Cells synchronized in S phase show increased rate of repair of UV damaged plasmids

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Abstract The capacity for nucleotide excision repair of cells synchronized in S phase and unsynchronized cells was compared by the host cell reactivation assay and the cell-free repair system. HeLa cells were transfected with in vitro damaged by UV irradiation pEGFP and the repair capacity was determined by the number of fluorescent cells. In the cell-free repair system, the repair capacity of protein extracts isolated from K562 cells was determined by measuring the transformation efficiency of UV irradiated pBlueScript incubated in the extracts. In both cases, the repair capacity of the cells synchronized in S phase cells was 30–50% higher than the repair capacity of unsynchronized cells.

Keywords: Nucleotide excision repair; S phase; Host cell reactivation; Cell-free repair system

1. Introduction

Repair of UV damage and a number of other helix distorting lesions is carried out by nucleotide excision repair (NER). Several lines of evidence suggest a connection between NER and the cell cycle progression. Thus, in response to DNA damage, checkpoints block the cell cycle to allow sufficient time for repair to take place [1]. NER is needed for activation of the DNA damage checkpoint in G1 and NER deficient mutants delay checkpoint activation until entry into S phase [2]. The basal RNA polymerase II transcription factor TFIIH contains the helicases XPB and XPD that take part both in NER and in the signal transduction pathway responsible for the G1/S transition [3,4].

Considerable effort was also made to answer the reverse question – whether NER activity depends on the stage of the cell cycle [5,6]. By measuring the rate of damage removal in synchronized mammalian cells treated with DNA damaging agents, it was found that the global genome repair efficiency was increased [7–11]. However, damaging cellular DNA in vivo blocks replication forks movement, cell cycle progression and induces apoptosis through activation of p53 [11–14]. This makes the approach unsuitable for studies of the cell cycle differences in constitutive repair rates and the problem is still poorly understood. The host cell reactivation (HCR) assay and the cell-free repair system allow measurement of the constitutive repair rates because the substrate for repair in these assays is exogenous in vitro damaged DNA and not the in vivo damaged cellular DNA. By using the cell-free repair system, we have previously shown that there was a well expressed difference in the repair capacity of quiescent and cycling cells, the latter being 2–3 times higher [15]. These data suggest that differences in NER rates may exist during the cell cycle.

In the present communication, we tried to establish whether cycling cells possess higher levels of NER activity during S phase. To this end, HeLa and K562 cells were synchronized in S phase and their repair capacity determined by HCR assay and the cell-free repair system. It was found that the level of repair of S phase cells was 30–50% higher than that of unsynchronized cells. A conclusion was drawn that in S phase, the cells possess an enhanced capacity for NER.

2. Materials and methods

2.1. Cells and treatment

K562 and XPA cells were grown in suspension in RPMI 1640 medium, containing 4.5 mg/ml glucose in the case of K562. HeLa cells were grown as monolayer in Dulbecco's modified Eagle's medium. The media were supplemented with 10% fetal bovine serum and antibiotics and the cells were grown in an atmosphere of 95% air/5% CO_2 at 37 °C. K562 and HeLa M cell lines were obtained from ATCC and XPA cells line – from NIGMS Human Genetic Cell Repository at Coriell Institute for Medical Research. HeLa and K562 cells were treated with 0.5 mM mimosine for 24 h [16]. HeLa cells were released in fresh medium and 1 h later were transfected with pEGFP for HCR assay. K562 cells were grown in fresh medium for 6 h to reach middle S phase and were used to prepare protein extracts.

To label uniformly genomic DNA, cells were incubated in the presence of 0.025 μCi/ml [^{14}C]thymidine (DuPont) for 24 h. To estimate the rate of DNA synthesis, cells were pulse labeled with 1 μCi/ml [^{3}H]thymidine (DuPont) for 30 min, collected, washed with phosphate-buffered saline (PBS) and the precipitated with 10% trichloroacetic acid radioactivity was counted. For FACS analysis cells were pelleted, washed with PBS, treated with 20 μg/ml RNase for 30 min at 37 °C and stained with 20 μg/ml propidium iodide at room temperature for 30 min. 2×10^4 cells/sample were analyzed with a Becton Dickinson (Facscalibur) cell sorter, using ModFit software (Becton Dickinson).

2.2. Plasmids and irradiation

Plasmids pBlueScript SK+ (Stratagene) and pEGFP-N1 (Clontech) were propagated in *Escherichia coli* strain XL-1Blue. The UV irradiation of the plasmids was carried out with a germicidal mercury lamp as described in [17]. Plasmid DNA was dissolved in 10 mM Tris–HCl, pH 8, and 1 mM EDTA (TE buffer) to a final concentration of 100 μg/ml. It was poured in Petri dishes to form 1–2 mm thick layer and UV irradiated for 2 and 3 min. Upon these irradiation conditions, the

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exposure rate was 8.3 $J\,m^{-2}\,s^{-1}$ as measured with an ultraviolet power energy meter Scientech 362 and pBlueScript SK+ and pEGFP-N1 received 1 and 1.5 $kJ\,m^{-2}$, respectively.

