

# DNA repair of pyrimidine dimers and 6-4 photoproducts in the ribosomal DNA

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## ABSTRACT

**The nucleolus is a unique structural component of interphase nuclei where the ribosomal genes, transcribed by RNA polymerase I (RNA pol I), are organized. In the present study, the repair of UV-induced photolesions was investigated in the ribosomal DNA (rDNA) in relation to RNA pol I transcription. We used hamster cells because their repair phenotype permits the separate analysis of the major photoproducts induced by UV light. Immunofluorescent labeling of UV-induced DNA repair and transcription sites showed that the nucleolar regions were deficient in DNA repair despite the presence of abundant RNA pol I transcription foci. Immunological staining indicated that various NER proteins, including TFIIH (subunits p62 and p89), p53, Gadd 45 and proliferating cell nuclear antigen are all enriched in the nuclei but distinctly absent in nucleoli. This lack of enrichment of NER factors in the nucleolus may be responsible for the inefficient repair of photoproducts in the rDNA. UV irradiation generates two major photoproducts, the cyclobutane pyrimidine dimers (CPDs) and the 6-4 photoproducts (6-4 PPs). The repair kinetics of these two lesions were assessed simultaneously by the immunological isolation of bromodeoxyuridine (BudR) containing excision repair patches using an antibody to BudR. We found that the repair of the photolesions was less efficient in the rDNA compared to that of the endogenous housekeeping gene, dihydrofolate reductase (DHFR). Gene specific repair of each of these two photoproducts was then measured separately in the rDNA and in the DHFR gene, which is transcribed by RNA pol II. The removal of CPDs was deficient in the rDNA as compared to the DHFR gene. On the contrary, 6-4 PPs were removed efficiently from the rDNA although somewhat slower than from the DHFR gene. The relatively efficient repair of 6-4 PPs in the rDNA is consistent with the notion that the 6-4 PPs are repaired efficiently in different genomic regions by the global genome repair pathway.**

## INTRODUCTION

The nucleolus is one of the important domains of interphase nuclei where the ribosomal DNA (rDNA) genes of all the chromosomes are clustered and the ribosome assembly takes place (1). In higher eukaryotic cells, the nucleolus lies at or near the nuclear envelope (2). The nucleolus contains RNA polymerase I (RNA pol I) that transcribes the rDNA, topoisomerase I, nucleolin and other proteins (3). The activity of RNA pol I is dependent upon the upstream binding factor (UBF), the promoter selectivity factor, the SL1 (4), and a growth dependent transcription initiation factor (TIF-IA) (5). These factors have been shown to be sufficient for the transcription of rDNA (6).

UV irradiation of cells at 245 nm generates two major photoproducts in the genomic DNA, the cyclobutane pyrimidine dimer (CPD) and the 6-4 photoproduct (6-4 PP). The major photoproduct is the CPD, which constitutes ~80% of the total lesions. Although the 6-4 PP is less frequent (~20% of total lesions), this adduct is of high biological importance and known to be much more mutagenic (7) than the CPDs (8). Earlier studies have shown that the CPDs induced by UV irradiation are removed at a much faster rate from the transcriptionally active DNA than from the overall genome (9). This efficient repair is due to the rapid removal of CPDs from the transcribing strand of RNA polymerase II (RNA pol II) transcribed genes through a transcription repair coupling (TCR) mechanism (10). Previous studies have reported deficient repair of CPDs in the rDNA genes in both hamster and human cells (11,12) suggesting a lack of TCR in genes transcribed by RNA pol I. Fritz and Smerdon (13) demonstrated a lack of strand-specific DNA repair of CPDs in the active chromatin fraction of rDNA in mouse Friend erythroleukemia cells. However, in those studies, the RNA pol I transcription was not assayed. Evaluation of transcription in addition to repair is essential for the detection of correlation between transcription and repair events. Furthermore, the effect of UV irradiation on RNA pol I elongation is not clearly understood. While the repair of CPDs in the rDNA has been studied previously, the repair of 6-4 PP has not yet been characterized in the rDNA.

In this study, we have assessed the repair of UV-induced DNA damage from the rDNA genes in hamster cells. This repair was compared with that of an endogenous essential gene, dihydrofolate reductase (DHFR). We used hamster cells which have certain repair characteristics that make them useful for studies assessing individual photoproducts. We analyzed

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repair of CPDs and of the highly mutagenic 6-4 PPs. This is the first time repair of 6-4 PPs has been assessed in the rDNA. In addition, the repair kinetics of both photoproducts, CPDs and 6-4 PPs were analyzed simultaneously by isolation of bromodeoxyuridine (BudR) containing excision repair patches.

In order to study the correlation between RNA pol I transcription and repair in the rDNA, the focal sites of transcription and repair were localized in intact chromatin isolated under physiological conditions. Sites of repair and transcription are simultaneously labeled in the nuclei after incorporation of appropriate brominated or biotinylated nucleotide precursors. Localization of transcription and repair sites by this immunological technique yields information about the fidelity of RNA pol I transcription after UV treatment.

There is increasing evidence for enrichment and recruitment of various repair and transcription factors to the nucleus and nuclear matrix after DNA damage. However, very little is known as to whether these factors also accumulate in the nucleolus. We have assessed the relative distribution of the tumor suppressor gene p53, subunits of the basal transcription factor TFIID, proliferating cell nuclear antigen (PCNA) and the DNA damage inducible protein Gadd45 in the nucleus and in the nucleolus after UV irradiation. The results show that these factors are abundant in the nucleus but not in the nucleolus after UV. We have also investigated the relative nuclear matrix association of the rDNA, and find that most of it is associated with this structure where the transcription and repair factories are also located. The lack of NER factors may be due to the special chromatin organization in nucleoli and could be an element in the defective repair of CPDs in the rDNA. The differences observed in the processing of 6-4 PPs and CPDs in the rDNA indicate the existence of distinct repair pathways for the repair of these two lesions.

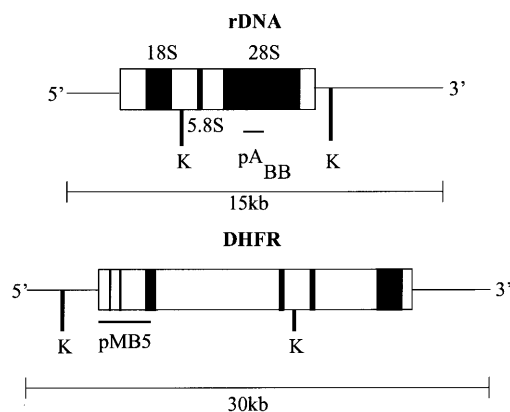
## MATERIALS AND METHODS

### DNA probes

The pMB5 probe originally described by Bohr *et al.* (14) was used to detect the 14 kb *KpnI* fragment of the 5' half of the Chinese hamster ovary (CHO) DHFR gene. The rDNA pABB probe (kindly provided by J. E. Sylvester, University of Pennsylvania, Philadelphia, PA) is a 1.4 kb human 28S rDNA which is homologous to hamster sequence. This DNA probe was utilized to detect the 7.2 kb *KpnI* fragment of rDNA. The genomic maps of the rDNA and DHFR genes as well as the positions of the DNA probes used in this study are shown in Figure 1.

