eagle's minimum essential medium; FCS, fetal calf serum; AAS, amino acid starvation; PBS, phosphate buffered saline; da, deoxyadenosine; dG, deoxyguanosine; dC, deoxycytidine; dT, deoxythymidine; HU, hydroxyurea; ara-C, 1-β-D-arabinofuranosylcytosine.
and assayed for their incision activity. The flow of cells through the cycle after replating was monitored autoradiographically or by scintillation counting of acid (trichloroacetic acid) precipitable radioactivity after incubating cells for 1 h in medium plus [3H]thymidine (1 µCi/ml 48-55 Ci/mmol, Radiochemical Centre Amersham) at various times after stimulation. In cultures synchronized by the AAS method DNA synthesis began around 10 h after refeeding with complete medium and, in cultures synchronized by release from confluence around 15 h after plating. Monitoring the flow of cells through the first cycle after trypsin release shows good synchrony with an S-phase index of 70% 21 h after seeding.

UV Irradiation and Assay of Incision Activity. For incision experiments DNA was uniformly prelabeled by incubating the cells for two to three generations (5–10 days) with [3H]thymidine (0.1 µC/ml). 1–2 × 10^6 cells were seeded in a series of 35-mm dishes in MEM with 10% serum (a mixture of fetal and newborn calf serum) with or without insulin. Drugs were added 90–120 min later as appropriate and the cells were incubated for a further 30–60 min. Irradiation was carried out in monolayer in phosphate buffered saline by means of a gerrnical tube emitting predominantly at 254 nm, at a dose rate of 0.25 Jm^-2s^-1. Prewarmed medium containing drugs and/or insulin, as appropriate, was added after irradiation and cells were incubated at 37°C for various periods. To assess incision activity during the first day, 30-min pulses of DNA synthesis inhibitors were given at different times after irradiation and the DNA breaks thus accumulated were determined. The alkaline lysis method of Erixon and Ahnstrom (2) was used to measure DNA breaks. At various times after irradiation, the attached cells were rinsed twice with cold PBS, drained, and placed on ice. Ice-cold alkaline sucrose solution (1 ml; 5% w/v sucrose, 0.01 M Na2 EDTA, 0.15 M NaCl, 0.1 M NaOH) was added to each dish. After 25 min incubation on ice in the dark, the alkaline sucrose was neutralized by the addition of 1 ml of 0.15 M KH2PO4, pH 4.5. The size of the DNA was reduced by sonication (MSE Instruments Ltd.) and samples were stored at -20°C. Hydroxyapatite (Boehringer Mannheim Ltd.) column chromatography was carried out as described previously (4). The extent of unwinding in alkali is a function of the frequency of DNA breaks, the number of DNA breaks being estimated from the calibration curves of X-irradiated cells. X Irradiation introduces a known number of DNA breaks (2.5 per 10^6 daltons per thousand rads (22)). The background break frequency for unirradiated cells is measured for each experiment and is usually low, but can vary slightly with the conditions of cell culture. In these experiments in the presence of DNA synthesis inhibitors a confluent culture has a background frequency of single-stranded DNA equivalent to about 0.5 breaks per 10^6 daltons and trypsin-treated cultures about 0.75 DNA breaks per 10^6 daltons. Addition of insulin to the medium does not effect this extremely low background level. The background is deducted from the estimated value of DNA breaks from each irradiated sample.

Measurement of Double-Strand DNA Breaks. Double-strand breaks were detected using the procedure of neutral filter elution essentially as described by Bradley and Kohn (23). 3H-Labeled human fibroblasts in different states of growth were UV irradiated and incubated with or without DNA synthesis inhibitors and with or without insulin as described above for the single-strand break assay. At the appropriate times 2 × 10^6 cells were loaded onto a Nuclepore 25-mm, 2.0-µm pore size polycarbonate filter (Bio-Rad Laboratories) on a filter support. The cells were lysed at pH 9.6, at room temperature in a solution of 0.05 M Tris, 0.05 M glycine, 0.025 M Na2-EDTA, 2% (w/v) sodium lauryl sulfate containing 0.5 mg ml^-1 freshly dissolved proteinase K (Boehringer Mannheim Ltd.). The DNA was eluted over a 15-h period and the radioactivity was counted as described (22).

