Repair of UV-induced pyrimidine dimers in the individual genes *Gart*, *Notch* and *white* from *Drosophila melanogaster* cell lines

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ABSTRACT

The excision repair of UV-induced pyrimidine dimers was investigated in three genes: *Gart*, *Notch* and *white* in a permanent Drosophila cell line *Kc*, derived from wild type *Drosophila melanogaster* embryonic cells. In this cell line *Gart* and *Notch* are actively transcribed, whereas *white* is not expressed. In all three genes UV-induced pyrimidine dimers were removed with the same rate and to the same extent: 60% removal within 16 hours, up to 80 – 100% in 24 hours after irradiation with 10 or 15 J/m² UV. These kinetics are similar to the time course of dimer removal measured in the genome overall. No difference in repair of the inactive *white* locus compared to the active *Gart* and *Notch* genes was found. Similar results were obtained using a different wild type cell line, *SL2*, although repair appeared to be somewhat slower in this cell line. The results are discussed with respect to the data found for gene specific repair in other eukaryotic systems.

INTRODUCTION

The role of Drosophila DNA repair pathways in mutagenesis is investigated in repair proficient and deficient flies by measurement of several genetic endpoints. Over the years many mutants have been described that are sensitive to UV and show an impaired excision repair pathway. Whereas evidence is accumulating in mammalian cells and yeast that the kinetics of excision repair of UV-induced damage is not uniform over the genome, no information concerning this aspect of DNA excision repair is available in Drosophila.

Studies in mammalian cell systems suggest that transcriptionally active genes are repaired preferentially. In UV-irradiated Chinese hamster cells pyrimidine dimers are more efficiently removed from the constitutively expressed housekeeping gene *dihydrofolate reductase* (*DHFR*) than from upstream, non-coding sequences and the bulk DNA (1,2). Preferential repair of this gene was also observed in human cells (3). In mouse *3T3* cells the active proto-oncogene c-*abl* is efficiently repaired whereas most of the dimers persist in the transcriptionally silent c-*mos* gene (4). In both primary and *simian virus 40* (*SV40*)-transformed human skin fibroblasts the housekeeping genes adenosine deaminase (ADA) and *DHFR* are repaired at a faster rate and to a greater extent than the non-transcribed locus *754* (5). In addition, evidence for preferential repair of actively transcribed genes has also been found in the lower eukaryote *Saccharomyces cerevisiae*. In this yeast strain the active mating type locus *MATα* is faster repaired compared to the identical inactive *HMLα* locus (6).

To investigate whether preferential repair forms part of DNA excision repair in Drosophila, the removal of UV-induced pyrimidine dimers was measured in the genes *Gart*, *Notch* and *white* in two diploid immortalized cell lines derived from embryonic cells of repair proficient (wild type) *Drosophila melanogaster*: *Kc* (7) and *Schneider's 2* (*SL2*; 8). The housekeeping gene *Gart* and the *Notch* gene provide examples of transcribed genes, whereas *white* was selected as an inactive gene.

MATERIAL AND METHODS

Cell culture and prelabelling of the cells

The *Kc* (7) and the *SL2* cell line (8) were kindly provided by Prof. Echalier (Université Pierre et Marie Curie, Paris). *Kc* and *SL2* cells were cultured at 24°C in D22 medium and Schneider's Drosophila medium (Gibco, Paisley Scotland) respectively, supplemented with 5% fetal calf serum (FCS; Gibco, Paisley Scotland) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (9,10). Routinely, cell populations were grown in 50 ml culture flasks (Nurtingen and Greiner, FRG) to high density and subcultured once a week (1:3) for the *Kc* cells and once every 10 days (1:2) for the *SL2* cells.

Prior to irradiation, cells were prelabelled with 0.1 μCi/ml ^{3}H-thymidine (100 mCi/mmol). After three days the medium was replaced by fresh label free medium and cell growth was continued for 16 hr. In the experiments on gene-specific repair, cells were transferred 1:1 to petri dishes (94 mm in diameter; P_{94}, Nurtingen and Greiner, FRG), whereas for the overall repair experiments, cells were seeded in P_{35} petri dishes (Nurtingen and Greiner) at the same density. Repair experiments with growing cells were started 24 hr later in case of the *Kc* cells and 4 days later in case of the *SL2* cells.