2.3. Host cell reactivation assay

A protocol for HCR assay to assess NER, using the green fluorescent protein as a reporter developed in our laboratory was used [18]. Transfection with 4 μg of each plasmid DNA was carried out with GenePorter II transfection kit (Gene Therapy Systems) following the manufacturer's recommendations. The number of fluorescent cells was counted 7 h post-transfection under fluorescent microscope.

2.4. Cell-free repair assay

A protocol for the cell-free DNA repair system developed in our laboratory was used [19]. Cell-free reactions contained 300 ng UV irradiated pBlueScript (ampicillin resistant) repair substrate, 300 ng unirradiated pEGFP (kanamycin resistant) internal control, 45 mM HEPES–KOH (pH 7.8), 70 mM KCl, 7.4 mM $MgCl_2$, 0.9 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 25 μM each of dGTP, dATP, dTTP and dCTP, 40 mM phosphocreatine, 2.5 μg creatine phosphokinase, 18 μg bovine serum albumin, and 100 μg protein extract in a final volume of 50 μl. They were incubated at 30 °C for 3 h. Repair was stopped by the addition of equal volume of 40 mM EDTA, and 100 mM Tris–HCl (pH 8.0). DNA was extracted with DNA extraction kit (Fermentas) and dissolved in 50 μl of TE buffer. Repair was assessed by the transformation efficiency of the plasmid DNA. Transformation was carried out by adding 20 ng plasmid DNA to 200 μl competent *E. coli*, strain XL-1Blue. Bacteria were plated on Petri dishes with solid LB medium (10 g Bacto tryptone; 5 g yeast extract; 10 g NaCl; and 15 g Bacto agar per liter) containing 100 μg/ml ampicillin or 50 μg/ml kanamycin and grown for 12 h at 37 °C.

3. Results and discussion

3.1. S phase synchronization increases host cell reactivation capacity of HeLa cells

Uniformly labeled with [^{14}C]thymidine exponentially growing HeLa cells were synchronized by treatment with mimosine, a widely used synchronizing agent that blocks the cells at the G1/S phase border and does not inflict DNA damage that is repaired by NER [16]. To follow the progression through S phase, cells were pulse-labeled with [^{3}H]thymidine at 2 h intervals after release from the block. The rate of DNA synthesis was expressed as the ratio of ^{3}H to ^{14}C counts. In parallel, samples were withdrawn for FACS analysis. The ^{3}H/^{14}C ratio steadily increased and reached a maximum at the sixth hour after release of the cells in S phase. The FACS analysis showed that at that moment about 90% of the cells were in S phase, while less than 30% of the cells in the exponentially growing culture were in S phase (Fig. 1A and B). This distribution made the exponential versus synchronized in S phase HeLa cells a suitable model system for studies of the constitutive NER rates, since the percentage of S phase cells in the synchronized population was up to three times higher than in the unsynchronized cells. One hour after release from the mimosine block, HeLa cells were transfected with UV irradiated and undamaged control pEGFP. Seven hours later, a period short enough not to exceed the duration of S phase and sufficient for the accumulation of the green fluorescent protein, the fluorescent cells were counted. Since upon transfection of the host cells with damaged plasmids the *egfp* gene would be expressed only if repaired, the higher the number of fluorescent cells, the higher their repair capacity. The results obtained with the restoration of the transcription of the GFP showed that there was about 30% increase in the repair capacity of the cells