Determination of NAD Levels. NAD was extracted from PBS-washed cells in 90% ethanol for at least 30 min on ice. Extracts were dried by evaporation at 37°C and stored at -20°C. The extracts were dissolved in water at a concentration equivalent to approximately 5 × 10^6 cells/ml, debris removed by centrifugation and NAD measured colorimetrically by the method of Slater and Sawyer (24). Briefly, a reaction mixture consisting of 5 ml dichlorophenol phenol (17.5 µg/ml, Sigma), 1 ml 0.1 M sodium phosphate buffer (pH 7.4), 0.1 ml phenazine methosulfate (1 mg/ml, Sigma), and 0.2 ml 90% ethanol was added to 0.2 ml of clarified cell extract and mixed. Fifty µl of yeast alcohol-dehydrogenase (6 mg/ml, Sigma A3263) were added and the loss of absorbance at 600 nm were measured as a function of time. Standard curves of NAD ranging 1–40 µM were constructed for each set of assays.

Single Cell Survival Curves. A suspension of cells from logarithmic or quiescent cultures was irradiated at a concentration of 10^6 cells/ml in Dulbecco's PBS at room temperature and with agitation. The suspension was exposed to increasing amounts of UV light, at dose rates of 0.01–1 Jm^-2s^-1. Irradiated and mock-irradiated control cells were plated out in petri dishes in quadruplicate after each dose in MEM 10% FCS with or without insulin (1 unit/ml). The dishes were incubated at 37°C in a CO2 incubator for 14 days at which time surviving colonies were fixed and stained. Only colonies with 50 or more cells were scored and the percentage of survival was calculated. The plating efficiency was enhanced from 5–10% to 15–30% by adding 10^6 UV-irradiated cells of the same strain to each dish (The UV-killed cells replaced the need to use feeder-layer plates.) Values for the survival curve parameters of D0 (the dose required to reduce the surviving fraction by the factor e^-1 in the linear part of a semilog curve) and D0 (the dose at which the straight line extrapolation of the experimental reaches 100% survival) were calculated with standard errors from the computed lines of best fit.

Chemicals. Fresh stock solutions of hydroxyurea (Boehringer Mannheim), 1,β-d-arabinofuranosylcytosine (Sigma), and 3-aminobenzamide (Sigma) were prepared in phosphate buffered saline at 10–100x the final concentration. Aphidicolin (Sigma) was dissolved in dimethyl sulphoxide at 2 mg/ml and stored desiccated at -20°C. All other chemicals were obtained from Sigma except where stated otherwise. Ten × stocks (at 10^-3 M in MEM) of dA, dG, dC, and dT were stored at -20°C.

RESULTS

Normal Human Fibroblasts Can Accumulate UV-dependent DNA Breaks in the Absence of DNA Synthesis Inhibitors. We have reported previously that CS cells accumulate incomplete repair sites following UV irradiation to an extent which is dependent on the time elapsed between plating and irradiating the cells (13). To better define the difference between CS and normal human fibroblasts as well as the effects of growth conditions on UV-induced DNA repair in cultured cells, we have measured the frequencies of repair-related single-strand DNA breaks in normal human fibroblasts in the presence and absence of DNA synthesis inhibitors. Cells were irradiated with 4 Jm^-2 of UV either in an asynchronously proliferating growth state or after synchronization in early G1 (about 13 h before the onset of DNA synthesis). Cells synchronized in G0/G1 resting stage (S-phase index, <0.02) were released from growth arrest either by serum stimulation, or by proteolytic detachment and plating in fresh medium at lower density. The rate of incision was measured by the accumulation of DNA breaks at different times in the presence of DNA synthesis inhibitors. Though there are slight differences, incision profiles are similar in each of the three growth states (Fig. 1). By contrast, in the absence of inhibitors, there are some clear differences between the cultures. Essentially no breaks are detected when logarithmically growing cells are irradiated (Fig. 1A), and very few are observed in serum-stimulated quiescent cells after irradiation (Fig. 1B), but a substantial number of breaks do appear, however, in cultures recently released from contact inhibition and irradiated in early G1 (Fig. 1C). When these contact released cells are irradiated much later in G1 (4 h before S-phase), fewer breaks accumulate in the absence of inhibitors, and in cells irradiated 24 h after release from quiescence (mid to late S-phase; S-phase index, 0.6), there is no accumulation of breaks under these conditions (data not shown).
Blasts stimulated either by fresh medium plus insulin or by trypsinization replating accumulate about three breaks/10⁹ daltons of the state of growth of the cells at the time of irradiation, the early S-phase of the ensuing cell cycle. In view of these results the proficiency of cells irradiated with UV in early S-phase. Synthesis of DNA is arrested prior to the S-phase and is resumed following a cell-permissive growth signal. The presence of DNA synthesis inhibitors makes it possible to determine the rate of DNA synthesis during this period. 