Repair of UV-induced pyrimidine dimers at the gene level was measured according to the procedure described by Bohr et al. (1).

Determination of T4 endonuclease V sensitive sites (ESS) in specific restriction fragments

At the time of irradiation the medium was removed and kept for later use at room temperature. The cells were washed with FCS free medium and irradiated using a Philips T.U.V. lamp (predominantly 254 nm) at a dose rate of 0.2 W/m^2. After irradiation, the cells were handled in yellow light to prevent photorepair: they were either lysed immediately or incubated for various periods of time in the dark, in the original medium supplemented with 10 μM bromodeoxyuridine (BrdU) and 1 μM fluorodeoxyuridine (FdU). BrdU/FdU is added to allow the separation of parental density DNA from newly synthesized, dimer free, DNA. Prior to lysis, cells were washed two times by pelleting with serum free medium. The lysate was incubated for 16 hr at 37°C with proteinase K and SDS at final concentrations of 150 μg/ml and 0.5% respectively. The DNA was extracted with phenol and chloroform. Nucleic acids were precipitated with ethanol, washed with 70% ethanol, dissolved in TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA) and incubated with 50 μg/ml RNase A for 1 hr at 37°C. After precipitation and resuspension in TE the DNA was restricted with the appropriate restriction enzyme, as indicated in the results, using digestion conditions recommended by the manufacturer and centrifuged to equilibrium in CsCl density gradients. Gradients were fractionated. Fractions containing parental density DNA were pooled and dialysed against TE. Based on specific radioactivity two 5 μg fractions of each DNA sample were taken: one was incubated with T4 endonuclease V, isolated according to Nakabeppu et al. (11) and the other with reaction buffer only. After electrophoresis in an alkaline agarose gel, the DNA was transferred to Hybond-N$^+$ (Amersham Internationalplc., Amersham, UK). Filters were hybridized at 42°C for 40 hr in a solution containing 40% formamide, 5×SSPE, 5×Denhardt, 1% SDS and 100 μg/ml denatured salmon sperm DNA using genes-specific probes labelled with α-^{32}P-dATP by nick translation (Amersham kit). After hybridization, the filters were washed 2 times with 2×SSPE, 1% SDS and exposed to Fuji X-ray films at −80°C using an intensifying screen. Band intensities were quantitated using a Biorad Video Densitometer Model 620. The average number of ESS per fragment was calculated from the densities of full-length fragments.

Determination of T4 endonuclease V sensitive sites in total cellular DNA

Removal of UV-induced pyrimidine dimers from total cellular DNA was measured by alkaline sucrose gradient centrifugation (12). The procedure, based on that of van Zeeland et al. (13) for mammalian cells is briefly as follows: Irradiated cells were washed by pelleting with cold Puck's saline A (150 mM NaCl, 5 mM KCl, 5 mM glucose, 40 mM $NaHCO_3$, 0.5 mM EDTA, pH 6.7) and the total amount of cpm was estimated. After a second washing step with Puck's saline A, the cells were washed with Low Salt buffer (LS; 10 mM Tris, 0.1 M NaCl, 10 mM EDTA, 1 mg/ml bovine serum albumin, pH 8.0) and resuspended in LS to a concentration of approximately 1000 cpm/μl. The cells (2 μl) were permeabilized by adding 2 μl High Salt buffer (HS; 10 mM Tris, 3.9 M NaCl, 10 mM EDTA, 1 mg/ml bovine serum albumine, pH 8.0) with 0.1% Triton X-100. After incubation on ice for 10 min the samples were diluted with 76 μl Neutral Salt buffer (NS; 10 mM Tris, 10 mM EDTA, 1 mg/ml bovine serum albumin, pH 8.0) and subsequently incubated for 15 min at 37°C with 1 μl T4 endonuclease V. The cells were lysed at 4°C by adding 30 μl lysis solution (3% sarkosyl, 10 mM Tris, 10 mM EDTA, pH 8.0). After denaturation with 100 μl 1 M NaOH (at least 1 hr), the samples were layered on 5 ml 5−20% linear alkaline sucrose gradients containing 2 M NaCl, 0.3 M NaOH and 10 mM EDTA and centrifuged in a SW 50 rotor at 20°C; unirradiated cells 120 min at 30 krpm (7×10^{10} rad^2/s) and irradiated cells up to 40 krpm and 16×10^{10} rad^2/s. The gradients were fractionated onto strips of Whatman No 17 filter paper, washed in 5% cold TCA, ethanol and acetone and dried. Radioactivity was assayed in a liquid scintillation counter. The average molecular weight of the single stranded DNA in each sample was calculated from the radioactivity profiles (14) and the frequency of ESS was estimated using the relationship:

$$\text{ESS} = 1 / \text{M(treated)} - 1 / \text{M(untreated)}.$$

Northern blot analysis

Cytoplasmic RNA was isolated by NP-40 lysis (15). The poly(A)$^+$ RNA fraction was selected using oligo (dT)-cellulose chromatography according to Maniatis et al. (16) and analyzed after Northern blotting onto Hybond-N$^+$ (Amersham International plc., Amersham, UK). Gene-specific fragments were labelled with α-^{32}P-dATP by nick translation (Amersham kit).

DNA probes

Southern analysis: A pEMBL 18+ plasmid, containing a genomic *XbaI* partial restriction fragment that includes the entire *Gart* gene and a few hundred bases of flanking sequences, was kindly provided by Dr. S.Henikoff (Fred Hutchinson Cancer Research Center, Seattle). A set of genomic clones, spanning the complete *Notch* gene, was a gift of Dr. S.Kidd (The Rockefeller Institute, New York). A genomic *white* clone was obtained from Dr. A.Pastink (Sylvius Laboratories, Leiden). The specification of the probes is shown in Figure 1.

Northern analysis: The 10.5 kb *XbaI* fragment of the genomic *Gart* clone mentioned above (Dr. S.Henikoff, Fred Hutchinson Cancer Research Center, Seattle), the 8.8 kb *HindIII* fragment of a genomic clone containing coding sequences from the 3′ end of the *Notch* gene (Dr. S.Kidd, The Rockefeller Institute, New York) and a 2.3 kb genomic partial *SalI white* fragment isolated from pGAw3 (17).

RESULTS

Transcriptional activity of the genes Gart, Notch and white in the Kc and the SL2 cell line

To investigate whether preferential repair of active genes exists in Drosophila, removal of UV-induced pyrimidine dimers was measured from three genes: *Gart*, *Notch* and *white*. Repair experiments were performed in two cell lines, *Kc* and *SL2*, derived from repair proficient (wild type) *Drosophila melanogaster* embryos. To determine the transcriptional status of the genes, poly(A)$^+$ RNA was isolated from both cell lines and analyzed by Northern blot hybridization. The results are shown in Figure 2.

The 10 kb *Gart* locus produces a primary transcript which is processed into a mature 4.7 kb mRNA encoding a protein with