### Labeling sites of DNA repair with BudR in cells

CHO B11 cells were grown in Hams F12 medium supplemented with 10% dialyzed fetal bovine serum and antibiotics. They were grown for 2 days in medium containing 0.5% serum prior to UV irradiation. Cells were irradiated with a UV dose of 20 J/m<sup>2</sup> and incubated with 50 μM BudR and 1 μM fluorodeoxyuridine (FudR) in complete medium for 2, 4 and 8 h after UV. After pulse labeling, cells were trypsinized, washed once in medium and fixed in aceto-methanol (1:3). The fixed cells were placed on acid cleaned slides. The slides were treated with 0.05% pepsin for 15 min at 37°C and washed several times in immunological buffer (IB: Tris 100 mM, NaCl



**Figure 1.** Genomic maps of the rDNA and DHFR genes. The 15 kb region including the rDNA gene and the 30 kb region including the DHFR gene and are shown. The letter K indicates the location of *KpnI* restriction sites. The filled black boxes indicate the exons. The positions of the DNA probes used in this study are shown as black solid lines below the gene.

150 mM, 0.05% Tween 20 pH 7.4). The slides were incubated at 37°C for 15 min in the above buffer containing 5% milk powder (Boehringer). The slides were then washed in IB and 100 μl of the primary antibody (antibromodeoxyuridine mouse IgG, 1:10 dilution in IB; Boehringer Mannheim) were applied and the slides were incubated at 37°C for 1 h. This was followed by three washes of 5 min in IB. Secondary antibody [antimouse IgG fluorescein isothiocyanate (FITC) conjugated; 1:100 dilution in IB; Boehringer Mannheim] was applied to the slides and incubated at 37°C for 1 h. After the incubation, slides were washed in IB three times and dehydrated through 70 and 90% alcohol. The slides were air dried and mounted with Vectashield mounting medium (Vector Lab) containing propidium iodide at a final concentration of 0.1 μg/ml.

### *In vitro* labeling of repair and transcription sites

**Encapsulation and lysis.** 2–4 × 10<sup>6</sup> cells per ml were washed three times in fresh PBS. The cells were encapsulated in 0.5% agarose (15) by mixing 4 vol of the cell suspension with 1 vol of molten agarose (2.5% prepared in PBS) in a conical flask, vortexed for 60 s and swirled many times on ice to form agarose microbeads. The cells in the agarose beads were lysed either with 0.05% Triton X-100 or Streptolysin O (SLO; Wellcome; 5 IU/ml of agarose beads) in a modified physiological buffer (PB). This buffer contains 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 65 mM potassium acetate, 65 mM potassium chloride, 1 mM Na<sub>2</sub>ATP, 1 mM dithiothreitol and 0.2 mM phenylmethyl sulfonyl fluoride (PMSF). The agarose encapsulated cells were incubated with SLO for 30 min at 4°C to allow binding. The cells were washed to remove unbound SLO, resuspended in an equal volume of PB and incubated at 32°C for 2 min to allow permeabilization. The permeabilized cells were washed in PB at 4°C. In case of Triton X-100, the cells were lysed with 0.05% Triton X-100 and incubated on ice for 15 min, washed in PB and processed for *in vitro* reaction.

**Irradiation with UV.** Two ml of encapsulated cells in 10 ml of PBS were irradiated with 40 J/m<sup>2</sup> [effective dose was 20 J/m<sup>2</sup> as agarose shields half of the radiation dose (16) in a 10 cm dish]. The encapsulated cells were briefly washed and incubated in complete medium for 10 min prior to lysis.

#### **In vitro labeling DNA repair and transcription sites**

The reaction was initiated by the addition of 10× concentrated mixture of triphosphates and MgCl<sub>2</sub> to give a final concentration of 0.1 mM CTP, GTP, Br-UTP (for transcription), 0.25 mM dATP, dCTP, dGTP, 25 μM biotin-16-dUTP (for repair), 1 mM ATP and 1.5 mM MgCl<sub>2</sub>. The encapsulated cells were incubated for 30 min at 37°C. In order to distinguish between RNA pol II transcription and that of RNA pol I and III, encapsulated and lysed cells were treated with the RNA pol II inhibitor α-amanitin (50 μg/ml) for 30 min on ice prior to the transcription assay.

#### **Immunological staining**

After the reaction, the encapsulated cells were washed several times to remove unincorporated nucleotides. The nuclear membrane was permeabilized (15 min on ice) in ice cold PB containing 0.5% Triton X-100 for 10 min and subsequently washed in PB (10 times the volume of agarose beads). The cells in the beads were then washed in PBS supplemented with 0.05% Tween 20. Sites containing Biotin were detected with Avidin-FITC (1:1000 dilution in PB, 4 h at 4°C; Sigma). Sites containing Br-RNA were indirectly immunolabeled using a primary antibody raised against BudR-BSA conjugate (anti-BudR mouse monoclonal IgG; 1:100 dilution, 4 h at 4°C, Boehringer Mannheim) which cross reacts only with single-stranded Br-RNA. After incubation with the primary antibody, beads were washed extensively with PB plus 0.05% Tween 20 and incubated with secondary antibody for 16 h at 4°C (sheep anti-mouse IgG Texas red conjugated; 1:500 dilution; Amersham). The encapsulated cells were then washed four times with PB followed by two washes in PBS alone. Vectashield mounting medium (25 μl) containing 0.1 μg/ml of DAPI was mixed with 10 μl of agarose beads and the mixture was placed on a glass slide and covered with a coverslip. Photographs were taken using a Zeiss Axiophot microscope using Kodak color film 400 ASA (100× oil immersion objective).