The UV-repair characteristics of early G₁ fibroblasts that were earlier stimulated to enter the proliferative cycle by trypsin detachment and replating in fresh medium. The presence of long-lived DNA breaks in these cells is of interest because of the suggested relationship between genome damage in particular stages of the cell cycle, mutation, and cell transformation (16–19, 21, 25, 26). For example, the protocol of Milo et al. (17) for human cell transformation in vitro, involves synchronizing cells by amino acid starvation followed by serum and HU and ara-C. However, the frequency of incomplete repair does not affect the rate of incision, measured in the presence of DNA synthesis inhibitors (open symbols). When inhibitors were used the cells were preincubated for 30 min before irradiation. The frequency of breaks in DNA was determined by alkaline lysis and hydroxyapatite chromatography. R, quiescent cell cultures were obtained two to three weeks after seeding, with at least two medium changes during the interim period. The contact inhibited cells were irradiated with 4 J m⁻² 3 h after serum stimulation. C, cultures obtained by seeding in fresh medium trypsinized quiescent cells. These were irradiated 3 h later. The results shown represent the mean of several experiments (A, at least 3; B, 6; C, over 10). At 15 min incubation standard errors of the mean break frequency per 10⁹ daltons were: A, ±1.4, ±2, ±0.9; B, ±3.4, ±2.3, ±0.6, ±2.8; C, ±2, ±2.1, ±1.8, ±2.4.

In the light of this finding we next compared excision repair proficiency of cells irradiated with UV in early S-phase. Synchronous S-phase populations were obtained either by collecting cells 10 h after release of AAS (17), or 16 h after trypsin release from contact inhibition (S-phase index, <30%). In early S-phase cells that had been synchronized by AAS, both incision and postincision steps are considerably diminished, compared with S-phase cells obtained by trypsin release of quiescent cultures. For example, their rate of incision is only about 70% of trypsin-released cells and, additionally, a greater proportion of the repair sites remain unsealed (65 and 27% with and without insulin, respectively). In early S-phase cultures previously released from quiescence incised sites are sealed promptly whether insulin is present or not. These findings may help to understand the aetiology of elevated rates of anchorage-independent growth reported in cultures synchronized by the AAS method (17, 19, 27).

Rate of Gap Sealing Is Constant for a Given Physiological State during the First Few Hours after Irradiation. The accumulation of detectable DNA breaks in the cells described above is most likely the result of a shift in the relationship between the rate of incision and the rate of gap sealing. The overall incision activity (breaks measured with inhibitors) and the frequency of unsealed gaps (without inhibitors) were determined over extended periods after irradiation. In the experiments shown in Fig. 2 quiescent cells were detached by trypsin and seeded into complete medium with or without insulin. Three h later the cells were irradiated with 4 J m⁻² and incubated further for up to 4 h. At different times after irradiation the frequency of DNA breaks was measured both in the presence of inhibitors and in their absence. Incision was estimated at different times from the number of DNA breaks accumulated during 30-min pulses of DNA synthesis inhibitors. This procedure (pulsing) is essential because continuous incubation with inhibitors interferes with the further operation of excision repair, and therefore leads to an underestimate of the real frequency of the incision events (4, 28). A 30-min period is required to overcome the necessary lack of preincubation with inhibitors and to achieve a near maximum accumulation of DNA breaks. Fig. 2 shows that the number of DNA breaks accumulated either in the presence or absence of inhibitors falls off very rapidly during the first 3–4 h after irradiation. This decline in incision activity with time appears to be a simple exponential one, probably due to substrate depletion. Most importantly, at different times after UV the decrease in the number of breaks accumulated without inhibitors closely parallels the decline in incision activity. Over the first 2.5 h a plot of the log of break number versus time is a straight line with a very similar slope for all four conditions examined—with and without inhibitors and with and without insulin. These results, along with the fact that insulin does not influence the incision rates of these cells in the various physiological states, strongly suggest that (a) our ability to detect open repair sites is due to a significant reduction in the rate of post-incision gap sealing; (b) the repair site completion time is influenced by the physiological state of the cells; (c) the repair site completion time for a given physiological state is constant up to about 3–4 h after irradiation. The longer patch repair time associated with certain growth conditions is, therefore, a result of a new steady state in the excision repair process. It is also clear that addition of insulin considerably enhances the trypsin effect and shifts the equilibrium further in the direction of incomplete repair sites.

