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QUARTERLY

Comparison of repair activity in different genomic regions*°

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We have developed a quantitative technique to determine repair activity at defined genomic regions. Cells were treated with hydroxyurea to inhibit the replicative DNA synthesis and were incubated with 5-bromodeoxyuridine (BrdUrd) to label the regions undergoing repair. In the course of the labelling, the regions that were more actively repaired would incorporate more BrdUrd than the regions that were less actively repaired. Thus the kinetics of BrdUrd incorporation in the different sequences would reflect the kinetics of reparation of the respective regions. The total BrdUrd-containing, repaired DNA was isolated by immunoprecipitation with anti-BrdUrd antibody, and after controlled sonication, it was used as a template in quantitative PCR in which the amount of the product was directly proportional to the amount of template. This approach was used to address the question whether DNA repair after UV irradiation occurs in an uniformly random manner, or with preferences for certain regions. We found that, in Ehrlich ascites tumor cells, the repair efficiency was higher at the 5′ end of the mouse β-globin domain than in the rest of the domain.

DNA reparation is one of the fundamental processes in the living nature. During recent years its enormous importance for the life of the cells, and for life at large has been recognised and it has become one of the most intensively investigated topics. Thanks to the efforts of many laboratories its complexity as well as its links to the other DNA metabolic pathways become increasingly elucidated. In particular, it has become clear that the process of DNA reparation is closely coupled to the process of DNA transcription in that the transcriptionally active genes are in many cases repaired preferentially [1–4] and that transcription and repair machineries share many common factors [5–8].

On the other hand, the relationship of DNA repair with another fundamental pathway of

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Abbreviations: BrdUrd, 5-bromodeoxyuridine; EAT, Ehrlich-Lettre ascites tumor; LCR, locus control region; NER, nucleotide excision repair; SDS, sodium dodecyl sulphate.

DNA synthesis, i.e. DNA replication, has been rarely discussed. There is strong evidence that such relationship does exist since quiescent human lymphocytes are not able to repair their DNA and they acquire this ability only in parallel with the ability to replicate DNA after stimulation with phytohemagglutinin [9]. In eukaryotic cells DNA is organised in the form of loops of 50–300 kb on the average, anchored to a nuclear structure called nuclear matrix [10,11]. Each such DNA loop represents a functional replication unit called replicon. There are well defined sites, or regions within each replicon, called origins of replication, where replication begins [12–15]. Two replication complexes (replisomes) are formed at each origin and they move in opposite directions replicating DNA outward from the origin untill they reach the ends of the replicon. Activation of groups of origins is controlled simultaneously which permits large eukaryotic genomes to complete replication in the course of a single S-phase [16, 17].

On the other hand, little is known about the mechanism of the reparative synthesis of DNA. This is rather surprising since repair is quite massive and under certain conditions comparable to the replicative DNA synthesis. Do there exist, similarly to replicons, certain functional units of reparation that could be called "reparons", do they have a structural equivalent similar to the DNA loops, are there DNA reparation origins, and do reparosomes, once formed, move along DNA as replisomes do? In an attempt to obtain an idea about the pattern of DNA repair in contagious DNA fragments topologically representing single loops we decided to study the repair rates of several adjacent regions of DNA in UV-irradiated cells. As a model we used the mouse β-globine gene cluster and a modification of the technique previously successfully used by us [15] and by others [18, 19] to follow the movement of the replication forks along DNA. In this method based on culturing the cells in the presence of BrdUrd (5-bromo-2′-deoxyuridine) for different time intervals and determining the amount of BrdUrd incorporated in the different DNA regions. The DNA that has incorporated BrdUrd as a result of repair was isolated by immunoprecipitation and used as a template in quantitative PCR to determine the relative abundance of the different sequences.

In this way we were able to demonstrate that there was a well defined zone located at the 5′ end of the β-globin cluster, which was repaired at a higher rate than the rest of the domain.