#### **Isolation of BudR containing repair patches**

**UV irradiation and separation of parental DNA.** CHO B11 cells were pre-labeled with 0.1 μCi of <sup>3</sup>H-thymidine (80 mCi/mmol, Amersham) and 10 μM cold thymidine for 2 days. The cells were then washed in PBS, trypsinized, reseeded and grown for 1 day in isotope-free medium. The procedures for UV irradiation and separation of parental DNA were essentially the same as that of Bohr *et al.* (14). Briefly, cells were irradiated with a UV dose of 20 J/m<sup>2</sup> (254 nm) and some cells were lysed immediately for the 0 h time point after UV irradiation. The cells incubated for different repair time points were grown in medium containing 50 μM BudR and 1 μM FudR. The cells were lysed after 2, 4, 8 and 24 h and the high molecular weight DNA was isolated. DNA (200 μg) from each time point was restricted with either *KpnI* or *EcoRI* (5–10 U/μg of DNA) and the parental DNA was separated from replicated DNA in neutral CsCl gradients.

#### **Immunoextraction and Southern hybridization**

The procedure for the immunoextraction of repair patches was as described by Leadon (17) and Kalle *et al.* (18) with small modifications. Briefly, 3 μg of DNA in 9 μl of TE was denatured with 1 μl of 1 N NaOH (30 min at room temperature). The denatured DNA was neutralized by the addition of NaCl and Tris-Cl pH 7.5 to a final concentration of 150 and 100 mM, respectively. Antibody to BudR (anti-BudR Mouse IgG, 1:50 dilution; Boehringer Mannheim) and 5 μl salmon sperm DNA (11 mg/ml; Sigma) were added to the DNA and this mixture was incubated overnight at 4°C. Incubation with the secondary antibody (biotinylated goat anti-mouse IgG, 1:50 dilution; Boehringer Mannheim) was performed for 4 h at 4°C.

Streptavidin coated magnetic beads were prepared 1 day prior to use. Magnetic beads were washed several times and equilibrated with 150 mM NaCl and 100 mM Tris-Cl pH 7.5 containing 5 mg/ml salmon sperm DNA. 200 μg beads in a total volume of 20 μl buffer were added to the DNA solution. The beads were gently mixed and incubated for 4 h at 4°C. After incubation, the beads containing the repair patches were separated using magnetic particle separator (MPC-E Dynal). The supernatant was carefully collected without disturbing the pellet. The pellet containing the beads was incubated with 0.5 N NaOH for 30 min to release the bound DNA. As a positive control, the same procedure was repeated without the antibodies. Aliquots (2 μl) of both pellet (BudR-containing, bound) and supernatant fractions (BudR-free, unbound) were scintillation counted to determine the percentage of bound DNA. The pellet and supernatant DNA fractions were adjusted to a volume of 100 μl with TBS buffer. The samples were applied on a pre-wet Hybond N-membrane using a slot blot apparatus. DNA was immobilized by vacuum drying at 80°C for 1 h and subsequently hybridized to gene specific probes.

#### **Analysis of repair kinetics**

A small proportion of <sup>32</sup>P counts was observed at the 0 h time point in BudR bound DNA fraction after hybridization with gene specific probes, although scintillation counting of this sample revealed no <sup>3</sup>H counts. Hence these <sup>32</sup>P counts could result from non-specific binding of the probe during hybridization. In order to correct for this non-specific binding, <sup>32</sup>P counts found in the BudR bound (Bo) fraction at the 0 h time were subtracted from both Bo and BudR unbound (UB) fractions at the 2, 4, 8 and 24 h time points. The relative amount of specific sequence enriched in the Bo was calculated by dividing the <sup>32</sup>P counts in the Bo with the total number of counts in both the Bo and UB DNA fractions.

#### **Gene specific repair assay for CPD and 6-4 PP in hamster cells**

The kinetics of removal of CPD and 6-4 PP were determined by following the procedures published earlier from this laboratory (19). Briefly, exponentially growing CHO B11 cells were UV-irradiated with 20 J/m<sup>2</sup>. The cells for the 0 h time point were lysed immediately, whereas those for the later time points were incubated in the culture medium containing 10 μM BudR and 1 μM FudR. The genomic DNA was isolated and restricted with the endonuclease *KpnI*. The parental DNA was separated from the replicated DNA by CsCl gradient centrifugation. For the detection of pyrimidine dimers in the parental DNA, 4 μg

aliquots of the DNA sample from each time point were treated with T4 endonuclease V or mock treated with endonuclease buffer alone. The samples were resolved on 0.5% alkaline agarose gels followed by Southern transfer and hybridization as previously described (19). Intensities of the bands representing full-length fragments were quantitated by densitometric scanning or by direct analysis of the support membranes on a PhosphorImager. The relative intensities of parallel bands representing full-length fragments were compared. Assuming the random distribution of CPDs in a homogenous population of DNA fragments, the adduct frequency per DNA fragment was determined by Poisson distribution (19).

The procedure for the induction and repair of 6-4 PPs was essentially the same as described by May *et al.* (19). CHO B11 cells were irradiated with 40 J/m<sup>2</sup> for the determination of 6-4 PP repair analysis. The parental DNA was isolated and restricted with the endonuclease *KpnI*. The DNA isolated from each time point was treated with photolyase and light which splits the pyrimidine dimers into monomeric form. The remaining non-dimer photoproducts were mainly 6-4 PPs. Samples treated with photolyase were preincubated at room temperature in the dark in the buffer containing 50 mM Tris–Cl pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 mM  $\beta$ -mercaptoethanol and 0.25  $\mu$ g photolyase/ $\mu$ g of DNA. The samples placed in a small reaction tube were irradiated in a monochromator at 405 nm for 30 min at room temperature. The photoreactivated DNA, purified by extraction with organic solvents, was divided into three parts. One part was reacted with T4 endonuclease to verify the presence of CPDs. The second part was reacted with ABC excinuclease and the third part was used as a control. All three samples were run parallel on an alkaline agarose gel, transferred to a nylon membrane and hybridized to <sup>32</sup>P labeled gene specific fragments. The conditions for treatment with ABC excinuclease were the same as described previously (19). Highly purified preparations of excinuclease UvrA, B and C were kindly provided by Dr A. Sancar (University of North Carolina School of Medicine, Chapel Hill, NC).

