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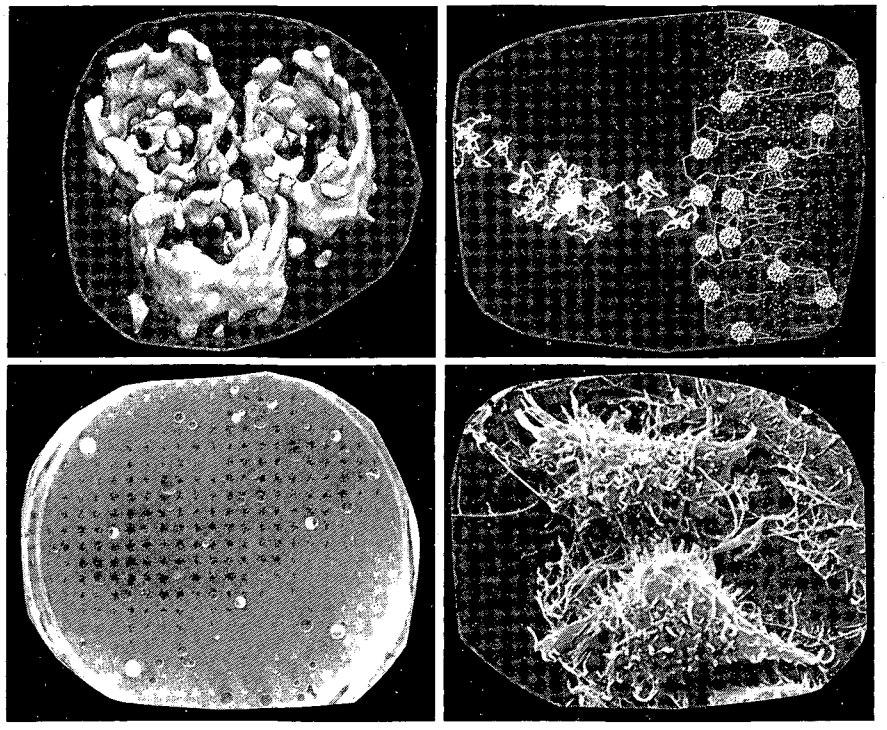

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The Production and Repair of Aflatoxin B_1-induced DNA Damage

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Running head: Repair of AFB_1 in specific genomic sequences

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Abstract

To investigate the influence of function or activity of a DNA sequence on its repair, we have studied excision repair of aflatoxin B_1 (AFB_1)-induced damage in the nontranscribed, heterochromatic alpha DNA of monkey cells and in the metallothionein genes of human cells. In confluent cells, AFB_1 adducts are produced in similar frequencies in alpha and in the rest of the DNA, but removal from alpha DNA is severely deficient, however, removal of AFB_1 adducts from alpha DNA is enhanced by small doses of UV. The repair deficiencies are not observed in actively growing cells. These results suggest that in confluent cultures, the highly condensed chromatin structure of alpha DNA hinders access of the repair system that acts on bulky adducts, but that this chromatin structure may be less condensed in growing cells due to DNA replication. We have also shown that there is preferential repair of AFB_1 damage in active genes. AFB_1 damage is efficiently repaired in the active human metallothionein (hMT) genes, but deficiently repaired in inactive hMT genes. The efficient repair of damage in the active hMT genes is due to selective repair on the transcribed strand of the genes. The transcriptional complex appears to direct repair to the transcribed strand of an active gene. Thus, the consequences of AFB_1-induced DNA damage most likely depend on the functional state of the DNA containing the damage.

I. Introduction

DNA is subject to damage produced by environmental agents, such as radiation and reactive chemicals, and by endogenous events occurring naturally in the cellular environment. Nucleotide excision repair is one mechanism in living cells by which several types of alterations in the DNA are recognized and the damaged regions are replaced by repair replication using the undamaged DNA strand as template (1,2). This process serves to protect the cells from the lethal, mutagenic and/or carcinogenic consequences of otherwise persisting DNA damage.