MATERIALS AND METHODS

Cells. Hyperdiploid Ehrlich-Lettre ascites tumor (EAT) cells were maintained *in vivo* in albino mice. They were transiently cultured in suspension *in vitro* in Minimum essential Eagle medium (Sigma) supplemented with 50 mM Hepes/NaOH, pH 7.2, and heparin (0.2 units/ml).

Ultraviolet irradiation and preparation of DNA. Cells were placed in Petri dishes as 3 mm layers and were irradiated with a germicidal lamp with maximum emission at 254 nm at a flow of 0.08 $J \cdot m^{-2} \cdot s^{-1}$ in an ice bath. Hydroxyurea was added to a final concentration of 10 mM immediately after irradiation and the cells were incubated for 1 h to inhibit DNA replication. BrdUrd (Sigma) and [*methyl*-^{3}H]thymidine ([^{3}H]dT, 87.7 Ci/mmol, Du Pont) were added to the medium to 50 μM and 20 μCi/ml final concentrations, respectively, and cells were incubated at 37°C for 4 h, still in the presence of hydroxyurea, to label the repaired DNA. After labelling the cells were washed with ice-cold phosphate-buffered saline (pH 7.3) and lysed in 1% SDS, 50 mM Tris, pH 8.0, 1 M NaCl, 50 mM EDTA. Proteins were digested with 100 μg/ml Proteinase K (Sigma) at 37°C overnight. DNA was deproteinized with phenol/chloroform (1:1, v/v) and with chloroform, and recovered by ethanol precipitation. DNA was dissolved in 10 mM Tris, 1 mM EDTA, pH 8.0, and soni-

cated with an ultrasonic homogenizer (Cole-Parmer) in an ice bath for various time intervals to give preparations of desired average fragment length.

Sonicated DNA was mixed with CsCl to obtain a solution with a density of 1.70 g/ml, and was centrifuged in a Beckman VTi65 rotor at 45000 r.p.m. at 20°C for 24 h. Gradients were fractionated and counted. The residual peak of the "heavy-light", newly replicated DNA was discarded and the fractions containing bulk genomic DNA were pooled together and dialysed against 10 mM Tris, 1 mM EDTA, pH 8.0.

BrdUrd-containing, repaired DNA was isolated by immunoprecipitation as described by Burhans *et al.* [13] with modifications; 200 μg of DNA was heat-denatured and incubated with 5 μl of monoclonal anti-BrdUrd antibody (Sigma) at room temperature for 1 h in 0.14 M NaCl, 10 mM sodium phosphate buffer, pH 7.2, 0.05% Triton X-100. Then 50 μg of rabbit anti-mouse IgG antibody (20 μl of antiserum, Sigma) was added and the incubation continued for 1 h at room temperature and overnight at 4°C. The precipitate was collected by 5 min centrifugation in Eppendorf microcentrifuge at 14000 r.p.m. and dissolved in 50 mM Tris, pH 8.0, 1 M NaCl, 10 mM EDTA. SDS and Proteinase K were added to final concentrations of 0.5% and 500 μg/ml, respectively, proteins were digested at 37°C for 3 h and extracted with phenol/chloroform (1:1, v/v) and chloroform. $MgCl_2$ was added to 10 mM concentration and the repaired DNA was recovered by ethanol precipitation.

Quantitative PCR. The sequence of the mouse β-globin gene cluster determined by Shehee *et al.* [20] was used for primer design. Eight oligonucleotides (Microsynth) from the region of the mouse β-globin gene cluster were used as 4 pairs of PCR primers. Primer sequences were as follows: TTA TCT GAA ACC TGC AGG GAC (forward primer of pair A, positions 10779–10799 in Shehee *et al.* [20], GCT CCC TTT CAA AAT CGT GTC (reverse primer of pair A, positions 11452–11472), TTA TGT TGG CCC CTC ATT CAG (forward primer of pair B, positions 26234–26254), AAA CTT CTC TTC CAG GGT AGG (reverse primer of pair B, positions 27230–27250), ACA AGT AAT CTG ATG GAG GTT (forward primer of pair C, positions 35276–35296), ACT AAA TGG CAA TCT GGA GTC (reverse primer of pair C, positions 35962–35982), AGC ACT TCA CAG TTC TCA AGC (forward primer of pair D, positions 39971–39991), CAC AAC TAC CTT TAT GGG TCC (reverse primer of pair D, positions 40768–40788).