**NAD Levels and Spontaneous Accumulation of DNA Breaks.** The existence of long-lived DNA breaks in UV-irradiated cells released from quiescence may be due to an alteration in the rate or efficiency of ligation. DNA ligase II activity is stimulated by ADPRT, a reaction which is induced by DNA strand breaks and which uses NAD as a substrate (29, 30). It is possible that releasing cells from the quiescent state affects ADPRT either by depleting the available pool of NAD or more directly by changing the activity of ADPRT. To examine these possibilities we measured intracellular NAD pools in normal fibroblasts under the different growth conditions, before and after irradiating the cells with 4 J m⁻² UV.

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**Fig. 1. The accumulation of UV-induced incomplete repair sites as a function of growth state and insulin treatment.** A, logarithmic cultures of normal human fibroblasts, labeled with [³H]thymidine were irradiated with 4 J m⁻² of UV light 3 h after seeding. The cells were incubated in growth medium either with insulin (1 unit/ml) (A, Δ), or without (B, O) for various periods in the presence of 10⁻⁵ M HU and 5 x 10⁻⁵ M ara-C (closed symbols), or without DNA synthesis inhibitors (open symbols). When inhibitors were used the cells were preincubated for 30 min before irradiation. The frequency of breaks in DNA was determined by alkaline lysis and hydroxyapatite chromatography. R, quiescent cell cultures were obtained two to three weeks after seeding, with at least two medium changes during the interim period. The contact inhibited cells were irradiated with 4 J m⁻² 3 h after serum stimulation. C, cultures obtained by reseeding in fresh medium trypsinized quiescent cells. These were irradiated 3 h later. The results shown represent the mean of several experiments (A, at least 3; B, 6; C, over 10). At 15 min incubation standard errors of the mean break frequency per 10⁹ daltons were: A, ±1.4, ±2, ±0.9; B, ±3.4, ±2.3, ±0.6, ±2.8; C, ±2, ±2.1, ±1.8, ±2.4.
We found that none of the conditions under which cells were grown before assay were associated with a significant depression of NAD levels. All cultures showed an NAD pool size of about 1 nmol/10^6 cells. The NAD pool is therefore unlikely to be a limiting factor underlying the accumulation of incomplete repair sites in certain growth conditions. However, in response to UV irradiation, the fall in NAD level correlates well with the frequency of DNA breaks accumulated either with or without DNA synthesis inhibitors (Table 1). In common with Jacobson et al. (31) we find that the greatest decline in NAD occurs in UV-irradiated cells incubated with HU and ara-C. Under these conditions when more than 10 breaks per 10^6 daltons of DNA accumulate, NAD levels fall to around 30% of unirradiated controls within 1 h. In the absence of HU and ara-C the biggest drop in NAD level (to about 60% of unirradiated control) occurs in cells released from quiescence in the presence of insulin, and no change in NAD levels is observed in proliferating cells. The results suggest that the presence of long-lived DNA breaks after UV can stimulate ADPR of nuclear proteins and that the rate of this process, as estimated from the relative decline in cellular NAD levels, is regulated by the number of DNA breaks present at a given time.

In order to assess the putative role of ADPR on the ligation step of UV-induced excision repair we have shown that in human fibroblasts and in HeLa cells (32), 3AB, which blocks the fall in NAD level (Table 1), has no effect on the sealing of breaks accumulated in the presence of repair synthesis inhibitors (6). Neither does 3AB change the rate of gap sealing of the UV-induced incomplete repair sites in 46BR fibroblasts, though these cells are sensitive to this agent (Table 2). From these data we can conclude that a ligase malfunction associated with ADPRT activity is unlikely to account for the accumulation of incomplete repair sites. In contrast to the effect of 3AB on retarding strand rejoining in cells treated with alkylating agents (29, 30, 33), it is clear that extensive poly ADP-ribosylation is not required for DNA strand rejoining during excision repair of UV damage in human fibroblasts.