Many aspects of excision repair in mammalian cells have yet to be characterized. Most of the information we possess about the processing of damage DNA in mammalian cells has been derived from experiments in which events occurring throughout the entire genome have been analyzed. Yet the genomic DNA can hardly be viewed as uniform. Besides specifying messenger and non-messenger RNAs, DNA also carries information for the regulation of transcription and for the regulation of replication, as well as signals for translation and RNA processing. Some DNA carries information related to maintenance and functioning of the chromosomes (e.g., in the DNA sequences associated with telomeres and centromeres). Interspersed in the coding sequences for proteins and ribosomal and transfer RNAs are introns, segments that apparently carry no utilized information in their sequences. Damage to these various types of DNA is likely to have differing consequences to the cell. The insertion of incorrect nucleotides during replication of damaged DNA can lead directly to alterations in proteins or changes

in the level of expressions of genes. While base changes may have dramatic effects when they occur in structural DNA, they might have little or no adverse consequences in intron or other non-informational DNA. Unrepaired lesions could exert powerful effects on cells by engendering recombination or gene rearrangements that could alter gene expression. Somatic cells might be able to dispense with proper repair of lesions in coding sequences of genes that have already fulfilled their developmental roles and become "silent", but at the risk of inappropriate regulation of their expression.

An increasingly important area of investigation of DNA damage and excision repair is whether all sequences in the eukaryotic genome are repaired with the same efficiency. It is known that the chromatin structure of expressed genes causes these sequences to be highly sensitive to digestion by DNase I (3), while non-expressed, tandemly repeated DNA sequences are tightly condensed chromatin structures (4). We are just beginning to learn to what extent these different chromatin structures affect the distribution of damage and repair in the genome. Damage produced by AFB_1 (5), furocoumarins (6) and N-acetoxy-2-acetylaminofluorene (7) are found predominantly in the internucleosomal linker DNA. Preferential binding of AFB_1 to ribosomal genes (8) and both preferential binding and removal of benzo(a)pyrene in the transcribed fraction of the genome (9) have been reported. Several groups have shown correlations between repair efficiencies and various structural features of mammalian chromatin such as the nuclear matrix (10), DNA loops (11), or relaxed chromatin structures (12). Evidence has

been presented for a more rapid removal of pyrimidine dimers from the highly amplified dihydrofolate reductase (DHFR) gene both in Chinese hamster ovary cells (13, 14) and in human cells (15), for preferential repair of UV damage in the expressed c-abl gene but not the inactive c-mos locus in mouse cells (16), and for more efficient repair of UV and AFB_1 induced damage in pSV2-gpt that is integrated into the genome of monkey cells (17). It has recently been shown that the removal of UV damage from both the hamster and human DHFR gene is more rapid in the transcribed strand of the gene than in the nontranscribed strand (18). Based on these results, it has been hypothesized that even though rodent cells show lower overall levels of excision repair of UV damage compared to primate cells, their resistance to UV irradiation is due to the selective repair of damage in essential genomic regions and that repair of a gene may be directly coupled with its transcription.

This paper summarizes the results of studies on the production and repair of AFB_1-induced DNA damage in specific DNA sequences using two different cell culture systems: African green monkey cells and a human fibrosarcoma. In both systems, AFB_1 was activated by treating cell cultures in the presence of an S9 microsomal fraction derived from rat liver (19).

II. Repair of AFB_1-DNA adducts in the alpha DNA sequence of African green monkey cells.

A. Confluent cultures

Alpha DNA is a highly repetitive DNA (20), found primarily near chromosome centromeres (21) in constitutive heterochromatin,

comprising 15-20% of the genome of African green monkey cells (22). It is generally considered to be a nontranscribed sequence (23) and no function for it has been identified. The repeating unit of alpha DNA in monkey cells is a 172 base pair (bp) monomer, which has been cloned and sequenced (24). Most of the monomers contain a single recognition site for the restriction endonuclease HindIII (25). The sequence does not differ significantly from total cellular DNA in base composition and nearest neighbor analysis.