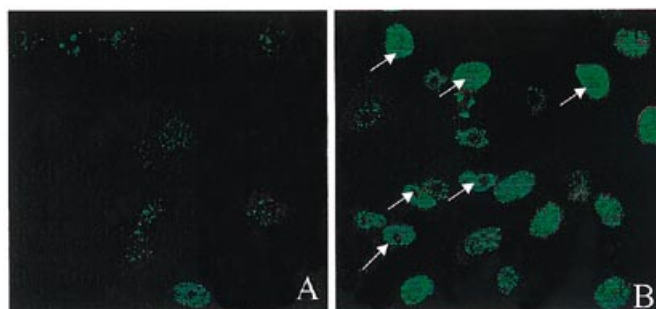
### Accessibility of rDNA

25–50  $\times 10^6$  cells were used for the preparation of loop and matrix DNA. The procedure was essentially the same as that of Dijkwel *et al.* (20). Different concentrations of loop and matrix DNA (100, 200, 500 and 1000 ng) in 100  $\mu$ l of TE were heat denatured and cooled on ice. The samples were then slot blotted onto Hybond N membrane (Amersham). The filters were hybridized to <sup>32</sup>P-labeled DNA probes. Conditions for hybridization and post-hybridization washings were the same as described previously (21). Filters were then exposed to Kodak X-Omat film at –80°C for 1–2 h. The autoradiographs were scanned using a densitometer.

## RESULTS

### Localization of DNA repair sites with antibodies to BudR

In order to localize the UV-induced repair sites in the interphase nuclei and the nucleolus, an indirect immunofluorescent technique involving BudR was used. The cells were irradiated with UV and incubated in the presence of BudR for 2, 4 and 8 h to allow the repair to occur. The BudR incorporated repair sites

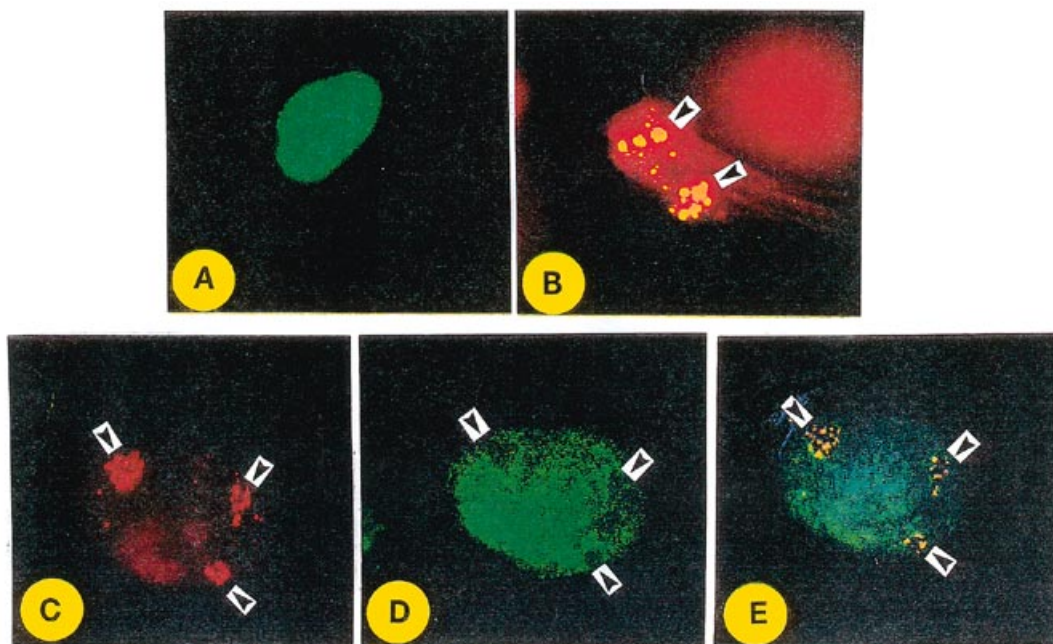


**Figure 2.** Labeling sites of repair in interphase nuclei of CHO B11 cells by antibody to BudR. Non-irradiated control cells showed no labeling (A). Note the homogeneous distribution of repair foci in cells pulse labeled with BudR for 2 h immediately after UV irradiation (B). Arrows indicate the nucleolar regions.

were detected with a FITC conjugated secondary antibody. Non-irradiated control cells did not show any repair labeling with antibodies to BudR (Fig. 2A). The majority of UV-irradiated cells (85–90%) showed bright repair labeling (green fluorescence) except in the nucleolar regions (indicated by arrows). In cells pulsed for 2 h with BudR immediately after UV, repair labeling was found homogeneously throughout the nucleoplasm (Fig. 2B) except in the nucleolar regions. BudR pulse labeling for different post UV incubation (2, 4 and 8 h) times enabled us to follow the progression of repair events within the interphase nuclei. However, the nucleoli were consistently found to be devoid of any repair labeling at all the time points analyzed after UV irradiation (data not shown).

### Immunological localization of DNA repair and transcription sites in chromatin

We next used a quasi *in vitro* chromatin assay to determine the correlation between the DNA repair and transcription sites after UV irradiation in the nuclear and the nucleolar regions. This technique involves the use of brominated and biotinylated nucleotide precursors and the incorporated nucleotides were indirectly detected by antibodies. In non-irradiated cells, transcription foci were found predominantly in the nucleoplasm (Fig. 3A). The transcription foci were stained with avidin-FITC and hence the foci appeared as green fluorescence. Several hundreds of distinct nascent RNA pol II spots were observed in each cell. The nascent RNA sites were compared with the relative distribution of DNA visualized by DNA-specific fluorochrome, DAPI (data not shown). Nascent transcripts were observed in regions of both low and high DNA concentration. When cells were treated with  $\alpha$ -amanitin, an inhibitor of RNA pol II, the transcription pattern was distinctly different. RNA pol II transcription was completely abolished in the nucleoplasm while distinct RNA pol I transcription foci (10–20) were observed in the nucleoli (Fig. 3B). The RNA pol I transcription sites were labeled with avidin-FITC. In order to show the DNA distribution, the cells were stained with propidium iodide and photographed using an Omega multi-fluor filter. The overlap of green fluorescence (FITC) and red (propidium iodide) gave a yellowish color of RNA pol I transcription sites in the nucleolar region. This active ribosomal transcription pattern in the presence of  $\alpha$ -amanitin was very