**Effect of Ribonucleosides and Deoxyribonucleosides on the Level of Unsealed DNA Breaks after UV.** We have shown previously (13) that in CS cells retarded gap sealing after UV can be alleviated by the provision of deoxyribonucleosides, implicating the DNA precursor pool size. We have therefore examined the effect of providing DNA or RNA precursors on the accumulation of repair gaps in normal human cells. Quiescent cells were seeded in complete medium with or without insulin and irradiated with 4 J/m^2 3 h later. Table 2 shows that provision of the four deoxyribonucleosides before and after UV substantially increased the rate of gap sealing, especially in the presence of insulin. Provision of 10^{-4} M dC, dT, or dG separately (data not shown) or the four ribonucleosides together (each at 10^{-4} M), did not affect the frequency of unsealed gaps. On the other hand, provision of deoxyadenosine alone reduced gap sealing; 60 min after UV the number of gaps was nearly doubled. Deoxyadenosine like HU causes breaks to accumulate in DNA after UV (34), an effect probably explained by its potency (as dATP) as a general allosteric inhibitor of ribonucleotide reductase (35).

To summarize, our data on the potentiation of spontaneous break accumulation by trypsin, insulin or both together, and the alleviation of this situation by DNA but not RNA precursors, strongly suggest that these mitogenic agents reduce the supply of DNA precursors below the level necessary to support

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**Table 1  Effect of cell growth state, insulin, repair synthesis inhibitors, and 3AB on NAD levels in UV irradiated cells.**

Experiments were performed as described in the legend to Fig. 1. The total intracellular content of NAD was determined in cells 60 min after 4 J/m^2 of UV and are the mean values obtained from at least two separate experiments. 3AB was present at a concentration of 5 mM for 60 min pre- and post-irradiation.

<table>
<thead>
<tr>
<th>Growth state</th>
<th>No Insulin</th>
<th>Insulin</th>
<th>Insulin and 3AB</th>
<th>HU + ara-C and 3AB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quiescent</td>
<td>77 ± 11</td>
<td>84 ± 12</td>
<td>45 ± 13</td>
<td>89 ± 30</td>
</tr>
<tr>
<td>Reseeded</td>
<td>83 ± 19</td>
<td>65 ± 4</td>
<td>95 ± 7.5</td>
<td>18 ± 5</td>
</tr>
<tr>
<td>Logarithmic</td>
<td>92 ± 4</td>
<td>104 ± 8</td>
<td>117</td>
<td></td>
</tr>
<tr>
<td>Logarithmic</td>
<td>96 ± 18</td>
<td>110 ± 6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* NAD pool size in unirradiated control is about 1 nmol/10^6 cells.
SLOW DNA REJOINING IN UV-IRRADIATED FIBROBLASTS

Table 2 Ribo- and deoxyribonucleosides, d4 and 3AB effects on UV-induced break accumulation in normal and 46BR fibroblasts

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Treatment</th>
<th>15 min*</th>
<th>30 min*</th>
<th>60 min*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic normal fibroblasts</td>
<td>None</td>
<td>100(3.9)*</td>
<td>100(5.3)</td>
<td>100(3.1)</td>
</tr>
<tr>
<td></td>
<td>dX&lt;sup&gt;c&lt;/sup&gt;</td>
<td>46</td>
<td>24</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>rX&lt;sup&gt;c&lt;/sup&gt;</td>
<td>133</td>
<td>100</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>dA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>117</td>
<td>100</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>3AB&lt;sup&gt;c&lt;/sup&gt;</td>
<td>85</td>
<td>90</td>
<td>105</td>
</tr>
<tr>
<td>46BR fibroblasts&lt;sup&gt;d&lt;/sup&gt;</td>
<td>None</td>
<td>100(6.3)*</td>
<td>100(5.5)</td>
<td>100(9.7)</td>
</tr>
<tr>
<td></td>
<td>3AB</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Incubation time.
<sup>a</sup> Quiescent normal fibroblast seeded in medium with or without insulin and irradiated with 4 Jm<sup>-2</sup> of UV 3–4 h after trypsinization. Proliferating cultures of 46BR were irradiated.
<sup>b</sup> Various deoxyribonucleosides (dX: dA, dG, dC, dT) and ribonucleosides (rX: adenosine, guanosine, cytosine, and uridine) at a final concentration of 10<sup>-4</sup> M each, and 3AB at 5 mM.
<sup>c</sup> Numbers in parentheses, control number of DNA breaks per 10<sup>9</sup> daltons DNA accumulated after UV in the absence of DNA synthesis inhibitors.

