Formation and Repair of Furocoumarin Adducts in α Deoxyribonucleic Acid and Bulk Deoxyribonucleic Acid of Monkey Cells†

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ABSTRACT: We have extended our previous finding that excision repair of furocoumarin photoadducts is deficient in the highly repetitive α DNA sequences in cultured African green monkey cells. The formation and removal from DNA of the individual photoadducts of 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen (HMT) were monitored by analysis of DNA hydrolysates using a high-pressure liquid chromatography procedure that separated the major adducts from each other and also resolved the two diastereomers of the most frequent monoadduct. The overall deficiency in removal of HMT adducts from α DNA was similar to that previously observed by us with 4′-(aminomethyl)-4,5′,8-trimethylpsoralen and angelicin. The two diastereomers of the furan–T monoadducts were formed in the same relative amounts in α DNA and bulk DNA whether photoaddition was in vivo or in vitro, and they were removed from cellular DNA at the same relative rates. Therefore, the deficient removal of furocoumarin adducts from α cannot be due to preferential formation of some adduct inherently refractory to repair. However, in vivo, the photochemical conversion of the furan–T monoadducts to diadducts was markedly reduced in α DNA, relative to that in bulk DNA. This indicates a possible conformational constraint in the internucleosomal DNA in α-chromatin which may account for the deficiency in repair.

We have recently shown that the 172 base pair (bp) highly repeated α DNA sequence in confluent African green monkey cells in culture is repaired less efficiently than the bulk of the genome after certain types of chemical damage (Zolan et al., 1982a). In particular, the absolute amount of repair of the photoadducts of the furocoumarin AMT[1] was only about 30% as great in α DNA as in the bulk of the genome, even though the initial levels of damage, the time course of excision repair, and the size of excision repair patches were all indistinguishable for the two DNA classes. In striking constrast, all aspects of repair were nearly identical for α and bulk DNA following damage by 254-nm ultraviolet light. The results obtained with UV_{254} indicate that α DNA sequences are not intrinsically poor substrates for the activities of repair enzymes. Instead, some features of the α sequence and its cellular chromatin conformation may inhibit the recognition and removal of furocoumarin adducts. The fact that furocoumarins can form a variety of different adducts in DNA raised the possibility that one or more of these may be refractory to repair. If, for some reason, the relative preponderance of certain adducts was different in α DNA, our results concerning their removal might be explained. Furthermore, the existence of adducts that are inherently poor substrates for cellular repair systems would be consistent with our observation that only one third of the isotopically labeled furocoumarin adducts were removed from total DNA in 24 h.

Straub et al. (1981) and Kanne et al. (1982) developed high-pressure liquid chromatography methods for separating adducts formed in vitro by a number of furocoumarins, and they identified the structures of the major species using various spectroscopic methods. They showed that the furocoumarin HMT (Issacs et al., 1977) forms several adducts with DNA. These adducts principally involve the furan side of the molecule; a cyclobutane ring is formed in a photochemical reaction between the 4′- and 5′-positions of the furan and the 5- and 6-positions of a pyrimidine. The furan-side adducts with thymine (furan–T monoadducts) are formed in much greater amounts than are those with cytosine (furan–C monoadducts). Each type of adduct can exist in one of two forms, depending upon whether the HMT molecule was intercalated on the 5′ or 3′ side of the pyrimidine nucleotide in the DNA. Each of the furan-side monoadducts can theoretically absorb another photon and react at their pyrone ends with a properly positioned pyrimidine in the complementary DNA strand, creating an interstrand cross-link. In vitro, more than 90% of the cross-links have been found to be of the thymine–HMT–thymine type (T–T cross-link), formed by reaction of a furan–T monoadduct with another thymine. Monoadducts are also produced by photoreaction at the pyrone side of the molecule, but these comprise only a very small fraction of the total and apparently cannot be converted to cross-links (Kanne et al., 1982).

We knew from our previous studies that differential formation and repair of cross-links could not account for our results with α DNA (Zolan et al., 1982a). However, it had been suggested that the ratio of formation of the two different furan–T monoadducts might be related to DNA sequence (Kanne et al., 1982), as in the case of the analogous furan–U monoadducts in RNA (S. Issacs, personal communication). Therefore, it was possible that they might form in different proportions in α DNA and bulk DNA. If this were the case, then, since the furan–T monoadducts are the predominant adducts, a difference in their susceptibility to enzymatic removal might explain the repair deficiency in α DNA. We therefore undertook a study of the formation and removal of various HMT adducts in African green monkey cells to examine the spectrum of adducts formed in vivo and the extent to which each is removed. In the course of our work we modified the previously reported HPLC procedure to provide

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[1] Abbreviations: AMT, 4′-(aminomethyl)-4,5′,8-trimethylpsoralen; HMT, 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen; HPLC, high-pressure liquid chromatography; UV_{254}, 254-nm ultraviolet light; UVA, long-wavelength ultraviolet light; Tris, tris(hydroxymethyl)aminomethane.

