Journal of Cell Science 107, 1745-1752 (1994)
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Sites in human nuclei where DNA damaged by ultraviolet light is repaired: visualization and localization relative to the nucleoskeleton

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SUMMARY

The repair of damage induced in DNA by ultraviolet light involves excision of the damage and then repair synthesis to fill the gap. We investigated the sites of repair synthesis using MRC-5 fibroblasts and HeLa cells in G_1 phase. Cells were encapsulated in agarose microbeads to protect them during manipulation, irradiated, incubated to allow repair to initiate, and permeabilized with streptolysin O to allow entry of labelled triphosphates; [^{32}P]dTTP was incorporated into acid-insoluble material in a dose-dependent manner. Incubation with biotin-16-dUTP allowed sites of incorporation to be indirectly immunolabelled using a FITC-conjugated antibody; sites were not diffusely spread throughout nuclei but concentrated in discrete foci. This is similar to sites of S phase activity that are attached to an underlying nucleoskeleton. After treatment with an endonuclease, most repaired DNA electroeluted from beads with chromatin fragments; this was unlike nascent DNA made during S phase and suggests that repaired DNA is not as closely associated with the skeleton. However, the procedure destroyed repair activity, so repaired DNA might be attached in vivo through a polymerase that was removed electrophoretically. Therefore this approach cannot be used to determine decisively whether repair sites are associated with a skeleton in vivo.

Key words: DNA repair, DNA damage, biotin-dUTP, nucleoskeleton

INTRODUCTION

When cells are irradiated with ultraviolet light (UV), damage is not introduced uniformly into the genome; subsequently it is removed from different parts of the genome at different rates (reviewed by Downes et al., 1993; Sage, 1993; Bootsma and Hoeijmakers, 1993). For example, 6-4 photoproducts are introduced preferentially into non-nucleosomal DNA (Mitchell et al., 1990) and pyrimidine dimers are removed more quickly from active (or potentially active) genes than inactive genes (Venema et al., 1992). Superimposed upon this, dimers are removed more quickly from transcribed strands compared with their complements (Mellon et al., 1987; Bohr, 1988; Venema et al., 1991; May et al., 1993; but see de Cock et al., 1992; Carreau and Hunting, 1992), biasing mutation towards the transcribed strand (Vrieling et al., 1991). Mutant xeroderma pigmentosum cells of complementation group C can remove damage only from transcribed strands (Venema et al., 1991), whereas UV-sensitive Cockayne syndrome cells of complementation groups A and B, which have normal repair capacity overall, are defective in the preferential repair of active genes (Venema et al., 1990).

Despite this detailed knowledge at the molecular level, relatively little is known about where damage is repaired in relation to the various sub-nuclear structures that are now understood to play such an important role in nucleic acid synthesis. Sites of replication and transcription, which can be immunolabelled after incorporation of the appropriate brominated or biotinylated precursors, are not diffusely spread throughout nuclei, but concentrated in discrete foci associated with an underlying nucleoskeleton (e.g. see Nakamura et al., 1986; Nakayasu and Berezney, 1989; Mills et al., 1989; Hozák et al., 1993; Jackson et al., 1993). We have now investigated whether UV-induced damage is also repaired at discrete sites.

The localization of sites of repair posed several problems. First, the high rate of S phase DNA synthesis in a growing population obscures any repair synthesis; therefore we studied human cells (i.e. MRC-5 fibroblasts or HeLa cells) synchronized in G_1 phase. Second, nascent nucleic acids are prone to aggregate, making it difficult to ensure that any associations seen are not generated artifactually (Cook, 1988). Third, polymerization probably occurs so rapidly (i.e. at tens of nucleotides/second) that during incubations long enough for incorporation of sufficient labelled precursors for detection, there is plenty of time to complete synthesis of the short region of ~30 bp around the damage and then for the repaired DNA to move away from the synthetic site (Huang et al., 1992). Fourth, the complexity of damage introduced by UV and the

variety of pathways for removing it further complicate matters (see above). We minimize some of these problems by encapsulating living cells in agarose microbeads (diam. 50-150 μm), before permeabilizing cell membranes using streptolysin O (Ahnert-Higler et al., 1989) in a physiological buffer. Such permeabilized cells can synthesize RNA and DNA at rates found in vivo (Jackson et al., 1988; Hassan and Cook, 1993); if polymerases had aggregated artifactually, we would expect rates to be reduced. Moreover, encapsulation allows permeabilized cells to be washed thoroughly to remove both endogenous pools of triphosphates and unincorporated precursors. Synthetic rates can be reduced by lowering the concentration of precursors to ensure that nascent molecules are elongated by only a few nucleotides so that synthetic sites are labelled. The encapsulating agarose also protects fragile cells from damage and permits them to be manipulated freely.

We find that repair synthesis does not occur throughout nuclei but is focally concentrated; this is similar to S phase synthesis, which occurs in 'factories' attached to the skeleton (Hozák et al., 1993). But unlike the S phase activity, DNA made during repair was (after manipulation in vitro) not as closely associated with the skeleton, suggesting that the repair activity might be unattached. However, as the procedure used removed repair activity, it remains possible that repaired DNA was originally attached in vivo through a polymerase that was subsequently removed by the procedure.