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even the limited requirements of polymerization in the excision repair process.

Breaks Accumulated during Pulses of DNA Synthesis Inhibitors Disappear More Slowly in Quiescent Cells Treated with Trypsin and Insulin: Provision of Deoxyribonucleosides Overcomes the Trypsin and Insulin Effect. If insulin and trypsin affect the rate of gap sealing after UV by reducing the size or availability of the dNTP pool then DNA breaks accumulated artificially (i.e., using inhibitors) should also be rejoined more slowly in response to treatment with these agents. To test this possibility cells in different states of growth were irradiated with 4 Jm<sup>-2</sup> and incubated for 30 min with HU and aphidicolin. Aphidicolin was used in preference to ara-C in this set of experiments because the inhibition of DNA polymerase α is rapidly and completely reversed following its removal. Inhibitors were removed, the cells were incubated further, and at various times the number of remaining DNA breaks was determined (Fig. 3). It is clear that, while synchronized S-phase cells (20 h after trypsin release) rejoined most of the accumulated breaks within 30 min of inhibitor removal, quiescent cultures treated with trypsin and irradiated in early G<sub>1</sub> rejoined the breaks much more slowly. The addition of insulin to the medium retards the rate of gap sealing in G<sub>1</sub> cells even further (though insulin has no effect on rejoining of the S-phase cells). The provision of deoxyribonucleosides increases the rate of gap sealing regardless of the type of culture. This finding provides further support for the idea that the effect of insulin and trypsin on break sealing is mediated via the DNA precursor pools.

Production of Double-Strand Breaks after UV in Cells Reseeded from Quiescence. We have shown that in cells reseeded from quiescence in the presence of insulin and then irradiated in early G<sub>1</sub> the reduced rate of gap sealing results in a high level of incomplete repair sites for a considerable length of time after irradiation. It has been shown (9, 10) that the accumulation of single-strand breaks in the presence of DNA synthesis inhibitors is associated with the appearance of DSB in a time and UV dose-related manner. In view of these results we have measured the generation of DSB, by means of the neutral elution technique (23), in fibroblasts under different growth conditions and treatments, some of which promote the accumulation of incomplete repair sites. The neutral elution behavior of uniformly prelabeled DNA is related to the frequency of DSB. We find that the rate of elution of radioactivity from the filters corresponds well to the frequency of long-lived DNA gaps revealed by alkaline lysis; the fastest elution rates are seen with DNA from cultures treated with inhibitors, followed by DNA from quiescent cells reseeded in medium plus insulin and finally, from cells seeded in medium without insulin (Fig. 4). Proliferating cells which do not accumulate incomplete repair sites do not generate double-strand breaks after UV and the amount of radioactivity retained on the filters is similar to the unirradiated control. When double-strand DNA breaks are generated under these conditions they occur in cells in pre-S-phase and can be detected as early as 60 min after irradiation.

Insulin Enhances the Cytotoxicity of UV in Quiescent Cells. The high steady-state level of DNA breaks which is observed in reseeded quiescent cells shortly after UV-irradiation might be expected to pose serious problems for cells trying to recover from the damaging effects of UV. For example, a level of 2.5 breaks 10<sup>9</sup> daltons of DNA represents approximately 2500 DNA strand discontinuities per genome. We have compared the cytotoxic effects of UV on quiescent and proliferating fibroblasts that were irradiated in suspension and then plated in the presence or absence of insulin (Fig. 5). Proliferating cells are much more sensitive to UV irradiation than are cells reseeded from a quiescent state. Treatment of proliferating cells with insulin does not alter their survival. Insulin significantly increases the sensitivity of cells reproduced from quiescence to UV.
irradiation. This effect manifests itself primarily as a decrease from 3.9 to 1.4 Jm⁻² in D₀. The greater UV sensitivity of insulin-treated AAS-synchronized cells was found also by Milo et al. (17) and it correlates with the higher level of unligated DNA breaks present in the cells.