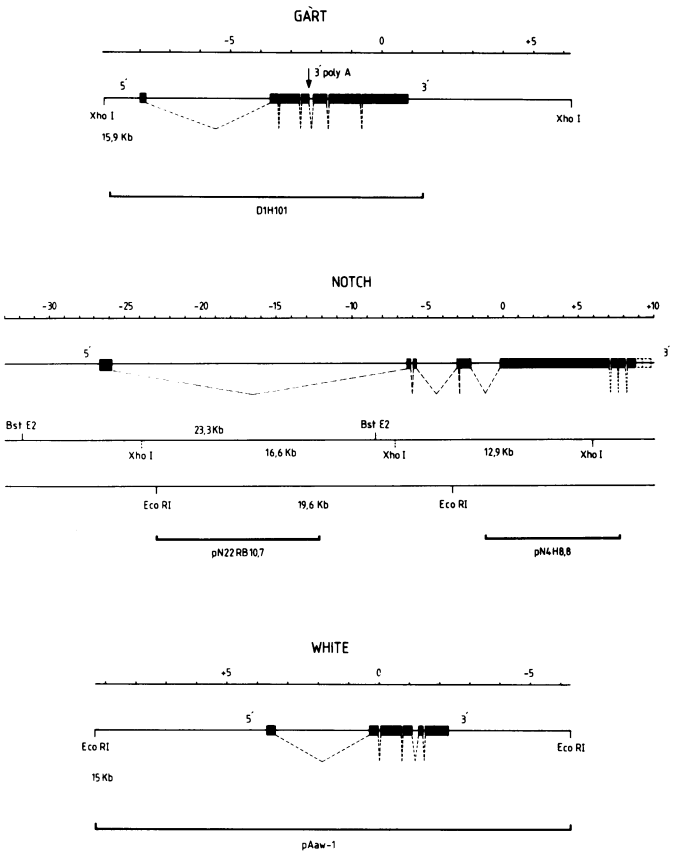

Figure 1. Molecular organization of the loci *Gart*, *Notch* and *white*. Filled boxes represent exons of the genes. The size in kb is indicated on the upper solid line. Genomic probes, matching the relevant genomic restriction fragments, are indicated by solid lines. The *Gart*, *Notch* and *white* maps were derived from ref. 20, 28 and 36 respectively.

three different enzymatic activities in the *de novo* purine biosynthesis pathway: phosphoribosyl-glycinamide formyltransferase (GART), phosphoribosyl-glycinamide synthetase (GARS) and phosphoribosyl-amidine cyclo-ligase (AIRS). Alternative processing of the primary transcript results in a 1.7 kb mRNA that codes for a smaller protein possessing only GARS activity (18,19). Expression of both transcripts was established in *Kc* and *SL2* cells. The 0.9 kb mRNA transcribed from the *cuticle* gene which is situated entirely within the first intron in the opposite strand of the *Gart* gene (20,21), was only found in adult flies. The complex *Notch* locus (22−26) corresponds to a region of approximately 38 kb that is transcribed as a single transcription unit and spliced into a major mRNA of 11.7 kb (27−30). Recently it has been shown that the distribution of this transcript coincides with a 300 kDa, EGF-like, glycosylated surface protein (31,32). The 11.7 kb *Notch* mRNA was found in *Kc* cells and adult flies.

In *SL2* cells the *Notch* function is apparently abolished due to a genomic rearrangement in the 5'region of the gene. In these cells transcription of *Notch* sequences does occur: an altered, presumably non-functional, transcript is produced (pers. comm. S.Artavanis-Tsakonas). Mutations at the *white* locus affect the degree of pigmentation of the adult eye, the ocelli, the testis sheath and the larval and adult Malpighian tubuli (33,34). This gene spans a region of about 6 kb (35,36). In both cell lines no

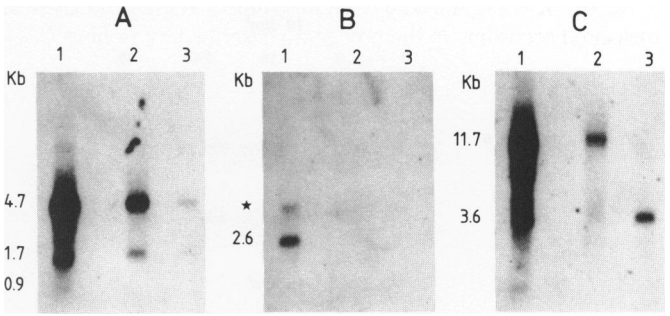

Figure 2. Northern blot analysis on the expression of the *Gart*, *Notch* and *white* genes. 5 μg poly(A)$^+$ RNA isolated from adult flies (*Berlin K*; 1), *Kc* cell line (2) and *SL2* cell line (3) was electrophoresed on a 0.8% agarose gel. The same filter was hybridized with the complete set of probes. Genomic probes used for hybridization are described in Material and Methods: A. *Gart*; B. *white*; C. *Notch*; (*) signal left of the 4.7 kb *Gart* mRNA from previous hybridization.

expression of the *white* gene is found, not even after five times longer exposure time (data not shown), whereas the few copies of the 2.6 kb transcript (37) present in the poly(A)$^+$ RNA from adult flies, are clearly detected.