MATERIALS AND METHODS

Cell culture and synchronization

Suspension cultures of HeLa cells were grown in minimal essential medium supplemented with 10% foetal calf serum. When labelling with [^{32}P]dTTP or [^{32}P]dGTP, cells were grown prior to synchronization in [*methyl*-^{3}H]thymidine (0.05 μCi/ml; ~60 Ci/mmol) for 18-24 hours to label their DNA uniformly, to allow corrections for slight variations in cell numbers. HeLa cells were synchronized using thymidine and nitrous oxide (Rao, 1968; Jackson and Cook, 1986b). Cells were first blocked in S phase (2.5 mM thymidine; 22 hours), washed thoroughly, regrown for 4 hours in fresh medium and >95% were arrested at mitosis using nitrous oxide at high pressure (8 hours) and regrown in fresh medium; G_1 phase cells were generally collected 2 hours later when <1% were in S phase. Confluent MRC-5 cells were maintained in Ham's F10 and 10% foetal calf serum for 3 days before they were encapsulated and regrown in fresh medium for 3 hours; then only 2-5% were in S phase.

Encapsulation and lysis

Cells were washed 3× in fresh PBS, encapsulated (10^6 to 10×10^6 cells/ml) in 0.5% agarose (Jackson et al., 1988) and lysed with streptolysin O in a modified physiological buffer (PB). This contains 10 mM Na_2HPO_4, 1 mM $MgCl_2$, 65 mM potassium acetate, 65 mM KCl, 1 mM Na_2ATP, 1 mM dithiothreitol and 0.2 mM phenylmethylsulphonyl fluoride (PMSF). Beads were incubated with streptolysin O (Wellcome; 5 i.u./ml per 10^6 cells) for 30 minutes at 4˚C to allow binding, washed to remove unbound streptolysin, resuspended in an equal volume of PB, re-incubated at 33˚C for 2 minutes to allow permeabilization and then rewashed in PB at 4˚C. In the experiments shown in Fig. 4, some samples were permeabilized with Triton (0.25%; 15 minutes; 4˚C; 0.5% Triton gave identical results) and then washed with PB (4×; 10 vol).

Irradiation with UV

A 1 ml sample of encapsulated cells in 10 ml PBS was irradiated (Sylvania germicidal tube; 2 and 15 J/m^2 per minute for doses of 0.2-5 J/m^2 and 15-100 J/m^2, respectively) in a 10 cm diameter glass Petri dish, washed in medium and generally regrown for 1 hour prior to lysis. During irradiation, cells at high concentrations shield each other from irradiation; however, when encapsulated cells (7.5×10^6/ml) are UV-irradiated (40 or 100 J/m^2), grown for 1 hour and permeabilized, [^{32}P]dTTP is incorporated (see below) at ~60 and 47% of the rate of unencapsulated controls. Therefore a dose of 40 J/m^2, which introduces one dimer or endonuclease-sensitive site every ~2.5 kb in unshielded cells (e.g. see Williams and Cleaver, 1979), introduces one site every ~5 kb under our conditions.

Labelling sites of repair

When labelling with [^{32}P]dTTP or [^{32}P]dGTP, encapsulated and permeabilized cells were pre-incubated (33˚C; 2 minutes) before reactions were started by addition of a 10× concentrated mixture of triphosphates and $MgCl_2$ to give final concentrations of 0.1 mM CTP, GTP, UTP, dATP, dCTP, dGTP (Pharmacia), 2.5 μM dTTP plus [^{32}P]dTTP (Amersham; ~3000 Ci/mmol; 100 μCi/ml) and 1.6 mM $MgCl_2$. (PB contains 1 mM ATP and 1 mM $MgCl_2$; equimolar concentrations of triphosphates and Mg^{2+} were used.) In the experiments shown in Fig. 4, final concentrations were 0.1 mM CTP, GTP, UTP, dATP, dCTP, 25 μM dTTP or biotin-16-dUTP, 2.5 μM dGTP plus [^{32}P]dGTP (Amersham; ~3000 Ci/mmol; 100 μCi/ml) and 1.6 mM $MgCl_2$. For Fig. 6, final concentrations were 0.1 mM CTP, GTP, UTP, dATP, dCTP, dGTP plus [^{32}P]dTTP (100 μCi/ml) and 1.6 mM $MgCl_2$. Inhibitors, if present, were pre-incubated (4˚C for 10 minutes, then 33˚C for 2 minutes) prior to addition of triphosphates. Reactions were stopped by removing samples and adding them to 2% SDS; after incubation (2 hours; 37˚C), ^{32}P incorporation into acid-insoluble material was measured by scintillation counting (Jackson and Cook, 1986b). For immunofluorescence, both dTTP and [^{32}P]dTTP were replaced by 25 μM biotin-16-dUTP (Boehringer), the $MgCl_2$ concentration was adjusted to maintain equimolarity of Mg^{2+} with triphosphates and reactions were stopped by washing in ice-cold PB (2×; 20 vol.). Variations from standard conditions are given in figure legends.

There were 0.2-1% (average 0.4%) S phase HeLa cells in different G_1 populations that contributed to a background level of DNA synthesis. As a result, UV irradiation (40 J/m^2) stimulated [^{32}P]dTTP incorporation 2-10 times, depending on this background. The experiments described in each figure were repeated at least 3 times, but each figure includes typical results from only one population.