PCR reaction mixtures contained 8 pmol each of the two primers, 100 μM each of the four dNTPs, 1 × *Taq* DNA polymerase reaction buffer (Stratagene, 10 mM Tris/HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1%, w/v, gelatin and other stabilisers), 1 unit of *Taq* DNA polymerase (Stratagene) and various amounts of control or repaired DNA. Thermal cycling was carried out in a GeneE thermal cycler (Techne) with a paraffin overlay as follows: 23 to 40 cycles at 94°C for 1 min, annealing temperature for 1 min, and 72°C for 1 min, with an initial denaturation step of 3 min at 94°C. The optimal annealing temperature for primer pair A was 62°C, for pair B – 64°C, for pair C – 60°C, and for pair D – 64°C.

For quantification of the reaction a master mix was prepared that contained all reaction components except the template. It was distributed into individual tubes and various templates were added to the tubes.

Labelling of DNA, agarose gel electrophoresis, blotting and hybridization were performed as described by Sambrook *et al.* [21].

RESULTS

The general approach developed in the present paper to assay DNA repair is based on the assumption that each repair event results in the synthesis of a short DNA fragment. If the damaged cells are cultured in the presence of BrdUrd, the precursor would incorporate into DNA at the sites of repair. If a given sequence

is repaired more actively than another, a higher percentage of the DNA fragments containing this sequence would incorporate BrdUrd compared with the fragments containing the other sequence which is repaired less effectively. Thus in the population of BrdUrd containing DNA fragments, the ratio between actively and less actively repaired DNA sequences will differ from this same ratio in the genome in that the actively repaired sequences will be overrepresented. The proposed approach is schematically depicted in Fig. 1. We used this approach to analyse the

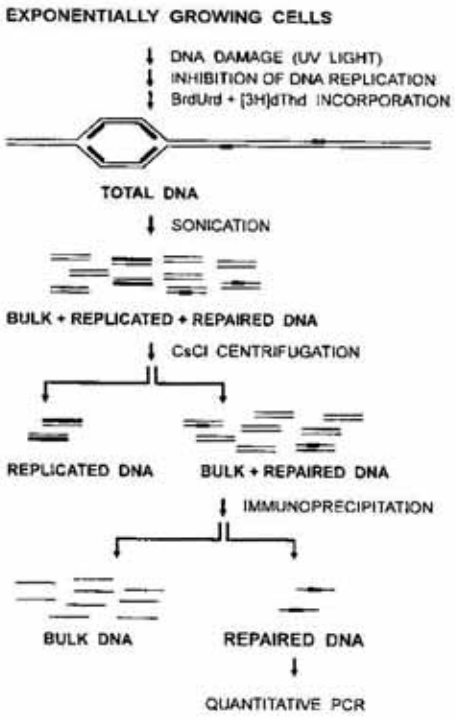

Figure 1. General scheme of the repair assay.