Alpha DNA can be isolated from cellular genomic DNA by separation of HindIII fragments on preparative 2% agarose gels. Alpha DNA and the remaining cellular DNA (which we refer to as bulk DNA) can then be excised and purified for further study. Zolan et al. (26) observed a deficiency in the excision repair of bulky chemical adducts in alpha DNA. The repair of furocoumarin photoadducts in alpha DNA was only about 30% of that in the bulk DNA, even though the initial levels of damage, the time course of excision repair and the repair patch size were indistinguishable for the two DNA classes. In contrast, damage produced by 254 nm UV was repaired equally well in alpha and bulk DNA. These results suggested that some feature of the alpha DNA conformation and/or its chromatin structure inhibited the recognition and repair of furocoumarin damage but not UV damage.

Since the primary AFB_1-DNA adducts and their secondary products can be identified chromatographically, the formation and removal of AFB_1-DNA adducts were also examined in cultured monkey cells (27). African green monkey cells were exposed to 0.25 uM $[^3H]AFB_1$ in the presence of a microsomal activation system for 30

min at 37° C. This produced modification levels of 0.8 to 1.2 AFB_1 adducts per 10^8 daltons. Repair synthesis elicited by AFB_1 adducts was deficient in alpha DNA sequences compared to that in bulk DNA, even though the initial levels of modification were the same for these DNAs. The removal of the primary initial adduct, 2,3-dihydro-2-(N^7-deoxyguanosyl)-3-hydroxyaflatoxin B_1, was deficient in alpha DNA (Table 1) and the kinetics of its loss resembled those we previously reported for the removal from total DNA in xeroderma pigmentosum cells of complementation group A (19). Spontaneous loss of the AFB_1 moiety or the concomitant loss of the guanine to yield an apurinic site account for these results. The formation of the more chemically stable secondary product, AFB_1-triamino-pyrimidine, occurred more rapidly and to a greater extent in alpha DNA than in the bulk of the genome, probably because of the slower removal of the primary product. Irradiation of cells with low doses of UV prior to or immediately after treatment with AFB_1 increased the rate and extent of removal of AFB_1 adducts from alpha DNA to the levels found in the bulk DNA (28). The degree of enhanced removal of AFB_1 is dependent on the UV dose and the time interval between irradiation and AFB_1 treatment. The UV enhancement is not inhibited by cycloheximide. Therefore, the formation of UV photoproducts or some intermediate in their processing alters the chromatin structure of alpha DNA thereby rendering bulky adducts accessible to repair enzymes.

B. Synchronized cultures

In actively dividing cells, the chromatin structure undergoes a number of changes to facilitate gene expression and prepare for DNA synthesis which might affect the processing of DNA damage (29, 30). When human fibroblasts are temporarily prevented from entering S-phase, the UV induced mutation frequency is decreased (31, 32) while cell survival is enhanced (33, 34, 35). Konze-Thomas et al. (36) reported that the UV induced mutation frequency was strongly influenced by the length of the period between irradiation and the beginning of S-phase. UV survival, however, has not been found to be dependent on the stage in the cell cycle in which the cells were irradiated (32, 36, 37).

It is important to note that the studies reported above on repair in alpha DNA have been carried out on confluent cultures. In order to determine whether the cell cycle influences repair of DNA damage, we compared the removal of lesions produced by treatment with AFB_1, N-acetoxy-2-acetylaminofluorene (NA-AAF), and ionizing radiation from alpha DNA sequences and the bulk of the genome in synchronized and exponentially growing cultures of monkey cells (38). Proficient removal of AFB_1 adducts in alpha DNA was observed in exponentially growing cultures (Table 1). However, as the cultures approached confluence, adduct removal from alpha DNA became deficient. Cells synchronized by subculturing confluent cultures exhibited proficient removal of AFB_1 adducts from both alpha and bulk DNA when treated in early G_1 or late S/G_2 while those cells treated in early S phase did not remove adducts from either alpha or bulk DNA. In contrast, radiation-induced thymine glycols were efficiently repaired in S phase but showed deficient

repair during G_2 (data not shown). We conclude that the accessibility of DNA lesions to repair in specific regions of the genome is influenced by the growth state, the cell cycle stage, and the type of damage introduced into the DNA.