Digestion and electrolution

After incorporation into streptolysin-lysed cells, nuclear membranes were permeabilized with 0.5% Triton (2× 10 minutes; 4˚C; 10 vol.) and washed (3×; 4˚C; 10 vol. PB). Beads were then incubated (33˚C; 20 minutes) with *Eco*RI (2500 units/ml) plus *Hae*III (500 units/ml) or DNase (500 units/ml) in PB and subjected to electrophoresis (0.8% agarose; 4 V/cm; 4 hours; Jackson et al., 1988) in PB. About 90 or 98% ^{3}H (i.e. chromatin) elutes after treatment with the two restriction enzymes or DNase, respectively (Jackson et al., 1990). For Fig. 6 nuclear membranes were not permeabilized with Triton; encapsulated cells were lysed with streptolysin, cut with *Eco*RI and *Hae*III plus *Alu*I (250 units/ml) as above, subjected to electrophoresis to remove 25% ^{3}H (i.e. chromatin), beads were recovered and incorporation of radiolabel (expressed as cpm in samples containing 2.5×10^5 cells) was determined as above.

Immunolabelling

Nuclear membranes were permeabilized (5-10 minutes) in ice-cold PB plus 0.25-0.5% Triton X-100, washed 4× in PB (10 vol.) and fixed (15 minutes; 4˚C) in fresh 4% paraformaldehyde in PB, washed 2× in PB and 2× in PB supplemented with 0.05-0.2% Tween-20 (Sigma) and 0.1% BSA. Sites containing incorporated biotin were detected using a goat anti-biotin antibody (Sigma; 1/1000 dilution; incubation for 4 hours at 4˚C) followed by donkey anti-goat IgG, conjugated with

FITC (Jackson Labs; 1/500-1/1000 dilution; incubation 16 hours at 4˚C). Beads were washed 4× with 10 vol. PB + Tween + BSA before samples (25 μl) were mounted under coverslips in Vectashield (Vector Labs) ± 4′,6′-diamidino-2-phenylindole (DAPI; Boehringer; 0.05 μg/ml).

Fluorescence microscopy

Conventional photographs were taken using a Zeiss Axiophot microscope (standard filter sets) fitted with an Optivar (×1.25) using T-Max black and white film, both push-processed to ASA 1600. The faint repair foci require a 5-10 times longer exposure than the corresponding S phase replication foci.

RESULTS

The kinetics of repair synthesis

HeLa cells in G_1 phase were encapsulated, UV-irradiated with various doses and re-grown for 1 hour to allow the first steps in the repair pathway to occur; then, after permeabilization with streptolysin O, the rate of incorporation of [^{32}P]dTTP into acid-insoluble material was measured. Mock-irradiated cells incorporate label at a low rate (Fig. 1A); this is probably due to: (i) contaminating S phase cells; (ii) mitochondrial DNA synthesis; and (iii) repair of pre-existing damage (see below). UV irradiation increases the incorporation in a dose-dependent manner (Fig. 1A,B). At the highest dose used (i.e. 100 J/m^2), the initial rate of incorporation is ~10% of the rate of S phase synthesis under similar conditions. Notwithstanding the fact that repair synthesis is relatively less affected than S phase synthesis by the low dTTP concentration used (see below), this illustrates the high repair capacity. After ~5 minutes, the initial rate declines, so that after ~15 minutes there is little further incorporation; this is probably because synthesis of the short patches around damaged sites is completed so rapidly, combined with relatively little initiation close to additional damage. If a nanomolar dTTP concentration is used, elongation is limited to only a few nucleotides and the initial rate is maintained for longer (e.g. Fig. 6). Most UV-induced incorporation is sensitive to aphidicolin, an inhibitor of DNA polymerase α/δ (Fig. 1A); the resistant activity is probably due to polymerases β and γ (see Kornberg and Baker, 1992, for a review of the effects of inhibitors).

After irradiation with 40 J/m^2, a dose used in most subsequent experiments and which introduces 1 dimer or endonuclease-sensitive site every ~5 kb (see Materials and Methods), irradiated cells take some time to organize maximal rates of repair incorporation; rates rise to a peak after 1 hour, then fall until entry into S phase obscures any repair incorporation (Fig. 1C). This dose is, in the biological context, extremely high, reducing the cloning efficiency to <1% (not shown).

Fig. 2 illustrates how the dTTP concentration affects rates of both UV-induced and S phase incorporation. Over this range of concentrations, these curves give apparent K_m values of 0.4 and 2 μM for repair and S phase synthesis, respectively, consistent with earlier results (e.g. see Dresler et al., 1988; Hassan and Cook, 1993). One consequence of this is that sub-micromolar dTTP concentrations increase the relative proportion of repair synthesis relative to S phase synthesis.

Visualization of sites of repair

Sites of S phase DNA synthesis can be immunolabelled after incorporation of biotin-16-dUTP into DNA (e.g. see Hozák et al., 1993; Hassan and Cook, 1993); sites of unscheduled DNA synthesis may be labelled similarly (Fig. 3). MRC-5 cells in G_1 were UV-irradiated, grown for 1 hour to allow repair to initiate, permeabilized and incubated with biotin-dUTP; then sites containing incorporated biotin were indirectly immunolabelled using a FITC-conjugated antibody. Mock-irradiated controls gave background labelling (Fig. 3A; this has been