DISCUSSION

We have explored the coordination or the lack of it between incision and postincision steps in the excision repair process in normal embryonic human fibroblasts irradiated with low doses of UV light. Particular attention has been paid to the physiological status of the fibroblast populations and especially to the dependence of excision repair completion on the availability of DNA precursors. In proliferating cells the postincision repair steps (excision, gap formation, repair replication, and ligation) are executed very rapidly such that the number of unligated single-strand breaks at any point during the repair process is very low. But when cells, synchronized by release from confluence, are irradiated in early G₁, it is clear that the steady state of excision repair has shifted so that now substantial numbers of incomplete DNA repair sites accumulate. The highest frequency of incomplete repair sites is found in quiescent cells stimulated to proliferate by proteolytic detachment followed by incubation in the presence of insulin.

Our data indicate that the repair patch synthesis step is the most likely cause of delay and it is the level of DNA precursors that limits the rate of gap sealing. Thus, providing precursor levels of deoxyribonucleosides after UV prevents the appearance of incomplete repair sites in populations of cells stimulated to grow from a quiescent state by mitogenic treatment. Adequate provision of the DNA precursor pool is essentially under control of the enzyme ribonucleotide reductase. In quiescent cells its activity is virtually undetected but increases dramatically with the onset of DNA synthesis and, in parallel, the DNA precursor pool size increases (35-38). The very small precursor pool size in quiescent cells is nevertheless sufficient to sustain normal repair synthesis after 4 Jm⁻², since incomplete repair sites do not accumulate (Fig. 1) (38). In this context it is important to note that inhibitors of ribonucleotide reductase, such as HU and daA, are most effective in inhibiting repair synthesis in quiescent cells (Table 2) (34, 38-40). Therefore it is possible that, like HU, trypsin and/or insulin treatment of quiescent cells affect the limited activity of ribonucleotide reductase resulting in the depletion of some or all of the dNTP pool components. The level of DNA precursor in the G₁ cells would then fall below the minimum needed to maintain a normal rate of repair synthesis, causing UV-induced DNA breaks to accumulate. Since our evidence is indirect it remains to be determined how trypsin and insulin influence the DNA precursor pools which play a role in excision repair. However, preliminary data⁴ suggest that both trypsin and insulin cause significant DNA precursor pool reduction.

Fibroblasts from two moderately UV-sensitive human conditions, CS and XP variant, show normal incision behavior and normal DNA repair synthesis, but the former have difficulty in recovering semiconservative DNA synthesis after UV and the latter display a severe retardation in the assembly of high molecular weight nascent DNA (41, 42). Under certain physiological conditions, however, excision repair in these cells can also be severely disturbed. For example, CS cells that were recently exposed to trypsin, and quiescent XP variant cells,

show an impaired rate of DNA strand rejoining after UV (3, 13). The results in this paper show that more extreme conditions are required to stress normal fibroblasts to display similar perturbation. In CS cells, as with normal fibroblasts, provision of DNA precursors can alleviate break accumulation. It is probable that underlying the stressed state in each case is the metabolism responsible for balanced DNA precursor supply. It is intriguing that this pathway should be especially susceptible to disturbance in these inherited conditions.

The analytical strategy we have used in this paper is a simple one and allows us to estimate the rates of incision and strand rejoining from the frequency of DNA breaks accumulated after UV in the presence or absence of repair synthesis inhibitors. This procedure also allows us to calculate the average time required to complete a repair site from the ratio of incomplete sites remaining at the end of a given period to the total number of incision events occurring during that period (Table 3). The accuracy of this estimate depends on using a brief incubation period of 15 min to ensure that the rate of incision, measured in the presence of HU and ara-C, is almost constant since longer incubations result in an underestimate of incision rate with the result that the calculated site completion time will be overestimated (4). The calculation also makes another simplifying assumption that all repair sites give rise to breaks in the presence of inhibitors. But even if this is not the case (43, 44) we believe that it effects only a small proportion of the incision events, and that a comparison is valid as long as the frequency of the inhibited repair sites in the various cultures is similar, as we show in Fig. 1. Our estimates underline the extent to which the physiological state of diploid human cells influences the excision repair process. In quiescent cells stimulated either by serum alone or by trypsin release, completion of a repair site takes about four and 18 times longer, respectively, than in a trypsin-treated proliferating culture. In all these cases the cells were irradiated 3 h after growth stimulation. 46BR, a naturally occurring human cell mutant which may be defective in DNA ligase activity (15), takes about 12 min to complete a repair site; 40X longer than a normal counterpart. For normal cells insulin is a potent effector in reducing the rate of gap sealing. In quiescent cells the effects of trypsin and insulin on postincision processes are additive; under these conditions it takes as long as 9 min to complete a repair site, while in the proliferating cell less than half a minute is required. Trypsin and insulin are two of the diverse range of mitogens that stimulate contact inhibited cultures to initiate DNA synthesis (45–47). At present, however, we can provide no rationale for the effects of trypsin or insulin on DNA synthetic metabolism, as reflected in our work by the temporary retardation of repair synthesis. These effects are, however, early responses to mitogen treatment of G0/G1 or early G1 cells, occurring long before replication is initiated.