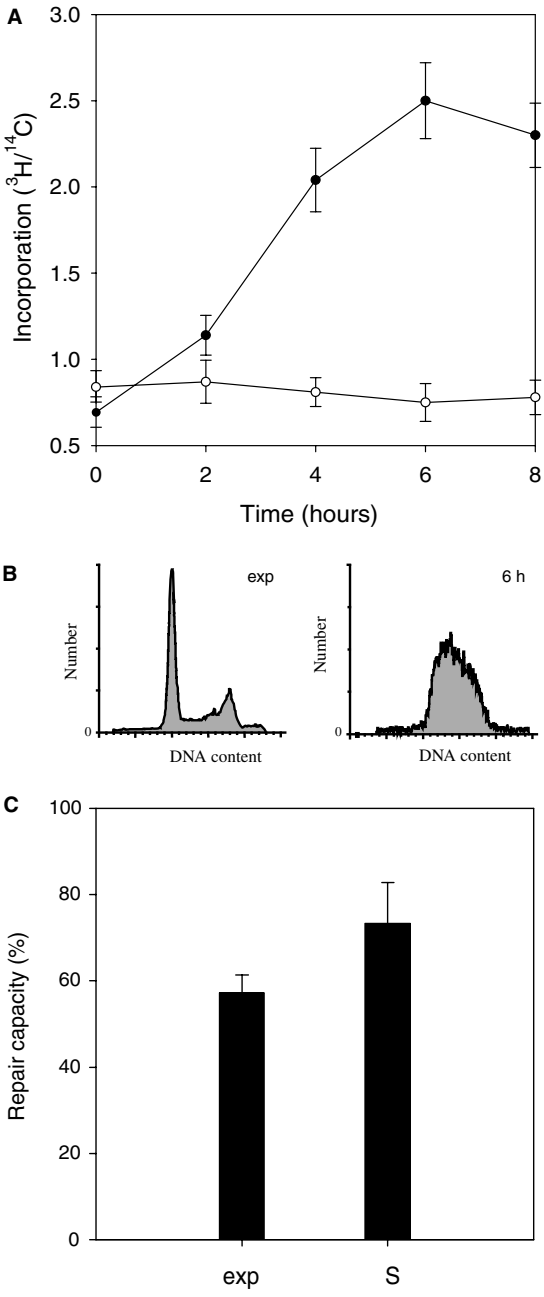

Fig. 1. HCR assay for DNA repair capacity of S phase and exponentially growing HeLa cells. (A) DNA synthesis rates of exponential (empty circles) and synchronized in S phase (filled circles) HeLa cells. Cellular DNA was pre-labeled uniformly with [^{14}C]thymidine for 24 h, the cells were treated with 500 μM mimosine for 24 h, transferred to mimosine free medium and the rate of DNA synthesis at the indicated time points measured by a pulse labeling with [^{3}H]thymidine for 30 min. Incorporation rate is expressed as the ratio of ^{3}H counts of the pulse to the ^{14}C counts of the total DNA. (B) FACS analysis of unsynchronized exponentially growing HeLa cells and HeLa cells at the sixth hour after release from the mimosine block. (C) HeLa cells were synchronized at the G1/S phase border. Synchronized cells and asynchronous cell cultures were transfected with undamaged pEGFP and with pEGFP damaged by UV irradiation as described in Section 2. Seven hours after transfection, the fluorescent cells were counted and repair capacity expressed as percentage of the fluorescent cells observed after transfection with the undamaged plasmid DNA. Legend: exp – repair capacity of exponentially growing HeLa cells; S – repair capacity of HeLa cells synchronized in S phase. Figures are means of five independent experiments. Standard deviations of the means are shown with error bars.

traversing in S phase (Fig. 1C). This increase was statistically significant ($P < 0.01$) and showed that cells remove the damage with increased efficiency during S phase. A similar result (not shown) was obtained when hydroxyurea was used in place of mimosine to synchronize the cells, which means that the effect was not due to the synchronizing agent.

3.2. Protein extracts of cells synchronized in S phase reproduced the increase in host cell reactivation rate

To confirm the results obtained by the HCR assay we applied the cell-free repair system, which is based on the introduction of in vitro damaged DNA into repair proficient protein extracts prepared from the cells whose repair capacity will be assessed. For these experiments, we chose to use K562 cells because they show similar to HeLa cell cycle distribution (54% in G1 and 29% in S phase), but are easily grown in the large quantity needed to prepare the extracts. The extent of repair was determined by the restoration of the colony forming ability of the damaged plasmids after transformation in *E. coli* [19]. Plasmids carrying UV lesions cannot replicate after transformation of *E. coli* in the absence of SOS induction and for this reason do not support colony formation on selective medium [20,21]. This permits to use the transformation efficiency of the irradiated plasmids incubated in protein extracts as a measure for the repair capacity of the respective cells. Transformation of *E. coli* with UV damaged pBlueScript and undamaged pBlueScript incubated in protein extracts isolated from K562 cells and the repair deficient XPA cells showed that the increase of the transformation efficiency of the irradiated plasmids was a result of nucleotide excision repair taking place in the K562 extracts during the incubation (Fig. 2A). To avoid variations due to differences in transformation efficiency and handling of the samples, all experiments were carried out with 1:1 mixture of pBlueScript, which confers ampicillin resistance and undamaged pEGFP-N1, which confers kanamycin resistance and the number of ampicillin resistant colonies was normalized against the number of kanamycin resistant colonies. The repair capacities of the extracts were expressed as percentage of the normalized number of ampicillin resistant colonies, taking the number of colonies obtained after transformation with undamaged pBlueScript as 100%. To avoid differences due to the nuclease activity of the protein extracts, the undamaged control pBlueScript was also incubated in the extracts under the same conditions as the irradiated plasmids. The results are presented in Fig. 2B. Similar to the situation in vivo, S phase extract showed between 40% and 50% increased NER capacity compared with the exponentially growing cells ($P < 0.05$).