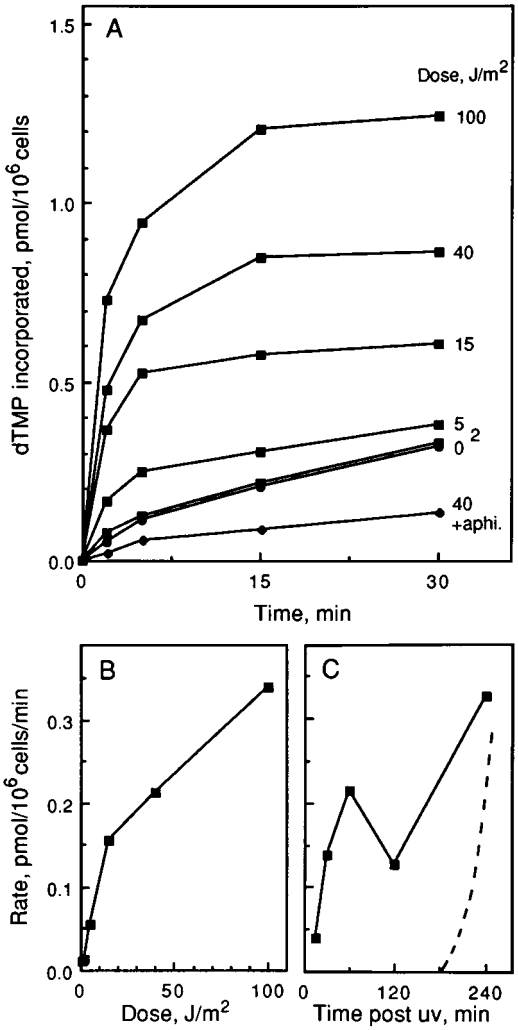

Fig. 1. UV-induced DNA synthesis in permeabilized HeLa cells. (A) Encapsulated G_1 cells were UV-irradiated with various doses, incubated for 1 hour in growth medium, permeabilized with streptolysin and the rate of incorporation of [^{32}P]dTTP into acid-insoluble material was measured. 10 μg/ml aphidicolin (aphi) was present as shown. (B) Dose-response curve. The initial rate of incorporation (measured between 15 s and 2 minutes in A) is plotted against UV dose. (C) Time-course of rate of repair incorporation following UV irradiation. G_1 cells were UV-irradiated (40 J/m^2), incubated for various times in growth medium to allow repair, permeabilized and the initial rate of incorporation of [^{32}P]dTTP into acid-insoluble material measured as in B. The broken line shows the increase in the incorporation rate as cells reach S phase; values were calculated using the maximum rate of S phase synthesis (Hassan and Cook, 1993) and the proportion of cells in the population that were brightly labelled with biotin-dUTP (i.e. were in S phase).

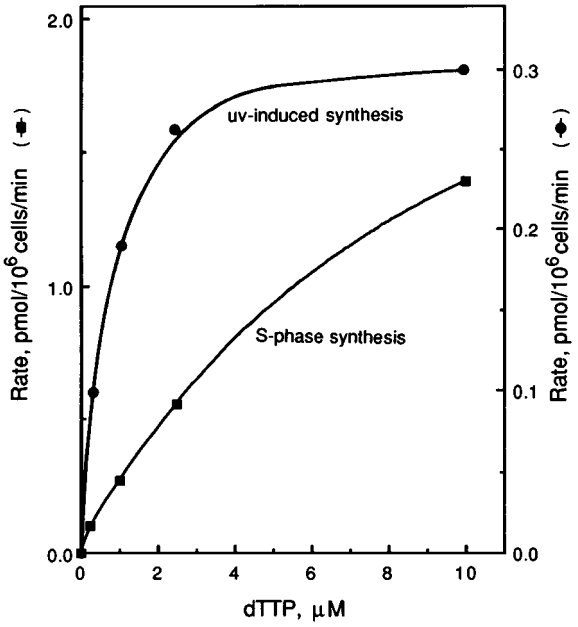

Fig. 2. The effect of dTTP concentration on rates of S phase and UV-induced DNA synthesis. Rates were measured as in Fig. 1 using a constant concentration of the other triphosphates, [^{32}P]dTTP (75 μCi/ml) as a label and either a UV-irradiated (40 J/m^2) G_1 or unirradiated, unsynchronized, population.

exposed for twice as long as Fig. 3B-E). Irradiated samples contained a large number of faintly fluorescing, but discrete, foci that increased in intensity with increasing dose (Fig. 3B-E). Use of a more sensitive CCD camera, rather than conventional film, allows doses closer to the physiological (i.e. 0.5-2 J/m^2) to be detected (not shown; see also accompanying paper: Jackson et al., 1994).

Growing HeLa cells after irradiation (40 J/m^2) to allow repair to initiate increases the intensity of these foci (Fig. 3F-I). Label is not diffusely spread throughout nuclei, reflecting the DNA concentration detected by DAPI (compare Fig. 3I and J). DNase treatment removes most incorporated biotin- and DAPI-staining material; aphidicolin treatment also abolishes labelling (see accompanying paper).

About 0.4% HeLa and 2-5% MRC-5 cells in unirradiated controls fluoresce brightly with patterns typical of S phase cells; the rest are unlabelled. Repair foci are easily distinguishable from replication foci seen in such contaminating S phase cells. One hour after irradiation (40 J/m^2), 80-90% of both cell types are relatively uniformly labelled with many repair foci and have intensities about one-tenth to one-fifth of those seen in the contaminating cells.

These images are of round cells encapsulated in beads and contain considerable out-of-focus 'flare' from above and below the focal plane; individual foci are then best seen at the edge of nuclei. MRC-5 cells, which are grown prior to encapsulation as monolayers, tend to have slightly flattened nuclei even after encapsulation and when favourably oriented they give less flare than the rounder, larger, HeLa nuclei (compare Fig. 3A-E with F-J). Therefore we tend to use MRC-5 cells for immunofluorescence and HeLa cells, which have fewer contaminating S phase cells in the G_1 population, for biochemical experiments.