III. Repair of AFB_1-induced damage in the human metallothionein genes.

A. Strand-selective repair of AFB_1 damage.

Metallothionein genes are a multigene family that are expressed in essentially every cell type, and their transcription can be further induced in response to a variety of exogenous stimuli, including heavy metals and glucocorticoid hormones. Their ubiquitous distribution and high inducibility make them an attractive model system for studying the relationship between gene expression and repair.

We focused on four members of the human metallothionein (hMT) gene family because of the different functional states they represent. The $hMTI_A$ and $hMTII_A$ genes are expressed at a basal level and their transcription is inducible, but these genes show different responses to inducing agents such as heavy metals and glucocorticoids (39). The induction of $hMTI_A$ occurs only in the presence of Cd^{++} but not in the presence of Zn^{++} or dexamethasone. The $hMTI_B$ gene is expressed only in some cell lines and may be tissue specific (40). In a cell line where it is not expressed, the 5'-flanking region of the gene is highly methylated, whereas it is not methylated in an expressing cell line. The $hMTII_B$ gene is a processed pseudogene flanked by direct repeats and possessing a

poly(A) sequence at the 3'-end (41). Therefore, within a single system, we can measure the repair of DNA damage in expressed genes for which transcription can also be differentially modulated, in a nontranscribed processed pseudogene, and in a gene whose expression may be tissue-specific.

Our basic approach to study repair in the hMT genes involves the physical separation of DNA regions containing bromodeoxyuridine (BrdUrd) substitution in repair patches from all other DNA using a monoclonal antibody against BrdUrd (42). Human cell cultures, labeled in their DNA with [^{3}H]thymidine, were exposed to a DNA damaging agent and then allowed to repair for various lengths of time in the presence of BrdUrd. Purified restriction enzyme-digested unreplicated DNA was reacted with a monoclonal antibody that binds BrdUrd in DNA, and total repair was assessed by the amount of ^{3}H-labeled DNA bound by the antibody. Repair in the hMT genes was determined by electrophoresing on agarose gels equal amounts of DNA from the bound (i.e., containing repair patches) and free (i.e., not containing repair patches) fractions and then quantitating by densitometry the intensity of hybridization that occurred to a specific restriction fragment in each fraction (43, 44). The amount of hybridization was then compared to the amount of total DNA in each fraction. Unlike methods that rely upon the loss of an endonuclease sensitive site from a sequence, this technique: (i) detects the repair synthesis event itself, thus eliminating the possible error produced if the loss of an endonuclease sensitive site reflects modification but not repair of a lesion; (ii) provides a sensitive measurement of repair since

only one, not all, the lesions in a DNA sequence need be repaired in order to detect repair in that sequence; (iii) allows direct comparison of repair of a variety of types of DNA damage; and (iv) makes it possible to simultaneously measure repair in more than one DNA sequence.

Repair in the hMT genes was examined using the plasmid $pZMTII_A$ which contains most of a cDNA probe of hMTII RNA. Because of the extensive homology (at least 80%) of the members of this gene family to the coding region of hMTII, this probe hybridizes to 12-15 different bands when genomic DNA is digested with EcoRI (45). These hybridizing bands correspond to DNA fragments containing the multiple members of the hMT gene family. Our studies focused on four members of this gene family: the active $hMTI_A$ gene, located on a 10 kbp fragment, the active $hMTII_A$ gene, on a 5.9 kbp fragment, the inactive $hMTI_B$ gene, located on a 14 kbp fragment, and the inactive $hMTII_B$ gene which, due to a polymorphic Eco RI site in its 5'-flanking region, is on two fragments of 4.8 and 4.6 kbp (45, 46, 47). The size of the transcriptional units of $hMTI_A$, $hMTI_B$, and $hMTII_A$ is approximately 1 kbp, while $hMTII_B$ is approximately 0.5 kbp.