repair pattern in the mouse β-globin gene cluster in EAT cells after irradiation with UV light. The mouse β-like globin genes are clustered in the order *5′-y-bh0-bh1-bh2-bh3-b1-b2-3′* (Fig. 2), comprising approximately 60 kb of DNA on chromosome 7 [20, 22]. During development the different genes are transcribed in a stage-specific- and tissue-specific manner, i.e. *y* and *bh1* are embryonic genes whose transcription is confined primarily to the yolk sac and foetal liver, *b1* and *b2* are expressed in erythroid cells in the bone marrow of adult animals, while *bh0, bh2* and *bh3* are β-like pseudogenes. The locus contains members of different repetitive families. The transcription of the individual genes is controlled by multiple control regions located 5′-, inside, and 3′- of the genes and the activity of the locus is controlled by a locus control region situated 5′- of the *y* gene. In agreement with transcription being restricted to erythroid tissues, specific DNase I hypersensitive sites are found in erythroid cells and are not present in nonerythroid cells [23, 24]. We chose to use as probes four unique DNA sequences of approximately 1 kb each, positioned 3′ of *y* (probe A), between *bh2* and *bh3* (probe B), between *bh3* and *b1* (probe C) and 3′ of *b1* (probe D) (Fig. 2). EAT cells were irradiated with a germicidal lamp with emission maximum at 254 nm for 2 min. UV irradiation is assumed to randomly damage genomic DNA and it was estimated that 2 min irradiation corresponds roughly to a dose of 10 $J \cdot m^{-2}$. There are data that such a dose would cause approximately 1 lesion/10 kb [25]. Cells were treated with 10 mM hydroxyurea for 1 h after the irradiation to inhibit the semiconservative DNA synthesis and were cultured with BrdUrd and [^{3}H]dT in the presence of 10 mM hydroxyurea for 4 h. The BrdUrd-containing, repaired DNA was isolated as described in Materials and Methods and in Fig 1. and used as a template in PCR.

Critical for the approach is the fragmentation of genomic DNA, since on the one hand very short fragments band poorly in CsCl gradients and are poor templates for PCR, but on the other hand, using longer fragments would compromise the accuracy of the mapping. We carried out a series of reactions to assess the effect of the length of DNA template on the results of quantitative PCR. To this end mouse genomic DNA was fragmented by sonication and fractions with decreasing average length were used as templates in parallel PCRs. Our

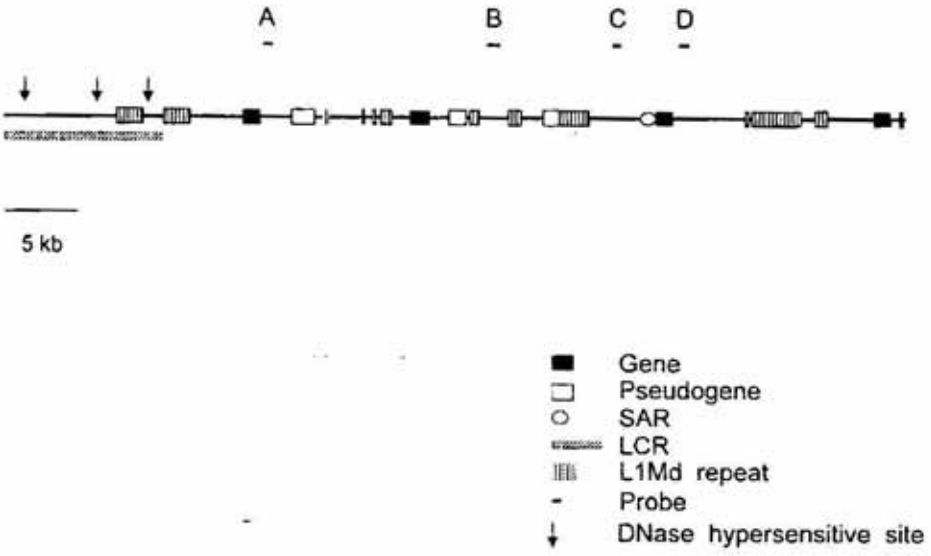

Figure 2. Physical map of the mouse β-globin gene domain.

The PCR generated probes are shown on top and are designated A, B, C and D.

results showed that the product to template ratio remained constant at the template DNA length decreasing down to about 2 kb, after which it began to decrease. Since 2 to 4 kb is a reasonable length for both CsCl density gradient fractionation and mapping, in the experiments described in this paper we used DNA fragmented by sonication to between 2 and 4 kb.