Biotin-dUTP is incorporated into repair sites efficiently

Replacement of dTTP by biotin-16-dUTP reduces S phase incorporation to 17% (Hassan and Cook, 1993). In contrast, the biotinylated analogue has little effect on repair incorporation (Fig. 4, compare +UV/SO with +UV/SO, bio-dUTP). During the short incubations used here, the incorporated biotin, which itself could become a target for repair (Huijzer and Smerdon, 1992), does not stimulate repair synthesis.

If residual incorporation by mock-irradiated G_1 cells was

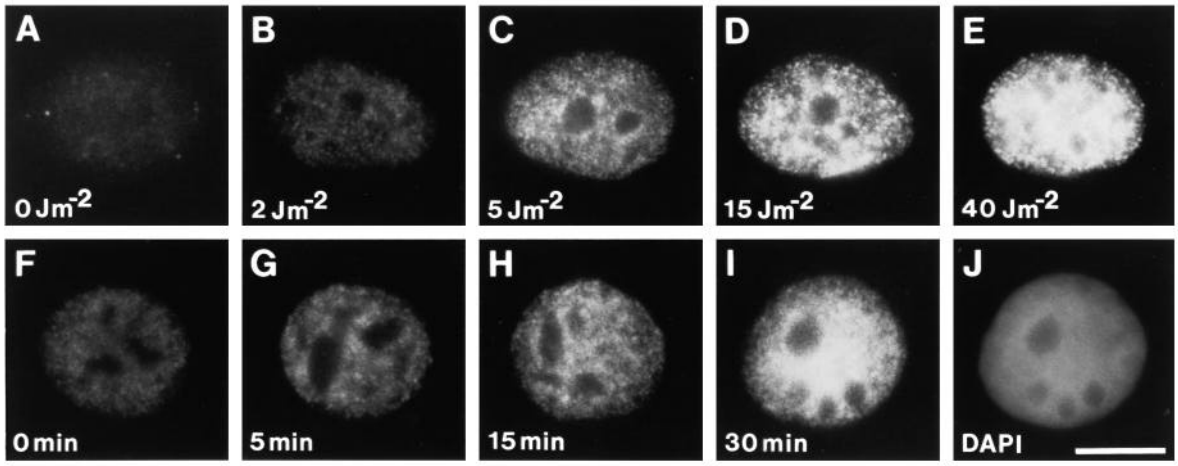

Fig. 3. Visualization of sites of unscheduled DNA synthesis in (A-E) MRC-5 irradiated with different doses and (F-J) HeLa cells at different times after irradiation. (A-E) Encapsulated cells in G_1 were UV-irradiated with the doses indicated, grown for 1 hour to allow repair to initiate, permeabilized with streptolysin and incubated with biotin-16-dUTP for 15 minutes; sites containing incorporated biotin were then immunolabelled (goat anti-biotin followed by a FITC-anti-goat antibody) before cells were photographed using similar exposures (except for that in A, for which the exposure was doubled). (F-J) As A-E, except cells were irradiated with 40 J/m^2 and grown for the times indicated. J illustrates DAPI staining of cell in I. Bar, 5 μm.

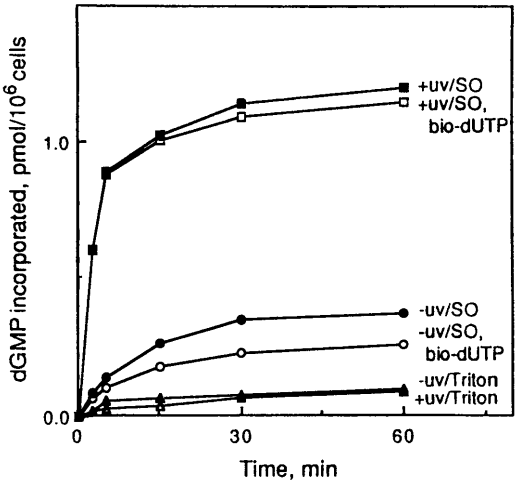

Fig. 4. The effects of lytic agent and biotin-dUTP on repair incorporation. Encapsulated G_1 HeLa cells were mock-irradiated or UV-irradiated (40 J/m^2; indicated by ±UV), incubated in growth medium (1 hour; 37˚C), lysed with Triton or streptolysin (SO) and the incorporation of [^{32}P]dGTP was determined in the presence of 25 μM dTTP or biotin-dUTP (indicated by bio-dUTP).

entirely due to contaminating S phase cells, biotin-dUTP should reduce incorporation to 17%. The higher value (Fig. 4, compare −UV/SO,bio-dUTP with −UV,SO) suggests that residual incorporation is due to a mixture of biotin-dUTP-sensitive and -insensitive synthesis (i.e. S phase plus mitochondrial and/or repair synthesis).

S phase and repair synthesis are differentially sensitive to Triton

Streptolysin-lysed cells continue S phase DNA synthesis at essentially the rate found in vivo, but Triton-lysed cells do so less efficiently (Hassan and Cook, 1993). As expected, the background synthesis by contaminating S phase cells is inhibited by Triton (Fig. 4; compare −UV/SO with −UV/Triton). However, UV-irradiation stimulates incorporation by streptolysin-lysed cells but has no effect on Triton-lysed cells (Fig. 4; compare −UV/SO with +UV/SO and −UV/Triton with +UV/Triton); presumably Triton removes some factor(s) required for repair.