Removal of T4 endonuclease V sensitive sites from defined genomic restriction fragments

Removal of UV-induced pyrimidine dimers from the active genes *Gart* and *Notch* was compared to the repair in the inactive *white* locus in *Kc* and *SL2* cells, according to Bohr et al. (1). The genomic organization of these genes is shown in Figure 1. Repair of the active *Gart* locus was analyzed in a 15.9 kb *XhoI* fragment that includes the entire 10.2 kb gene. Digestion with *XhoI* generates in addition two *Notch* fragments: a 16.6 kb fragment at the 5' end of the gene, entirely situated within the first intron, and an adjacent 12.9 kb fragment encompassing most part of the coding sequences. To measure removal of pyrimidine dimers from these fragments, the same filters used for repair analysis of the *Gart* gene were rehybridized with *Notch* specific probes. Repair of the inactive *white* gene was analyzed in a 15 kb *EcoRI* fragment including the entire locus. Filters containing *EcoRI* digested DNA were rehybridized with the 5' *Notch* specific probe to analyze repair in a 19.6 kb *Notch* fragment. This fragment overlaps most part of the 5' *XhoI* fragment.

Growing cells were exposed to 10 or 15 J/m^2 UV and incubated for various periods of time (0, 4, 8, 16, 24 or 48 hr) in the dark. The number of induced dimers per Dalton of DNA at time zero was found to be similar in all fragments analyzed, and was also comparable to the dimer frequency in the genome overall (data not shown). Autoradiograms visualizing removal of ESS from the restriction fragments containing *Gart*, *Notch* and *white* sequences are shown in Figure 3. For all fragments the intensities of the bands after T4 endonuclease V treatment were still strongly reduced after repair-periods of 4 or 8 hr. After 16 hr incubation, the intensities of these bands recovered and after 24 hr virtually no difference in intensities is visible between the treated and untreated fractions, indicating the nearly complete removal of pyrimidine dimers in the fragments. The cumulative results of several repair experiments on the rate of removal of UV-induced pyrimidine dimers in *Kc* cells are shown in Table 1. The kinetics of repair after doses of 10 or 15 J/m^2 were very similar, and therefore data for both doses were pooled. The repair