Quantitative PCR have been used to map DNA replication origins [26], to detect amplified oncogenes [27], to quantify HIV-1 proviral DNA [28], to identify gene deletions [29], etc. PCR follows the theoretical course of exactly doubling DNA at each cycle, up to a limit. The boundaries within which the reaction is quantitative depend on the experimental conditions, the type of template and even on the type of thermal cycler. The successful application of this technique depends critically on the number of cycles. We carried out experiments with total genomic mouse DNA as a template using the four pairs of primers we have designed and found out that the reaction was quantitative up to 28–30 cycles when using 50 ng of template DNA. In the experiments described in this paper we used 25 cycles. We found that in this case the template to product ratio remained constant over a broad range of template concentrations (Fig. 3). To determine the PCR products we ran aliquots of the reactions mixtures on agarose gel, transferred DNA to nylon membrane and hybridised the membrane with DNA probes labelled *in vitro* with ^{32}P representing the same PCR products purified by electrophoresis. Signals were scanned and quantified and it was estimated that the standard deviation of the results obtained in three independent experiments by this method did not exceed 30% for any of the used DNA sequences. Computer assisted search showed that there were no unexpected deviations from the average in the frequency of pyrimidine dinucleotides in the vicinity of the probes. The results showed that there was increased repair activity at the 5′ end of the mouse β-globin gene cluster (Fig. 4).

DISCUSSION

Nucleotide excision repair is an essential pathway for removing bulky base modifications from DNA. This mechanism involves endonucleolytic cleavage at two phosphodiester

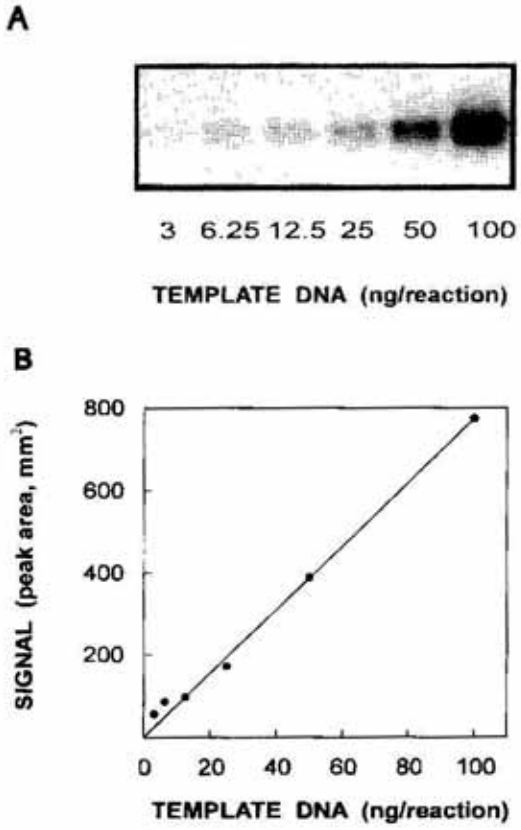

Figure 3. Template/product relationship of PCR.

Panel A. Different amounts of sonicated genomic DNA were used as templates in PCR to amplify sequence A (25 cycles). DNA was blotted and hybridised with the labelled *in vitro* fragment A. Panel B. Hybridisation signals in panel A were quantified by scanning with Quick Quant II Auto Scanner (Helena Laboratories) and plotted against template DNA.

bonds, one 3′ and the other 5′ of the site of damage, followed by excision of the DNA damage as the component of a single stranded fragment [8]. The excised fragment is replaced by DNA repair synthesis and finally DNA continuity is re-established by ligation. In mammalian cells, the major sites of incision are at the 5th phosphodiester bond 3′ and the 24th phosphodiester bond 5′ to the lesion. Thus, whatever the character of the damaging agent, the net result is the *de novo* synthesis of a 29 nucleotide long DNA fragment. The uniformity of the repair process opens the possibility to determine the repair rates by directly comparing the amounts of labelled precursors incorporated into different DNA fragments.