Attachments of repaired DNA to the nucleoskeleton

Evidence is accumulating that polymerases involved in S phase DNA synthesis are attached through a large factory to a nucleoskeleton (Cook, 1991; Hozák et al., 1993). This inevitably raises the question of whether the repair activity is also attached. Earlier evidence has been interpreted to suggest that repair synthesis both does, and does not, take place at such a skeleton (e.g. see McCready and Cook, 1984; Harless and Hewitt, 1987; Mullenders et al., 1988). Therefore, we re-investigated whether repaired DNA and the repair activity were attached, using the approach that had been used to demonstrate attachment of the S phase activity (Jackson and Cook, 1986a).

HeLa cells were UV-irradiated, grown for 1 hour, lysed with streptolysin and incubated for 1 minute with the lowest concentration of [^{32}P]dTTP that was practicable, before nuclear membranes were permeabilized with Triton. After incubation with restriction enzymes, cut chromatin can be electroeluted from beads to leave a residual fraction associated with a nucleoskeleton. If the active repair enzyme is associated with the skeleton, repaired DNA (now labelled with ^{32}P) and repair activity should both resist elution.

Table 1. DNA made during repair is easily detached from the nucleoskeleton

	pmol dTMP incorporated/10^6 cells (% resisting elution)		
	G_1		Unsynchronized
	−UV	+UV	−UV
A (8-10% DNA remains)	0.005 (35%)	0.024 (23%)	0.048 (99%)
B (75-85% DNA remains)	0.003 (63%)	0.017 (45%)	nd

HeLa cells were labelled with [^{3}H]thymidine and some were synchronized in G_1 phase. Next, they were encapsulated, mock-irradiated or UV-irradiated (40 J/m^2), regrown for 1 hour, lysed with streptolysin and the amount of [^{32}P]dTTP incorporated in 1 minute into acid-insoluble material was determined using 0.025 μM dTTP. This value is expressed as pmol dTMP incorporated/10^6 cells. Cells were then treated (A) with or (B) without Triton to permeabilize nuclear membranes, chromatin in beads was cut with restriction enzymes and some beads were subjected to electrophoresis to remove all but (A) 8-10 or (B) 75-85% chromatin (measured by retention of ^{3}H). The amount of ^{32}P resisting elution from beads is shown in parenthesis. About 40 bp are extended at an S phase fork under these conditions (Hassan and Cook, 1993). nd, not done.

An unsynchronized population of cells are included as a control (Table 1, unsynchronized, −UV); 99% incorporated label resists elution, consistent with S phase DNA synthesis occurring at a skeleton. DNA made during repair behaves differently: 23% of the incorporated label resisted elution but this still represents a two- to three-fold enrichment relative to bulk DNA (Table 1; A, G_1, +UV). These results are simply explained if repair sites (and therefore incorporated label) are not as closely associated with the skeleton as S phase replication However, two factors complicate interpretation. The first stems from the rapidity of elongation. Under these conditions, ~40 bp are made at each S phase replication fork and the elongation rate during repair is probably higher, given the lower K_m of the repair activity (Fig. 3). This means that there is ample time for damage to be repaired at a skeleton and then for most incorporated label to detach from the repair site. The second concerns the Triton used to permeabilize nuclear membranes to allow access of endonucleases to chromatin; it eliminates most repair activity (Fig. 4). Therefore, it remained possible that incorporated label was attached through a polymerase in vivo but Triton then detached the enzyme and its associated template, so we repeated the experiment without using Triton to permeabilize membranes. Fortunately, restriction enzymes can pass through the nuclear membrane to cut chromatin efficiently, but fewer chromatin fragments elute out of the nucleus (Jackson et al., 1988). Now, >75% chromatin remains but only 45% of the label at repair sites resists elution (Table 1, B, G_1 +UV); presumably label introduced by repair, which is concentrated in transcriptionally active regions (see accompanying paper), is preferentially accessible to digestion. These results show that, even in the absence of Triton, repaired DNA does not resist elution like the S phase activity.

The residual DNA made in an unirradiated G_1 population (Table 1; G_1, −UV) probably arises from mitochondrial DNA synthesis, background repair synthesis and contamination by S phase cells. The relative contribution of each of these to the total will depend on the K_m values of the relevant enzymes, the size of the repair patch and proximity to a skeleton.

Immunofluorescence confirms the difference in behaviour of S phase and repair synthesis (Fig. 5). After lysis, the contaminating S phase cells in the population incorporate biotin-dUTP to yield brightly fluorescing foci (Fig. 5D). As expected, cutting chromatin with restriction enzymes followed by electrophoresis removes few of these S phase foci (Fig. 5E; Fig. 5J shows that most chromatin has eluted). However, most UV-induced foci are removed by electrophoresis (Fig. 5B) along with the chromatin (Fig. 5G). Removing nearly all DNA with DNase removes nearly all incorporated biotin and DAPI-staining material (Fig. 5C,H).

Attachments of repair activity to the nucleoskeleton

We next determined whether repair activity remained in beads after cutting and elution, again using no Triton and the lowest triphosphate concentration practicable (i.e. 0.025 μM [^{32}P]dTTP).

In the particular experiment shown, UV irradiation stimulated ~4× the activity in G_1 cells (Fig. 6; compare −UV/−cut/−E with +UV/−cut/−E); incorporation still continued to increase after 30 minutes, due to the low dTTP concentration (compare Fig. 6, curve +UV/−cut/−E with Fig. 1A, curve 40 J/m^2). However, elution eliminated most activity, irrespective of whether chromatin was cut (Fig. 6, +UV/−cut/+E and +UV/+cut/+E); the activity, which cannot be removed from beads by washing, is either removed or destroyed by electrophoresis. Unfortunately, then, this approach cannot be used to determine whether activity is associated with a skeleton, as it destroys the activity.