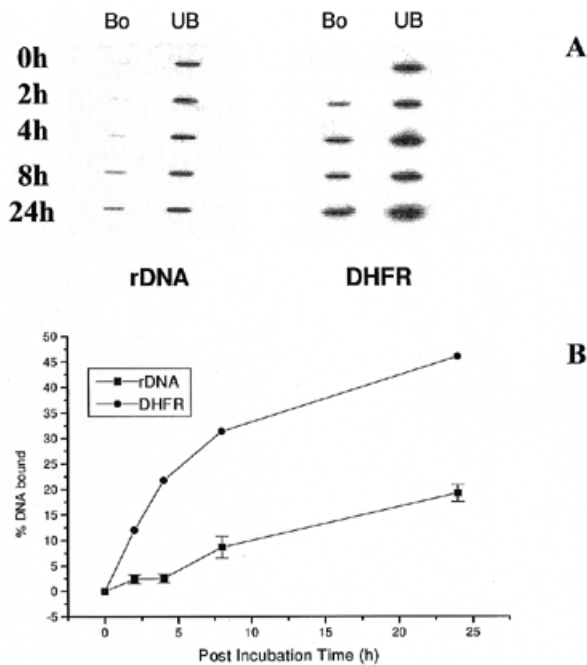


**Figure 3.** Visualization of transcription and repair foci in intact chromatin of B11 cells isolated under physiological conditions. Encapsulated cells in  $G_0$  were irradiated with  $20 \text{ J/m}^2$  of UV permeabilized with SLO and incubated with biotin 16-dUTP (DNA repair) and Br-UTP (transcription). Sites containing incorporated biotinylated and brominated nucleotides were immunolabeled with avidin-FITC (repair) and anti-BudR antibodies (transcription). (A) Non-irradiated cell showing bright transcription sites distributed uniformly. Note the enrichment of RNA pol I transcription foci in nucleoli regions of  $\alpha$ -amanitin treated cell (B). In UV treated cells, transcription elongation by RNA pol II is greatly reduced while 10–20 bright transcription foci are found in nucleoli (C). Note the absence of repair foci in nucleoli regions exhibiting transcription foci (D). The photograph of the same cell showing the DNA distribution with DAPI staining (E). Arrows indicate the nucleolar regions.

similar to the transcription pattern seen in UV-irradiated cells (Fig. 3C). After UV exposure, the intensity of extra nucleolar labeling of RNA pol II transcription was greatly reduced while bright transcription foci were found at the nucleolar regions (Fig. 3C). The RNA pol II transcription foci were not completely abolished in UV treated cells (Fig. 3C) as they were in the  $\alpha$ -amanitin treated cells (Fig. 3B), but were much smaller and less intense than the transcription foci in the nucleoli (Fig. 3C). The repair foci were completely absent in this region (compare the bright spots marked by arrows in Fig. 3C with the regions in Fig. 3D) despite the presence of bright transcription foci in the nucleoli. The DNA staining pattern of the same nucleus with DAPI showed that the transcription foci were found mainly in regions with high DNA concentration after UV irradiation (Fig. 3E). The patterns of transcription and repair labeling were carefully analyzed in more than 100 cells. In the nucleoplasm, the majority of the transcription and repair sites were found to co-localize. In the UV-treated cells, distinct RNA pol I transcription foci were found in the nucleoli while the intensity of RNA pol II sites were much less than in non-irradiated cells. The nucleolar labeling of transcription found in the UV-treated cells was very similar to that of  $\alpha$ -amanitin treated cells. It was very difficult to visualize the RNA pol I transcription foci without blocking RNA pol II transcription with either  $\alpha$ -amanitin or UV as previously reported by Jackson *et al.* (22).

### Repair in rDNA genes

*Enrichment of rDNA in BudR containing excision repair patches.* In order to determine the efficiency of rDNA gene repair of both photoproducts (CPDs and 6-4 PPs) we used an immunoextraction assay which separates the repaired DNA from the total genomic DNA. This procedure involves the use of BudR, which is incorporated in the place of thymidine during the excision repair synthesis after UV exposure. The scheme for isolating the BudR containing repair patches is described in detail in the Materials and Methods section. A number of pilot experiments were carried out to optimize the concentration of antibodies and Streptavidin coated magnetic beads. The extraction was found to be optimal at an antibody dilution of 1:50 (data not shown). The same concentration was used for both primary (anti-BudR) and secondary (biotinylated goat anti-mouse IgG) antibodies. Streptavidin coated magnetic beads were used at a concentration of  $200 \mu\text{g}$  in  $20 \mu\text{l}$  of TBS buffer per reaction. Omission of either primary or secondary antibody failed to separate the repair patches. Using the optimal concentrations of antibodies and beads, 18–20% of the total DNA was separated as BudR containing repair patches. This percentage of isolation of repair patches from the total DNA is in good correlation with the results of earlier studies using other assays to measure the kinetics of overall genome repair (14). The efficiency of immunoextraction was assayed by immunoblotting of the bound Bo and unbound UB DNA fractions using alkaline phosphatase conjugated antibodies to

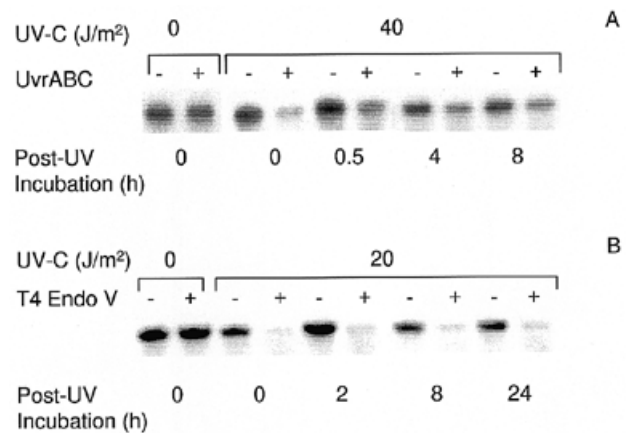


**Figure 4.** (A) Comparison of the relative enrichment of the rDNA and DHFR specific sequences in the BudR containing (bound, Bo) and non-BudR containing DNA (unbound, UB) fractions at different times after UV treatment. Note the reduced binding of the rDNA to the repair patches during the first 4 h. The numbers indicate the time points after UV treatment. (B) The percentage of rDNA and DHFR sequences enriched in the isolated BudR containing DNA fractions was calculated by dividing the  $^{32}\text{P}$  counts in BudR bound fraction (Bo) with the total number of counts in both the Bo and UB DNA fractions. The symbols represent the average values of three independent determinations.

BudR. Only the pellet fraction containing the Bo DNA gave a positive signal to alkaline phosphatase detection.

The efficiency of repair of UV-induced photo lesions was determined by measuring the relative enrichment of a given DNA sequence in the BudR containing repair patches. Representative slot blots hybridized with the *DHFR* and the rDNA genes are shown in Figure 4A. The average values of rDNA enriched in the Bo and UB fractions obtained from three independent determinations are shown in Figure 4B. Southern hybridization analysis showed enrichment of only 1–2% of rDNA at 4 h after UV irradiation. The percentage of rDNA in the pellet fraction gradually increased to 8 and 17% after 8 and 24 h, respectively. The relative percentages of *DHFR* and rDNA genes found in the repair patches are given in Figure 4B. The repair of rDNA genes was much less efficient than that of the *DHFR*, which showed up to ~46% enrichment of repair label after 24 h of UV treatment.