Strand-specific probes for the hMT genes were generated using the two phage RNA polymerase promoters, SP6 and T7, which are contained on $pZMTII_A$ and are oriented in opposing directions. The direction of transcription of the $hMTII_A$ gene in vivo is from the BamHI site to the PvuII site. Linearizing the plasmid with HindIII and using T7 RNA polymerase and ribonucleotides produces an RNA probe specific for the transcribed strand, while using BamHI and

SP6 RNA polymerase generates an RNA probe specific for the nontranscribed strand. Nick translation of this plasmid produces a DNA probe that will hybridize to both strands. For repair analysis, the membranes containing the DNA from the bound and free fractions were sequentially hybridized with the RNA probes for each strand of the hMT genes and with the nick-translated DNA probes for both strands. The membrane was "deprobed" between each hybridization.

Human cells were exposed to 80 uM AFB_1 and then allowed to repair for various lengths of time in the presence of BrdUrd. As we had previously found using a probe which hybridizes to both strands of the hMT genes (48), repair of AFB_1 damage was faster in the active $hMTI_A$ and $hMTII_A$ genes than in total DNA. However, repair was approximately 3-times slower in the inactive $hMTI_B$ and $hMTII_B$ genes compared to the genome overall. Using probes for either the transcribed or nontranscribed strands, we found that the more efficient repair of AFB_1 damage in the active genes was due to a much faster repair on their transcribed strands than on the nontranscribed strands (Table 2). The nontranscribed strands of the active genes showed repair kinetics similar to total DNA. In contrast to the active genes, both strands of the inactive hMT genes showed deficient repair of this damage. Therefore, repair of AFB_1-induced DNA damage occurs at three different rates: a fast rate on the transcribed strands of active genes, a slower rate, similar to the genome overall, on the nontranscribed strands of active genes, and a rate even slower than that of the genome overall, which occurs on both strands of inactive genes.

B. Involvement of transcription in strand-selective repair of AFB_1.

We previously found that incubation of cell cultures with either dexamethasone or $CdCl_2$, which induce transcription of the hMT genes five-fold or ten-fold respectively, selectively increased the rate of repair in the induced genes, but not the uninduced genes (48). Therefore, in order to ascertain whether transcription of the gene itself leads to strand-selective repair, we examined what effect modulating transcription of the hMT genes had on this repair. Transcription of the $hMTII_A$ gene, but not the $hMTI_A$ gene, was induced by incubation of cell cultures in 0.1 uM dexamethasone for 15 hr prior to exposure to 80 uM AFB_1. The initial rate of repair on the transcribed strand of $hMTII_A$ gene was faster following induction than when the gene was not induced (Table 3). Repair on the nontranscribed strand of the $hMTII_A$ was not affected by induction with dexamethasone and showed a rate of repair similar to total DNA. The effect of inducing transcription on the strand-selectivity of repair was specific to the gene being induced, since repair on either strand of the active, but uninduced $hMTI_A$ gene and and the inactive $hMTI_B$ and $hMTII_B$ genes were not affected by induction with dexamethasone. Therefore, increasing the rate of transcription of a gene specifically increases the rate of repair on the transcribed strand of that gene.

IV. Conclusions and perspectives

One of the most striking features of the interactions of specific DNA sequences with damaging agents and repair systems is

its complexity. In nondividing cells, something about the organization of alpha DNA severely restricts the cell's ability to remove furocoumarin and AFB_1 adducts. Yet, overall frequencies of these adducts were the same in alpha and bulk DNA. It appears that repair systems for these adducts are somehow excluded from interacting with the DNA although the damaging agents are not. In actively dividing cells, the repair deficiencies are eliminated or greatly reduced. Repair of AFB_1 in inactive hMT genes was also deficient compared to the genome overall. In contrast to the findings with nonexpressed DNA, repair of AFB_1 damage in active genes is much more efficient than in the genome overall. This more efficient repair was due to faster repair on the transcribed strands of the genes.