DISCUSSION

Properties of the repair activity

Several in vitro systems are available for studying UV-induced DNA synthesis (e.g. see Smith and Hanawalt, 1978; Ciarrocchi et al., 1979; Dresler et al., 1982; Wood, 1989), but none uses physiological conditions during assay. Therefore, we adapted our system for studying S phase DNA synthesis to the study of repair. G_1 cells are encapsulated in agarose microbeads, irradiated and grown to allow repair to initiate, before cells are permeabilized with streptolysin O in a physiological buffer; labelled precursors are then efficiently incorporated into DNA (Fig. 1A). Although encapsulation is not necessary for the basic assay, the lysed and now fragile cells can be washed repeatedly both to deplete endogenous pools and to allow the kinds of experiment with many steps, as performed in this study. This activity has been characterized further in the accompanying paper (Jackson et al., 1994) and is similar to one studied previously (Dresler et al., 1982, 1988).

The repair activity differs in several respects from S phase (replicative) activity. (i) It has a lower apparent K_m (i.e. 0.4 μM compared with 2 μM; Fig. 2). (ii) At moderate triphosphate concentrations, the repair activity quickly declines (Fig. 1A), presumably because the repair patch is so small and there is so little initiation in vitro (see accompanying paper), whilst S phase synthesis continues for at least 1 hour (Hassan and Cook, 1993). (iii) Whilst biotin-dUTP inhibits S phase activity (Hassan and Cook, 1993), it has little effect on repair synthesis (Fig. 4). (iv) After fragmenting chromatin, the S phase activity resists elution but the repair activity is removed (Table 1).

Visualization of repair sites

After incorporating biotin-dUTP, sites of repair can be indirectly immunolabelled; they are not diffusely spread through-

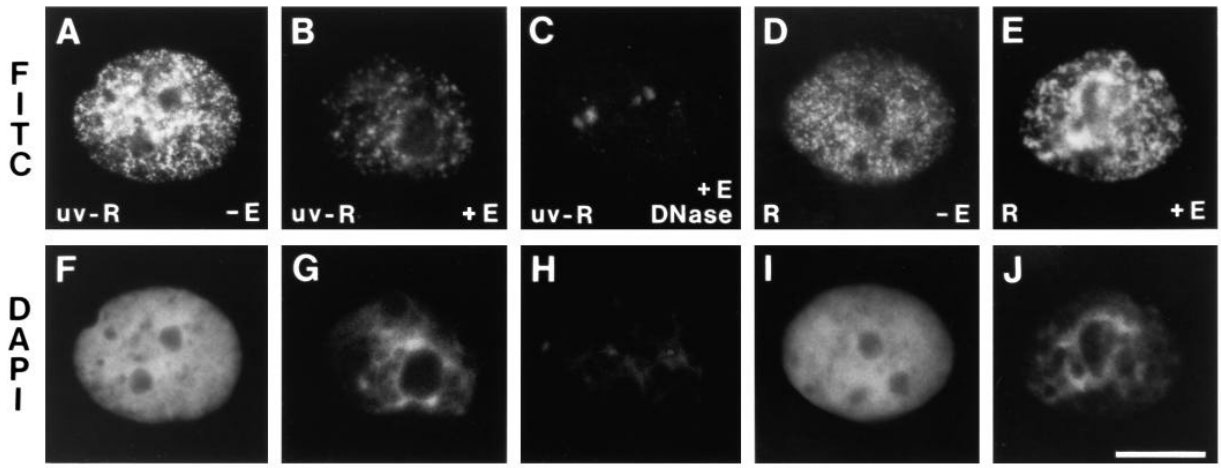

Fig. 5. DNA made during UV-induced replication (UV-R) is not as closely attached to the nuclear sub-structure as that made during S phase replication (R). In each case, the lower panel illustrates DAPI fluorescence of the cell above. Bar, 5 μm. (A-C) Encapsulated MRC-5 cells in G_1 were UV-irradiated (40 J/m^2), grown for 1 hour to allow repair to initiate, lysed with streptolysin, incubated with biotin-dUTP (15 minutes), nuclear membranes were permeabilized with Triton and three samples were treated as follows. (A) A control sample was stored at 0°C (indicated by −E). (B) Some beads were incubated with *Hae*III plus *Eco*RI and subjected to electrophoresis to remove ~90% chromatin (indicated by +E). (C) Other beads were treated with DNase and subjected to electrophoresis to remove ~98% chromatin (indicated by +E, DNase). Finally, sites containing incorporated biotin were immunolabelled with FITC and photographed using conventional film. (D,E) Unsynchronized and encapsulated MRC-5 cells were lysed with streptolysin, incubated with biotin-dUTP (30 minutes) and sites containing incorporated biotin were immunolabelled as above. The cells illustrated have patterns typical of early S phase; foci in E have collapsed on to nucleoli.

out nuclei, reflecting the DNA concentration, but locally concentrated (Fig. 3B,G). As only ~30 nucleotides are incorporated during repair around each damaged site (Huang et al., 1992), too little biotin is incorporated to be detected and each focus must contain many repair patches. Foci probably reflect repair of 6-4 photoproducts, as ~80% of these are removed within 3 hours, whereas dimers persist for longer (Mitchell and Nairn, 1989; Sage, 1993). Perhaps some foci also reflect repair of thymine-rich areas, which are hotspots both for UV-induced damage and for biotin-dUTP incorporation.