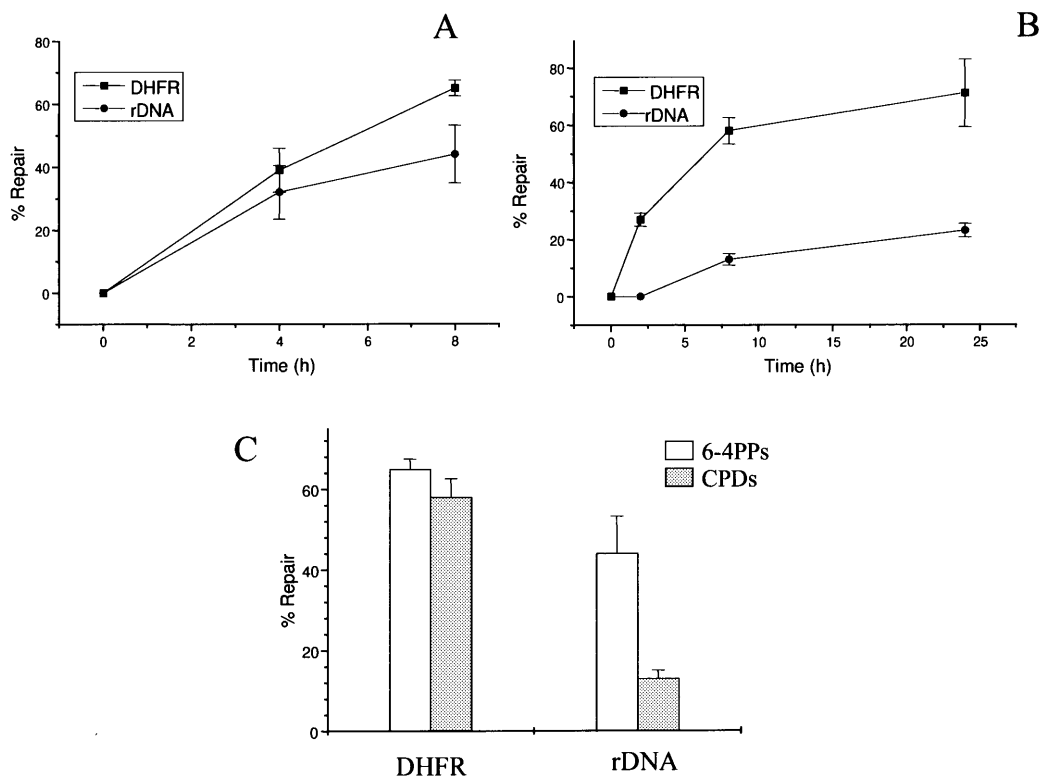
*Gene-specific repair analysis of CPDs and 6-4 PPs in rDNA.* As the repair patches generated during the repair of CPDs and 6-4 PPs are of similar size, it is difficult to distinguish between the repair of these two lesions detected by the immunoextraction assay. Therefore, the repair of each of the two photo-products was assayed separately. The repair rates of CPDs and



**Figure 5.** Representative autoradiographs of 6-4 PP (A) and CPD (B) removal at different post incubation times after UV from the rDNA genes in hamster cells. The cells were irradiated with 40 and 20 J/m<sup>2</sup> of UV-C irradiation and the DNA was isolated at different post incubation times. The DNA was purified in two rounds of CsCl gradient centrifugation to remove the replicated DNA. The repair kinetics of CPD and 6-4 PP are assayed as described earlier (19).

6-4 PPs were determined at different post-incubation times in the rDNA using the procedures that were published earlier from this laboratory (19). CHO cells were irradiated with 20 and 40 J/m<sup>2</sup> for the determination of CPDs and 6-4 PPs, respectively. The initial frequency of CPDs per 10 kb DNA was 1.03 for rDNA and 1.28 for the dihydrofolate reductase (*DHFR*) gene after a UV dose of 20 J/m<sup>2</sup>. The initial frequency of 6-4 PP for 10 kb DNA was found to be 1.05 and 0.93 for rDNA and *DHFR* genes, respectively, after a UV dose of 40 J/m<sup>2</sup> under our experimental conditions. This suggests that the frequency of 6-4 PP is ~30% of total lesions introduced after 40 J/m<sup>2</sup> UV irradiation. The representative autoradiographs for the removal of CPDs and 6-4 PPs from the rDNA genes are shown in Figure 5A and B. The quantitation of the autoradiographic signals showed that the repair of CPDs is defective in the rDNA compared to the *DHFR*, 17 versus 70% after 24 h (Fig. 6B). In contrast, the removal of 6-4 PPs is only slightly less effective in the rDNA than in *DHFR*. The repair of 6-4 PPs from rDNA is much more efficient than that of CPDs (Fig. 6A). Comparison of the relative removal of both 6-4 PPs and CPDs after 8 h of UV irradiation indicates that the repair of both photo products is efficient in the *DHFR* gene while the repair of CPDs is much less efficient than that of 6-4 PPs in the rDNA (Fig. 6C).

*Distribution of rDNA in loop and matrix DNA.* Fractionation experiments were carried out to determine the relative accessibility of rDNA in higher order chromatin organization. Loop and matrix associated DNA fractions were separated by digestion of nuclei with *EcoRI* and extracted with either 2 M NaCl or 10 mM lithium diiodosalicylic acid (LIS). LIS extracts a majority of histone and non-histone proteins from the nuclei in a low salt buffer containing 0.125 mM spermidine (20). Nuclear matrix associated DNA (MAD) was found to constitute 5–7% of the total genomic DNA. Slot blot analysis of loop and matrix DNA showed that 67% of the rDNA was found in



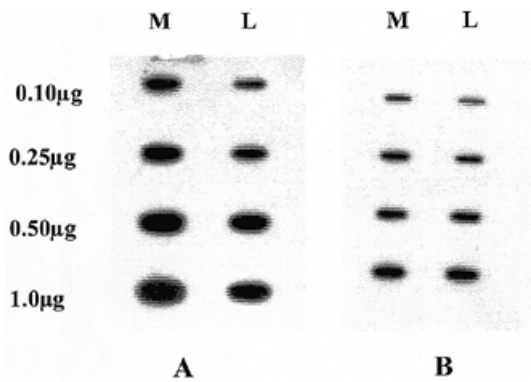
**Figure 6.** The percent repair of 6-4 PP (A) and CPD (B) from the rDNA and DHFR genes in hamster cells at different post incubation times. Bars indicate the standard error of the mean of three independent determinations. (C) Histogram showing the relative removal of 6-4 PP and CPDs from the DHFR and rDNA genes 8 h after UV irradiation.

the insoluble matrix fraction. Extraction of the chromatin with another protocol using LIS rather than NaCl yielded similar results indicating that this enrichment of rDNA was not an experimental artifact (Fig. 7A and B). The sequential hybridization of the same filter with the probe for the *DHFR* gene showed that ~20% of the *DHFR* gene sequences were attached to the nuclear matrix. This indicates that the enrichment of rDNA in the matrix fraction is ~3-fold higher than that of the *DHFR* gene.