These differences in repair between active and inactive sequences and transcribed and nontranscribed strands of a gene suggest that there may be several hierarchies of repair efficiency within the genome. At one level, the chromatin conformation associated with a DNA sequence would obviously provide barriers that would restrict accessibility of repair enzymes to the damaged DNA. In this context, it is easy to envision transcriptionally active DNA to be in a chromatin configuration more readily repaired than more condensed, nontranscribed sequences. Thus, the more compact chromatin structure associated with alpha DNA may account for the deficient repair of certain bulky chemical adducts from this DNA sequence. However, cycling cells may simply maintain alpha chromatin in a more accessible state, perhaps due to its location at centromeres. Alterations in the chromatin structure

associated with genes in various functional states could also explain the overall differences in repair we have observed between the transcribed and nontranscribed hMT genes. Thus, a more compact chromatin structure may also be associated with regions surrounding nonexpressed, processed pseudogenes, such as $hMTII_B$, and developmentally regulated genes, such as $hMTI_B$, when they are in a highly methylated, nonexpressed state. The observation that repair of AFB_1 damage in the $hMTI_B$ gene is deficient may also provide important insights into the processes involved in the activation of proto-oncogenes that are developmentally regulated. At times in development when these genes are not expressed, their chromatin structure may render them more prone to genetic alterations because of a deficiency in repair. Subsequent transcriptional activation of the altered gene could result in an mutated gene product that might then contribute to the malignant phenotype.

It is unlikely, however, that increased chromatin accessibility alone would mediate the strand-selective repair in active genes, especially considering our observations that by increasing the rate of transcription of the hMT genes, repair on the transcribed strand of a gene was affected. Repair on the nontranscribed strand and on both strands of inactive genes continued at rates similar to the genome overall. Thus, it seems much more likely that these differences in repair are directly related to the involvement of the transcriptional complex in the initiation of repair. The transcriptional complex could direct repair to the transcribed strand of a gene in at least two ways: repair enzymes could be associated with the RNA polymerase complex,

so that repair proteins which recognize DNA distortions "scan" the strand that is being transcribed. Alternatively, the difference in repair may be related to different effects of the DNA lesions in the respective strands on the transcription process itself. AFB_1-induced DNA damage is known to cause termination of transcription (49). It is possible that only lesions on the template strand of a gene have this effect. An enhancement of repair activity at sites where transcription is blocked would explain both preferential repair in active genes and its strand differences. Thus, increasing the rate of transcription of the hMT genes increases the density of RNA polymerase II molecules on the gene (50, 51). This, in turn, would increase the chances that the transcriptional complex would encounter a lesion on the transcribed strand.

IV. Acknowledgments

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Table 1

Removal of AFB_1-DNA Adducts From Actively Growing Cultures of African Green Monkey Cells

Stage when cells were treated	Percentage of initial adducts repaired by 12 hr	
	Alpha	Bulk
G_0 (confluent)	10	45
Exponential	37	42
G_1	23	30
S phase	6	9
Late S/G_2	36	49

Table 2

Strand-selective Repair of AFB_1-induced DNA Damage in the Human Metallothionein Genes

	Percent repaired by 4 hr post-treatment			
Strand Probed	$hMTI_A$	$hMTII_A$	$hMTI_B$	$hMTII_B$
Both	33	38	6	7
Transcribed	57	69	7	6
Nontranscribed	12	14	6	7

Table 3

Effect of Induction of Transcription of the $hMTII_A$ Gene on Strand-selective Repair of AFB_1-induced Damage

Treatment	Strand Probed	Percent repaired by 4 hr post-treatment: $hMTII_A$	Percent repaired by 4 hr post-treatment: Total
AFB_1 (80 uM)			16
	Transcribed	71	
	Nontranscribed	14	
AFB_1 (80 uM) + dexamethasone (0.1 uM)			17
	Transcribed	92	
	Nontranscribed	13	

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