These results show that repair, like replication and transcription, is compartmentalized; what is the basis for this compartmentalization? S phase replication occurs in factories attached to a nucleoskeleton (Cook, 1991; Hozák et al., 1993), but as these are not built until the end of G_1 (i.e. after the stage studied here; Hozák and Cook, 1994), they cannot provide the basis of this compartmentalization. It seems more likely that repair initially takes place at transcription sites (see accompanying paper). The visualization of sites of repair opens up the possibility of seeing which of the many proteins that have been suggested to play a role in repair are found at these sites (see also Jackson et al., 1994).

Is repair activity associated with a skeleton?

As the polymerases involved in S phase replication are found in factories attached to the nucleoskeleton, are active repair enzymes also attached? Several factors make this a particularly difficult question to answer. (For some early attempts, see McCready and Cook (1984), Harless and Hewitt (1987), Mullenders et al. (1988).) For example, damage is repaired at different rates in transcribed and non-transcribed sequences (e.g. see Downes et al., 1993; Sage, 1993), which are associated to different degrees with the skeleton (Cook, 1988) and which have different sensitivities to detachment by nucleases. Second, the repaired patch is so short (~30 nucleotides) and the rate of elongation by polymerases so rapid (i.e. many nucleotides/second) that there is ample time during most experiments for damage to be repaired at a skeleton and then for the patch of DNA to detach from it. The problem is compounded because nascent DNA is prone to aggregate artifactually, so giving the impression that polymerases are attached (Cook, 1988). Aggregation seems a likely explanation of some results, in view of the unphysiological conditions used and the known propensity of DNA polymerase to aggregate with sub-nuclear structures (Martelli et al., 1990). The use of physiological conditions and the retention of most of the polymerizing activity of the living cell provide some assurance that any attachments seen here are not generated artifactually.

One simple interpretation of our results is that the active polymerases involved in repair, unlike the S phase enzymes, are not attached; repaired DNA and repair foci are more readily detached nucleolytically from underlying structures than DNA made during S phase (Table 1; Fig. 5). However, repaired DNA is still enriched two- to three-fold more than bulk DNA in the residual attached fragments, implying that synthesis might occur at, or close to, an attachment point (Table 1). If repair polymerases were indeed unattached, this enrichment could still be explained if repair took place preferentially in small chromatin loops that were less likely to be cut nucleolytically and removed than the majority. Indeed, small loops are probably transcriptionally active (Jackson et al., 1990) and repaired preferentially soon after irradiation (Jackson et al., 1994). This interpretation would be consistent with data supporting the coupling of transcription with repair (Downes et al., 1993; Bootsma and Hoeijmakers, 1993), the difficulty of detaching repaired patches from nuclear matrices (Mullenders et al., 1988) and the association of those patches with a 'cage' in nucleoid spreads (McCready and Cook, 1984). Unfortunately, and even though we limit elongation to a few tens of nucleotides, a significant fraction of repaired DNA probably has time to dissociate from its site of synthesis during our labelling period. Moreover, as the electrophoretic removal of chromatin destroys the repair activity, we are unable to assess whether the repair complex resists detachment (Fig. 6); it remains possible that polymerases attached in vivo are removed by electrophoresis, so allowing the repaired DNA to detach. In view of these problems, we believe that a new approach is needed to determine decisively whether sites of repair synthesis are associated with a skeleton in vivo.

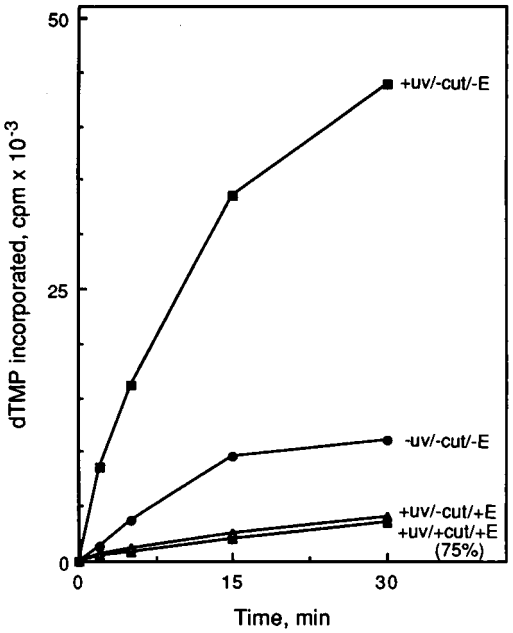

Fig. 6. Electroelution removes repair activity. Encapsulated G_1 HeLa cells were mock-irradiated or UV-irradiated (40 J/m^2; indicated by ±UV), incubated in growth medium (1 hour; 37˚C), lysed with streptolysin, incubated ± restriction enzymes (indicated by ± cut), stored on ice or subjected to electrophoresis (indicated by ±E), and the incorporation of 0.025 μM [^{32}P]dTTP was determined. After cutting and elution, 75% chromatin resisted elution. Mock-irradiated controls that were cut and eluted (i.e. –UV/+cut/+E) gave initial rates ~75% of those of their uncut counterparts (not shown), reflecting the combined effects of the loss of endogenous repair activity in most cells plus the resistance to elution of activity in S phase contaminants (which is halved by irradiation; see +UV/+cut/+E).

We thank Jon Bartlett for his help, and the Cancer Research Campaign, The association of the University of Leiden with Euratom (Contract number B16E166NL) and NATO for support.

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(Received 24 January 1994 - Accepted 18 March 1994)