*Immunostaining of repair and transcription factors in the nucleus and nucleolus.* The relative distribution of various factors directly or indirectly involved in DNA damage processing was next analyzed to determine their relative abundance in the nucleus and the nucleolar regions. The subunits of TFIIH (p62 and p89), p53 and Gadd45 were all enriched in the nucleus after UV irradiation, but distinctly absent or undetectable in the nucleolus (data not shown). The protein PCNA, which is an important component in the repair resynthesis step of NER, was specifically enriched after UV damage in the form of bright fluorescent foci in the nucleus of quiescent cells (Fig. 8B). In the non-irradiated cells, PCNA staining was found to be much less intense (Fig. 8A). However, the nucleoli regions were found to be devoid of any PCNA staining after UV damage.

## DISCUSSION

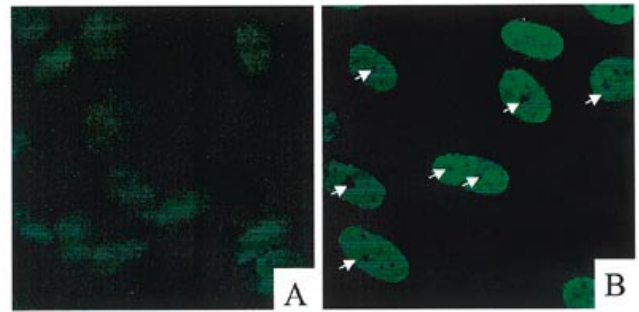
In this study, we have attempted to determine the correlation between RNA pol I transcription and DNA repair of photo lesions in the rDNA genes by immunofluorescent and gene specific repair assays. Although there have been previous assessments of repair in the rDNA, it has never been assayed in conjunction with the analysis of transcription. Earlier studies have dealt only with the repair of CPDs, while the fate 6-4 PP, which is more mutagenic than CPD, has not been examined in the rDNA. Our immunofluorescent results indicate that there is efficient RNA pol I transcription but minimal repair of photo-products in the nucleoli. In this study, we have used a quasi *in vitro* assay for measuring repair and transcription for two reasons. First, labeling *in vivo* with brominated and biotinylated precursors is impractical, as endogenous NTP and dNTP pools may greatly affect the uptake. Second, the rate of elongation is so rapid that many transcripts will be completed during several minutes required to give detectable labeling. Therefore we labeled the transcription sites *in vitro* after permeabilizing the cells with either Triton X-100 or Streptolysin O. Jackson *et al.* (22) using a similar assay have recently shown that essentially all RNA polymerases active *in vivo* remain active *in vitro*. It is also well established that the *in vitro* system we have utilized in the present study is analogous to nuclear run-on and is unable to initiate but only can elongate the transcripts that are initiated before permeabilization. The



**Figure 7.** Enrichment of rDNA genes in the matrix fraction. Chromatin extracted with 2 M NaCl was digested with *EcoRI*. Different concentrations of fractionated loop (L) and matrix (M) DNA were slot blotted and hybridized with the rDNA probe to determine the relative enrichment of sequences in the two fractions. Matrix DNA shows 1.5–2-fold enrichment of rDNA genes as compared to loop fraction (A). Hybridization patterns of the same filter were shown with the DHFR gene (B).

elongation step alone is labeled in this assay by pulse labeling with Br-UTP. Therefore it is unlikely that the transcripts we detect after UV irradiation are made before UV irradiation. Under the same irradiation conditions, we found that the RNA pol II elongation is greatly reduced in a dose dependent fashion with UV irradiation. Our observations indicate that the elongation of RNA pol I is relatively unaffected by UV compared to RNA pol II elongation. The pattern of nucleolar transcription observed after UV irradiation is very similar to that obtained after treatment of cells with  $\alpha$ -amanitin. In contrast to nucleolar regions, nuclear regions comprising RNA pol II transcribed genes show greatly reduced transcription but abundant repair sites after UV. The gene specific repair assays show that both photoproducts are repaired less efficiently in the rDNA than the *DHFR* gene. Interestingly, the repair of 6-4 PP, which probably does not require transcription coupling, is much more efficient than the repair of CPDs in the rDNA.

In the present study, we could not detect BudR repair labeling in nucleolar regions after a UV dose of 20 J/m<sup>2</sup>. Although 6-4 PPs seem to be removed very efficiently from rDNA, the frequency of 6-4 PPs induced by this UV dose was insufficient to visualize the repair sites. The molecular weights of the antibodies used for transcription and repair assessment in our assays are similar. Hence, the absence of repair labeling in the nucleoli is not due to the limited accessibility of antibodies used for repair detection. Several studies have demonstrated that rDNA is deficient in the repair of CPDs in both rodent and human cells (11,12). Using strand-specific probes, Christians and Hanawalt (12) demonstrated the lack of TCR of CPDs in the rDNA of Cockayne syndrome (CS) (complementation groups A and B) and Xeroderma pigmentosum (XP) (complementation group C) cells. Fritz and Smerdon (13) analyzed the repair of CPDs in the transcriptionally active and inactive fractions of the rDNA separated on the basis of psoralen binding. They found a lack of TCR in the rDNA genes of mouse Friend erythroleukemia cells. However, the repair deficiency of the rDNA is not universal as yeast cells can efficiently remove the CPDs from their rDNA (23). In contrast



**Figure 8.** Immunological analysis of PCNA distribution in control (A) and UV treated cells (B). The cells were grown on a coverslip, irradiated with 20 J/m<sup>2</sup> UV and fixed in ice cold methanol after 2 h. The slides were stained with a primary antibody (mouse IgG1a) to PCNA and a secondary FITC conjugated antibody to mouse IgG1a. Arrows indicate the nucleolar regions.

to the repair of CPDs, Stevnsner *et al.* (11) have shown that in hamster cells, intrastrand adducts induced by MMS are efficiently repaired in the rDNA genes. MMS forms simple alkylation lesions in DNA, which are largely repaired by base excision repair (BER). This suggests that the repair deficiency in rDNA is limited to bulky lesions removed by NER. An exception to this statement, however, is that the removal of cisplatin induced interstrand crosslinks is efficient in rDNA (11). These complex lesions are thought to be removed by a combination of excision and recombination repair pathways. Thus, rDNA does not appear to be deficient in recombinational DNA repair.