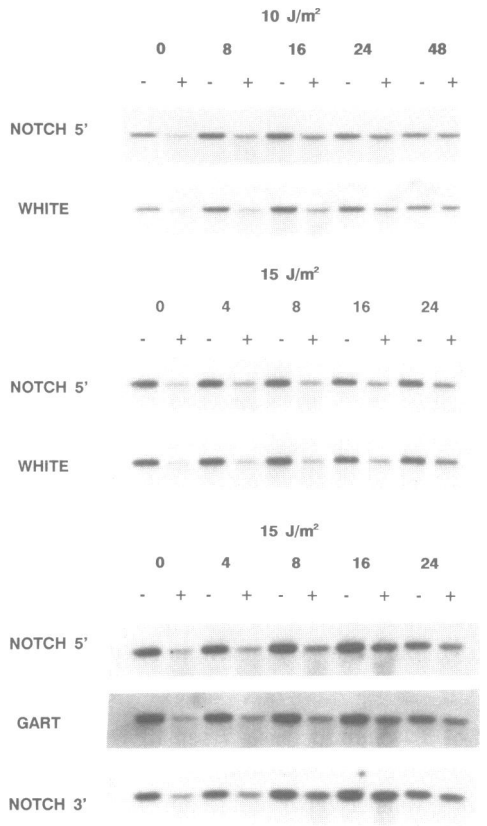

Figure 3. Autoradiograms showing the removal of T4 endonuclease V sensitive sites from defined genomic restriction fragments. Each panel shows films derived after hybridization of the same Southern blot with different gene specific probes. Panel A: 10 J/m^2 (0, 8, 16, 24, 48 hr repair time); 19.6 kb *EcoRI Notch 5'*, 15 kb *EcoRI white*. Panel B: 15 J/m^2 (0, 4, 8, 16, 24 repair time hr); 19.6 kb *EcoRI Notch 5'*, 15 kb *EcoRI white*. Panel C: 15 J/m^2 (0, 4, 8, 16, 24 hr repair time); 16.6 kb *XhoI Notch* 5', 15.9 kb *XhoI Gart*, 12.9 kb *XhoI Notch* 3'.

data from the *Kc* cells for all three *XhoI* fragments, the one containing the *Gart* sequences as well as both spanning the *Notch* gene were identical. The same repair kinetics were also found for the 15 kb *EcoRI* fragment containing the inactive *white* gene and the 19.6 kb *EcoRI Notch* fragment. Four hr after UV irradiation essentially no repair was measured. After 8, 16 and 24 hr of incubation, 15%, 57%, and 78% removal of dimers was found. At 48 hr, up to 89% of the induced pyrimidine dimers were removed.

Growing *SL2* cells exposed to 10 or 15 J/m^2 UV showed comparable results. Restriction fragments encompassing transcriptionally active *Gart* or *Notch* sequences as well as the fragment containing the nonexpressed *white* locus, were found to be repaired with the same rate and to the same the extent: up to 70% of the dimers were removed 24 hr after irradiation (data not shown).

Removal of T4 endonuclease V sensitive sites from total cellular DNA in Drosophila melanogaster cell lines

Removal of UV-induced pyrimidine dimers in total cellular DNA was assayed by sedimentation in alkaline sucrose gradients. Growing *Kc* and *SL2* cells were irradiated with 10 or 15 J/m^2 UV and repair was analyzed after incubation for various periods of time (0, 8, 16, 24 and 48 hr) in the dark. The average induction

Table 1. Percentages removal of pyrimidine dimers from defined genomic restrition fragments and total cellular DNA in growing *Kc* cells. Cells were irradiated with 10 or 15 J/m^2. Since there appeared to be no difference in repair kinetics between both doses, data were pooled. Repair kinetics in the 5' end of the *Notch* gene were derived from the 16.6 kb *XhoI* fragment and the overlapping 19.6 kb *EcoRI* fragment.

	Post-irradiation time (hr)	Percentage dimers removed (S.E.M.)	Number of experiments
GART	4	6 (3)	8
	8	15 (3)	8
	16	57 (3)	8
	24	78 (5)	8
	48	89 (4)	8
NOTCH 5'	4	9 (4)	8
	8	22 (3)	8
	16	65 (5)	8
	24	72 (3)	8
	48	88 (5)	2
NOTCH 3'	4	10 (6)	4
	8	15 (11)	4
	16	70 (7)	4
	24	80 (13)	4
	48	76 (6)	2
WHITE	4	13 (4)	3
	8	27 (2)	3
	16	63 (5)	3
	24	75 (6)	3
	48	82 (5)	1
GENOME OVERALL	4	–	
	8	13 (7)	6
	16	60 (12)	6
	24	83 (5)	6
	48	94 (2)	6

rate was about 15 ESS/10^9 Da per J/m^2. This frequency is comparable to those reported for primary cell cultures of different *Drosophila melanogaster* strains (12), *Xenopus* (38) and two types of mammalian cells (13). The repair data for *Kc* cells are also shown in Table 1: 8 hr after irradiation only 13% repair was found. At 16 hr, however, 60% of the pyrimidine dimers were removed, going up to 83% after 24 hr. Complete repair was found after 48 hr. In *SL2* cells the overall repair was found to be similar to the rates of dimer removal in the gene specific fragments analyzed: 67% after 24 hr.