In the present paper we studied the DNA repair pattern in a 60 kb domain of mouse DNA, containing the entire β-globin cluster, after exposure to UV. This was done in an attempt to gain insight into the pattern of DNA repair over a DNA region comparable in length with the matrix attached DNA loops, and most probably representing one such loop itself. This domain was chosen because it is not transcribed in nonerythroid cells, its primary structure is known and it has been well characterized. We envisaged that by extrapolation we would be able to map the sites within the loop where repair synthesis first begins, i.e. to map the origins of DNA reparation as we have done in the past with the origins of DNA replication [15]. We worked with EAT cells in which β-globin genes were not expressed and in this way any observed difference of the rate of repair could not be attributed to the so called "transcription-coupled repair". We did not find any significant differences in the repair rates over the entire cluster with the exception of the 5′-most DNA region, which was repaired with higher efficiency. We are not able to offer a firm explanation for the observed elevated repair rate at the 5′ end of the mouse β-globin gene cluster. The role of the different levels of chromatin structure in repair has been discussed in the literature. Thus it has been reported that lesions in the linker DNA are repaired with priority over lesions in the nucleosome cores [30, 31]. Higher order structure also can play a role in repair efficiency. Several papers have reported enhanced repair activity of some types of damage in the nuclear matrix DNA of humans [32, 33]. Terleth *et al.* [34] have also reported that the difference in repair rates between the closely related HMLα and MATα loci in yeast resided in their different chromatin structure. However, at present it is not clear whether the proximity of a DNA sequence to the nuclear matrix results in its preferential repair, or alternatively, the proximity of a DNA sequence to the transcription complex located at the nuclear matrix is responsible for its enhanced re-

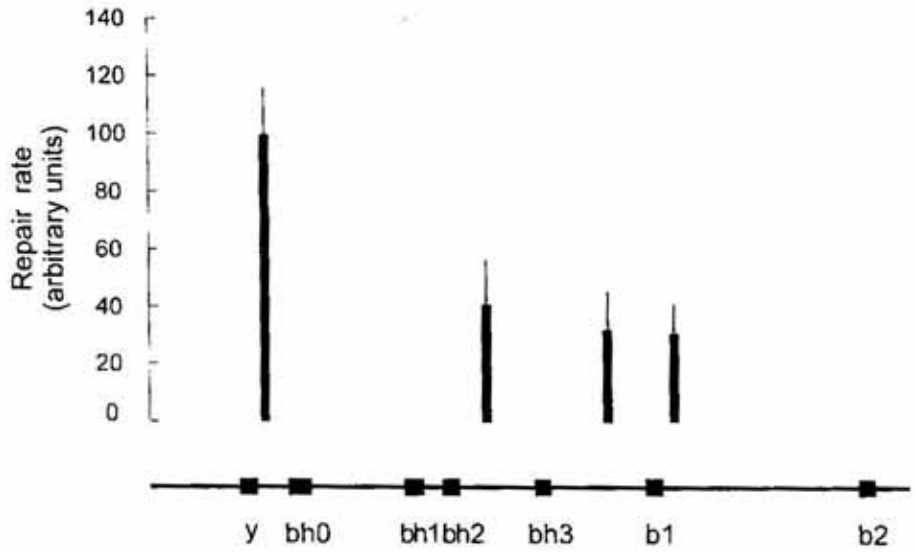

Figure 4. Repair efficiency in the different zones of the mouse β-globin gene.

EAT cells were irradiated with UV light for 2 min to receive 9.6 J/m^2 and allowed to repair for 4 h in the presence of 10 mM hydroxyurea. The repaired DNA was isolated as described in Materials and Methods. PCR were carried out with 50 ng of repaired DNA as template, using as primers amplifying sequences A, B, C and D, respectively. Parallel PCR were carried out with each pair of primers using various amounts of genomic DNA as a template to prepare calibration curves as in Fig. 3. The signals were quantified against these curves and expressed in arbitrary units taking the averaged signal obtained with primer pair A as 100. They are expressed as columns placed on top of the physical map of the region at the positions of the respective probes. The results are mean of three experiments and the standard deviations are shown by error bars on top of the columns.

pair regardless whether the sequence is transcribed or not. There are some data that the β-globin cluster topologically represents a single DNA loop anchored at the nuclear matrix. The area of higher repair activity detected by the novel BrdUrd method described in this paper lies close to the 5′ end loop anchor site. This could mean that the transcription-independent repair preferentially occurs at repair factories localized on the nucleoskeleton.

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