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better resolution of the furan–T monoadducts. Our results indicate that the two furan–T monoadducts are formed in the same proportions in α DNA and in bulk DNA, both in vitro and in vivo. Studies with total cellular DNA indicate that both furan–T monoadducts are removed from DNA by cultured cells. However, a smaller proportion of these monoadducts is converted to cross-links in α DNA than in bulk DNA, implying that the chromatin structure of these highly repeated DNA sequences has an unusual conformation in living cells.

Experimental Procedures

Cell culture, treatment of cells with labeled and unlabeled HMT, isolation of α DNA and bulk DNA, measurement of repair synthesis, and measurement of total HMT damage were all as previously described (Zolan et al., 1982a).

Chemicals and Drugs. A stock solution containing 1 mM fluorodeoxyuridine and 10 mM 5-bromodeoxyuridine (P-L Biochemicals) was stored frozen. Unlabeled HMT (HRI Associates, Emeryville, CA) was dissolved in ethanol at a concentration of 1 mg/mL and stored in the dark at 4 °C. ^{3}H-Labeled HMT (HRI associates) was stored in the dark at 4 °C. Cells were treated with an appropriate amount of stock solution of drug diluted with phosphate-buffered saline (Smith & Hanawalt, 1976).

Preparation of Samples for HPLC. Samples were prepared by previously unpublished procedures developed in this laboratory by N. Calvin. Purified DNA at ≤100 μg/mL was treated with 2 μg/mL DNase II (Sigma; 725 units/mg) in 100 mM NaOAc and 2 mM $MgSO_4$, pH 4.6, for at least 12 h at 37 °C. The solution was adjusted to pH 7.0 with 2 M Trizma base (5 μL/200 μL of the initial sample volume), phosphodiesterase II (Boehringer Mannheim; 4 units/mL; 8 μL/200 μL of sample) was added, and the sample was incubated for 5–8 h at 37 °C. Tris, 1 M, pH 8.5 (10 μL/200 μL of sample), and bacterial alkaline phosphatase (Sigma, 303 units/mL; 4 μL/200 μL of sample) were added, and the sample was incubated at 37 °C for 12 h. The sample was stored lyophilized at −20 °C in the dark, then redissolved in H_2O, and centrifuged in a microfuge (Beckman) to remove particulate matter just before analysis. When α DNA and bulk DNA were prepared for HPLC analysis, the method of Maxam & Gilbert (1980) was used to elute the DNAs from agarose gels prior to digestion by the procedure described above, since the electroelution was found to release factors that inhibitied the subsequent enzymatic hydrolysis.

HPLC Analysis of Samples. A Beckman Model 322 liquid chromatograph equipped with a Beckman 4.6 × 250 mm, 5-μm Ultrasphere reverse-phase octadecylsilane (ODS) column was used. Elution with the solvents described below was at 1 mL/min. Fractions were collected and assayed for radioactivity by scintillation counting in a 1:2 mixture of Triton X-100 and Omnifluor (New England Nuclear). Since the samples collected from any given HPLC analysis varied in volume and solvent concentration, the ^{3}H values of each elution profile were corrected by the amount of quenching of a [^{3}H]thymidine sample assayed under the same conditions, relative to "standard" conditions of 1 mL of aqueous sample plus 10 mL of scintillation cocktail.

Samples were adjusted to pH 3 with 2 N HCl immediately before loading. Two methods of elution from the column were used: *Method A* was essentially as described by Kanne et al. (1982). The aqueous solvent was 10 mM glycine hydrochloride, pH 3, and the organic solvent was pure methanol (Baker, HPLC grade). Samples were injected with the column equilibrated in aqueous solvent. After a 10-min wash in aqueous solvent, the organic proportion of the eluting solvent was raised to 100% over 60 min. *Method B* was our modification of method A. The organic solvent was a 3:1 mixture of acetonitrile (Baker, HPLC grade) and methanol. Five minutes after injection, the organic proportion was raised to 13% over 10 min and held constant at 13% for 65 min. The organic proportion was then raised to 70% over 45 min and then to 100% over 10 min. Elution times using this procedure were dependent upon both temperature and ionic strength. The column temperature was maintained at about 24 °C. For samples that had been digested in a large volume of buffer and then concentrated before analysis, the initial buffer wash and the 13% organic isocratic portion of the elution program were increased by 10 min each, in order to elute the salts prior to elution of the adducts.