The frequency of lesions induced by different agents appear to vary in the rDNA (11) as compared to other regions of the genome. The overall organization of chromatin and the sequence complexity may influence the induction of DNA damage. As compared to the DHFR, fewer CPDs are induced in the rDNA after UV exposure, and the greater GC content of the rDNA could also be a possible explanation. In corroboration with our earlier study (11), analysis of isolated repair patches (Fig. 7) showed very reduced repair of the rDNA sequences. This assay does not discriminate between the removal of CPD and 6-4 PP, and measures repair of both photolesions. In the present study, we observed only 2% binding of the rDNA to the repair patches as compared to 22% of the *DHFR* probe during the first 4 h (Fig. 7). These results are quite consistent with the analyses of CPD and 6-4 PP repair using the gene specific repair assay for each individual photoproduct. The results of immunoextraction correlate well with the T4 endonuclease analysis of CPDs, although the percentage of the DHFR gene bound to the isolated repair patches was comparatively less than that of the repair assayed by T4 endonuclease method. The repair of photolesions in the rDNA genes measured by the immunoextraction assay was slightly more efficient than the repair of CPDs measured by gene specific repair assay. The difference in repair rates observed between the immunoextraction and the gene specific repair assay could in part be due to different doses of UV. Also, the repair patches generated by the removal of 6-4 PPs and CPDs in GC-rich regions of the genome would be expected to be low in BudR due to the low thymidine content. The rDNA is



relatively GC rich. The low level of rDNA binding observed during the early hours after UV may be due to the poor BudR extraction of these GC rich regions with reduced thymidine content. Using a similar approach, Kalle *et al.* (18) extracted 60% of the total DNA containing repair patches in human cells. On the contrary only 17–20% of the total DNA is isolated from hamster cells. This difference is expected since hamster cells are deficient in overall genome repair of CPDs.

The lack of TCR of CPDs in the rDNA may be attributed to the enzymatic differences between RNA polymerases I and II as well as to their differential location in the nucleus. There are many copies of rDNA in mammalian cells. If only a fraction of the rDNA genes are transcribed, it would probably explain the lower repair efficiency of CPDs in the rDNA. However, Conconi *et al.* (24) have shown that majority of the rDNA genes are transcribed in exponentially growing cells. As cells in exponential growth phase were used in the present study, the lack of transcriptional activity is probably not the cause of the lack of repair of CPDs. In mammals, transcription by RNA pol II requires the co-ordinated action of seven accessory proteins for accurate initiation of transcription (25). TFIIF is the most complex of all these proteins with many diverse enzymatic activities (26). The efficient strand specific repair of genes transcribed by pol II is due to the association with basic transcription factor TFIIF that plays dual roles in both transcription and repair (10). While this large multi-subunit complex is required for RNA pol II transcription as well as for DNA repair, only three components, RNA pol I, UBF and the promotor selectivity factor (SL1) are required for transcription of rDNA (6). No evidence has been obtained yet for the association of TFIIF or the NER proteins with RNA pol I. In corroboration with this, we find that immunostaining of nuclei with antibodies to different repair proteins and transcription factors show homogeneous distribution in the nucleoplasm, but no presence in the nucleolar regions of both control and UV-treated cells. The lack of enrichment of NER factors in rDNA might be responsible for the reduced repair of CPDs in the rDNA as the repair factors from the nucleoplasm have to diffuse slowly through the membrane into the nucleolus. Other structural changes in rDNA or the nucleoli that affect the regulation of DNA repair cannot be ruled out.

Recent studies showed that the 6-4 PPs are removed equally efficiently from all over the genome without any bias to the transcriptionally active genes (19,27). It thus appears that 6-4 PPs are repaired without transcription coupling, and are repaired via the general genome repair pathway. There have been many debates as to whether or not the repair of 6-4 PPs and CPDs requires the same enzymatic machinery. The differences observed between the removal of CPDs and 6-4 PPs in the rDNA raise the possibility that these two lesions are processed by distinct repair pathways. Some cell lines of human and rodent origin substantiate this possibility where the repair capacity of one of the two major photoproducts is found to be much more efficient than the other, indicating that different pathways can be used for 6-4 PP and CPD removal. The hamster cells have proficient global repair of 6-4 PPs but the removal of CPDs is confined only to the transcribed strand of active genes. In hamster UV 61 cells, which belong to rodent complementation group 6 (homolog of human CS group B cells) the 6-4 PP repair pathway is intact while the CPD repair pathway is severely impaired (28). Furthermore, an XP-

A revertant cell line displays proficient 6-4 PP repair without any detectable level of repair of CPDs (29). In corroboration with these experimental evidences, the present study indicates that the excision repair capacity may vary for 6-4 PP and CPD in the rDNA.

A technical difficulty in detecting the induction and repair of 6-4 PPs is that a higher dose of UV-C irradiation (40 J/m<sup>2</sup>) than the one used for CPDs (10–20 J/m<sup>2</sup>) is required to generate enough lesions for detection. It is well known that the active RNA pol II elongation is important for TCR pathway in normal cells. Venema *et al.* (30) have shown that TCR of CPDs in the adenosine deaminase (ADA) gene is efficient in normal cells after a UV dose of 10 J/m<sup>2</sup> while the TCR is completely abolished in the ADA gene at 30 J/m<sup>2</sup>. This shows that at higher UV dose, like the one used for 6-4 PPs detection, RNA pol II elongation is impaired affecting the TCR pathway. Hence the efficient repair of 6-4 PPs observed at higher UV dose is most likely due to the fact that the global repair pathway overrules the TCR pathway. To determine whether there is 6-4 PP removal via TCR, a sensitive gene specific assay using the low dose of UV must be developed.

The 6-4 PP is an important determinant of the lethal and mutagenic effects of UV irradiation in biological systems (7). The removal of this mutagenic lesion appears to correlate well with early DNA repair responses of mammalian cells including incision, repair resynthesis and the removal of replication blocks. Considering the mutagenic potential of 6-4 PP, the integrity of 6-4 PP repair pathway may be critical for preventing the mutational load that may affect the maintenance of rDNA and the ribosome assembly in the nucleolus. Further studies are required to understand the mechanisms responsible for the differential processing of 6-4 PPs and CPDs in the rDNA.

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