A possible explanation for the elevated NER levels in S phase may be the close connection between NER and the process of DNA replication. Thus, DNA polymerases, DNA ligases, RPA, PCNA, and RFC are taking part in both DNA replication and DNA repair [22]. Also, DNA replication proteins are maximally expressed at the G1/S border and in early S phase and DNA repair genes are maximally expressed in middle S phase when the DNA synthesis rate is highest, suggesting that repair is functionally linked to S phase progression [23]. Steady-state levels of mRNA encoding DNA polymerase ε are elevated twofold during the G1/S phase transition [24]. However, such an increase in NER rates could not be expected since NER is taking place in all phases of the cell cycle, while DNA synthesis outside S phase normally does not exist. Here, we directly show that the capacity for NER increased in S phase in comparison with the other phases of the cell cycle. A suggestion could be made that in addition to the mechanisms that ensure induction of repair and cell cycle block when

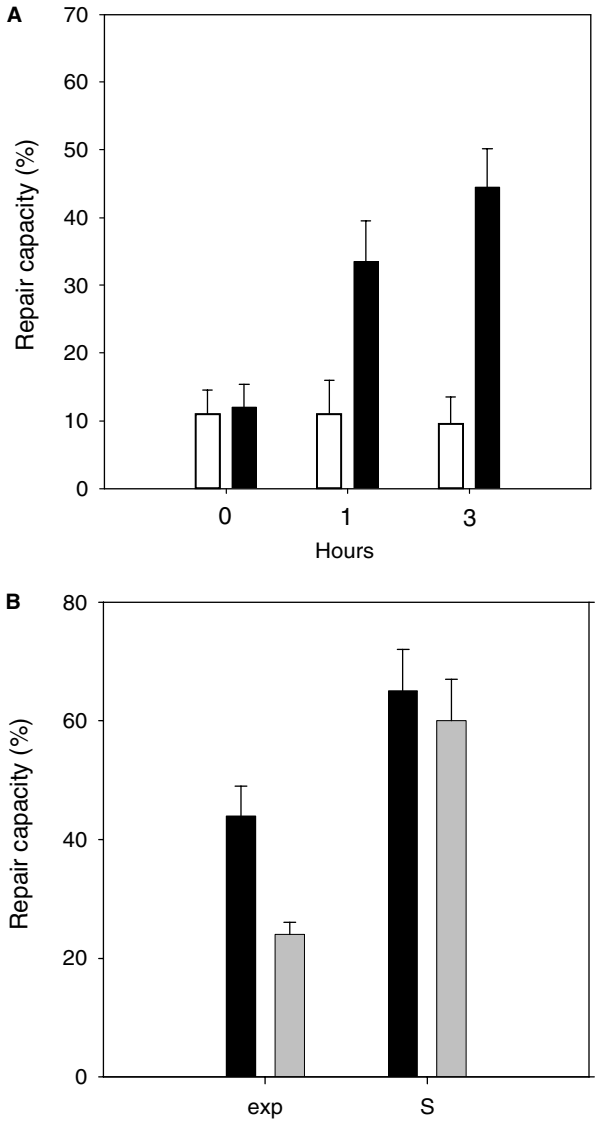

Fig. 2. NER in protein extracts isolated from synchronized in S phase or exponential K562 cells and XPA cells. (A) 1:1 (wt:wt) mixture of UV irradiated pBlueScript DNA and unirradiated pEGFP-N1 DNA was incubated in the repair deficient XPA (empty columns) and K562 extracts (filled columns). At 1-h intervals aliquots were withdrawn, plasmid DNA was isolated and used to transform competent *E. coli* cells, which were then grown on selective media. The number of ampicillin resistant colonies was normalized against the number of the kanamycin resistant colonies, and expressed as percentage from the reaction where both plasmids were not irradiated. (B) 1:1 (wt:wt) mixture of UV irradiated pBlueScript DNA and unirradiated pEGFP-N1 internal control was incubated in K562 extracts. After 3 h the reactions were stopped, plasmid DNA was isolated and used to transform competent *E. coli* cells. The repair capacity of the cells was determined as in (A). In parallel, the rate of DNA synthesis is shown as the ratio of ^{3}H counts from a 30 min [^{3}H]thymidine pulse to ^{14}C counts from 24 h uniform labeling of cellular DNA with [^{14}C]thymidine. Legend: filled columns – repair levels; shadowed columns – DNA synthesis; exp – exponentially growing cells; S – cells collected 6 h after release from G1/S mimosine block. Figures are means of three independent determinations. Standard deviations of the means are shown with error bars.

damage is inflicted, in S phase, when genome stability is most vulnerable, the cells increase their capacity to remove DNA damage.

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