There are several reasons for believing that long-lived DNA breaks arising from UV-induced excision repair have severe biological consequences, and the correlation between increased cytotoxicity and the presence of long-lived DNA breaks has been shown in several systems, regardless of whether breaks were generated either spontaneously or with inhibitors of DNA synthesis (Fig. 5) (6, 12, 22, 40). We have shown that double-strand DNA breaks are generated in UV-irradiated human fibroblasts at a frequency that correlates well with the abundance of long-lived single-strand DNA breaks (Fig. 4) (8, 12). Double-strand DNA breaks or gaps undergoing repair in mammalian cells may provide the necessary substrate for mitotic recombination within the first cycle (48, 49). Confirmation of this view comes from studies with X-irradiated human and rodent cells where recombination events such as translocation (50), chromosome fragmentation and ring formation (51) have been shown to occur, in G0 independently of S-phase. In addition the work of Zajac-Kaye and Ts'O (52) and Tezaghi and Little (53) has shown a clear relationship between the production of DNA breaks with subsequent chromosome rearrangements and increased rates of transformation. The increased level of DNA breaks reported in this paper in the trypsin released cultures subsequently irradiated in early G1, may help to explain the several-fold increase in X-ray-induced transformation and translocation found in rodent and human cells trypsinized shortly after mutagen treatment (21, 50).

In human fibroblasts the relationship between induction of long-lived repair sites by trypsin and insulin, and the promotion of genetic instability with long term biological consequences must remain speculative. However, it is worth pointing out that the successful transformation of human cells as measured by the development of anchorage-independent growth, reported by Milo et al. (16, 17), involves a protocol which we have shown results in the production of long-lived DNA breaks. This information may help to explain the finding that insulin augments X-ray-induced transformation of BALB/3T3 and 10T1/2 mouse quiescent cells (20, 21). The increased incidence of the amplification of the DHFR gene is another example in which insulin promotes genomic instability in replated 3T3 cells (54).

**Table 3** 

<table>
<thead>
<tr>
<th>Cell type and growth state</th>
<th>m/# (Relative DNA break frequency, without/with inhibitors)</th>
<th>Time for repair at a single site, t (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal fibroblasts (logarithmic)</td>
<td>Without insulin</td>
<td>With insulin</td>
</tr>
<tr>
<td>Normal fibroblasts (quiescent)</td>
<td>0.025</td>
<td>0.31</td>
</tr>
<tr>
<td>Normal fibroblasts (quiescent, reseeded)</td>
<td>0.1</td>
<td>0.45</td>
</tr>
<tr>
<td>Normal fibroblasts (quiescent, reseeded)</td>
<td>0.45</td>
<td>0.61</td>
</tr>
<tr>
<td>46BR fibroblasts (logarithmic)</td>
<td>0.8</td>
<td>12</td>
</tr>
</tbody>
</table>

* n and m, respectively, the DNA break frequencies observed after 15-min incubation with or without DNA synthesis inhibitors (see Fig. 1). We assume that the parameter represents the total number of incision events performed by the cell in that period (4). The parameter m, measured without inhibitors, represents those repair sites that were incised at the end of the 15-min incubation period and have not yet been sealed. t, the time it takes to complete repair at a single site can be estimated from the relationship t/15 = m/n.

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Slow DNA Rejoining in Ultraviolet-irradiated Human Diploid Fibroblasts Treated with the Mitogens Trypsin and Insulin

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