DISCUSSION

DNA repair pathways in *Drosophila melanogaster* have been biochemically studied in primary cell cultures of DNA repair proficient and repair deficient flies. These investigations have been focused on measurements of the removal of DNA damage from the genome overall. The objective of this study was to extend the reported data on the excision repair of UV-induced pyrimidine dimers to specific genomic sequences. Repair experiments were performed in two immortalized cell lines generated from embryonic stages of repair proficient (wild type) *Drosophila melanogaster* strains: *Kc* (7) and *SL2* (8) to reduce the variability due to heterogeneous cell populations.

Since in mammalian cells and also in yeast evidence is accumulating for the preferential repair of transcriptionally active genes, repair kinetics for the active *Gart* and *Notch* loci and the inactive *white* gene were compared. The transcriptional status

of these three genes in *Kc* cells was assessed by Northern blotting. Restriction fragments containing gene specific sequences were selected such that repair analyses in different genes could be performed using the same Southern blot. In this way variability due to unequal loading of separate gels is excluded and comparison of relative repair rates is much more accurate. Repair was measured in growing cells which were exposed to 10 or 15 J/m^2 254 nm UV. Both doses were reported to have no lethal effect on repair proficient (wild type) *Drosophila melanogaster* cell lines (39,40). The recovery of DNA synthesis after irradiation in the *Kc* cell line was comparable to that determined for human fibroblasts (data not shown; A.R. Lehmann, pers. comm.) and it can be assumed that the used UV doses have no severe effect on the DNA repair mechanism. Indeed, in all experiments performed nearly complete removal of the induced pyrimidine dimers is obtained after the 24 hr repair-period. The number of induced dimers per Dalton of DNA in all restriction fragments analyzed was found to be similar to the dimer frequency in the genome overall. This result confirms previous reports on the random distribution of UV-induced pyrimidine dimers over the genome (1,5,41) and allows direct comparison of repair kinetics in the genome overall and in specific sequences. The results show that in growing *Kc* cells the inactive *white* gene is repaired with the same kinetics as the active *Gart* and *Notch* loci. Moreover, in this repair proficient (wild type) cell line all three genes are repaired with the same rate and to the same extent as the genome overall. Similar results were obtained using the repair proficient (wild type) *SL2* cell line. In this cell line repair of the active *Gart* and *Notch* genes was also identical to the repair of the inactive *white* gene and the genome overall. These results are in contrast to what is found in other systems under similar experimental conditions. In *Saccharomyces cerevisiae* a 2.5 fold difference in the rate of repair between the active *MAT*α locus and the inactive *HML*α locus was found (6). This difference is less prominent then in rodent cells in which repair is apparently restricted to active genes (2) but it resembles the difference in repair kinetics between active and inactive genes in human cells. For example, in confluent human fibroblasts cells (VH16) only 51% of the dimers are repaired after 24 hr in the inactive *754* locus,whereas about 70% of the dimers are removed in 8 hr from the active ADA gene (5). These results suggest that transcription is a prerequisite for rapid repair of UV-induced pyrimidine dimers. It needs however further clarification whether the observed preferential repair is due to the action of a seperate transcription-coupled repair mechanism or is just the result of an open, more accessible chromatin structure of transcriptionally active genes. In Drosophila *Kc* and *SL2* cells no evidence was found for a mechanism that directly couples repair to transcription. The results obtained with Drosophila can be explained in perspective of the functional organization of the DNA in chromosome structures. Evidently all of the Drosophila genome is repaired efficiently, whereas in mammalian cells a large part of the genome is repaired slowly. Those genes in mammalian cells that are repaired slowly are invariably non-transcribed and possibly this slow repair is associated with the late replication-associated repression mechanism: the facultative hetero-chromatinization of tissue specific genes (42,43) as reflected in R and G banding patterns. Such patterns have not been observed in Drosophila chromosomes (44). Even if such a repression mechanism exists in Drosophila tissues, this may not be activated in the immortalized Drosophila cell lines which are derived from embryonic cells.

The Drosophila data presented here support the idea that preferential repair is not restricted to transcriptionally active sequences. This is in agreement with the findings that preferentially repaired domains extend beyond the borders of transcribed genes (45).

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