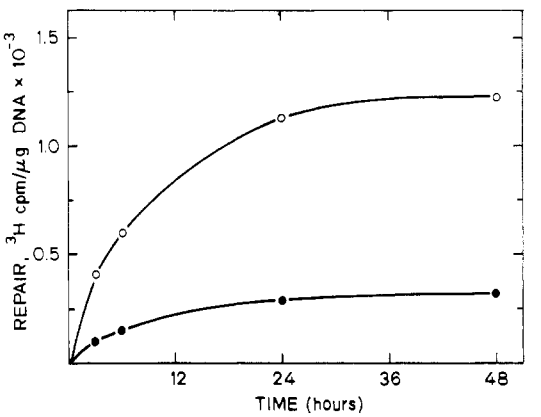

FIGURE 1: Time course of excision repair in α DNA and bulk DNA in cells treated with HMT and UVA as described in the text and incubated in medium containing 30 μCi/mL [^{3}H]dThd, 50 μM BrdUrd, and 5 μM FdUrd. The repair synthesis was measured in both α DNA (●) and bulk DNA (○) at the times shown.

Results

Deficient Repair of HMT Damage. We first determined the characteristics of repair replication and HMT adduct removal in α DNA and bulk DNA. Confluent BS-C-1 cells were treated with 30 μg/mL HMT and 7.5 kJ/m^2 long wavelength ultraviolet light and incubated for various times, and the amount of repair replication per unit DNA was determined in α DNA and bulk DNA. Repair replication in α DNA was about 20% of that in bulk DNA over the entire time course (Figure 1). Thus, HMT damage elicits less repair replication in α DNA than in bulk DNA, in a manner quantitatively similar to the results obtained previously for other furocoumarins (Zolan et al., 1982a).

To confirm that the lower amount of repair synthesis measured in α DNA after HMT damage reflected the removal of fewer adducts from α DNA, confluent cells were treated with 0.2 μg/mL [^{3}H]HMT and 3.75 kJ/m^2 UVA and incubated for 8–48 h. The loss of bound ^{3}H from α DNA was about 20% of that from bulk DNA (Figure 2), in agreement with the difference found for repair replication between α DNA and bulk DNA.

Analysis by HPLC of HMT–DNA Photoadducts Generated in Vitro. Purified BS-C-1 DNA (109 μg) was mixed with 0.7 μg of [^{3}H]HMT in a total volume of 1.5 mL, and the solution was irradiated with 16.8 kJ/m^2 UVA. The DNA was repurified and enzymatically hydrolyzed. The sample was then analyzed by using the HPLC elution conditions described by Kanne et al. [(1982) method A, described under Experimental Procedures]. The elution profile (Figure 3) agreed well with the published data, both with respect to elution times of the various components and in the relative amount of each species

Table I: Analysis of HMT-DNA Photoadducts Generated in Vitro[a]

peak[b]	adduct[c]	^{3}H (cpm) total DNA method A	^{3}H (cpm) total DNA method B	^{3}H (cpm) α DNA method B	total ^{3}H (%) total DNA method A	total ^{3}H (%) total DNA method B	total ^{3}H (%) α DNA method B	adducts/base pair × 10^{-6} total DNA method A	adducts/base pair × 10^{-6} total DNA method B	adducts/base pair × 10^{-6} α DNA method B
1	T-T cross-links	2263	2555	331	18	22	17	730[d]	900	690
1a		976	223	107						
2	furan-T monoadducts	13182	8568	1803	72	69	70	2940	2800	2860
3	furan-C monoadducts	1401	788	189	7.7	6	7	320	250	290
4	pyrone monoadducts	422	374	139	2.3	3	5	80	120	210

[a] Samples of the single preparation of DNA described in the legend to Figure 3 were analyzed separately by HPLC methods A and B and used to prepare α DNA, which was then analyzed by method B. [b] As shown in Figures 3 and 4. [c] Assignments based on Straub et al. (1981) and Kanne et al. (1982). [d] These values were calculated from the measured activity of the [^{3}H]HMT (6.9×10^{15} cpm/mol), the overall level of DNA modification (45 070 cpm/μg of DNA), and the percent total cpm for each species.

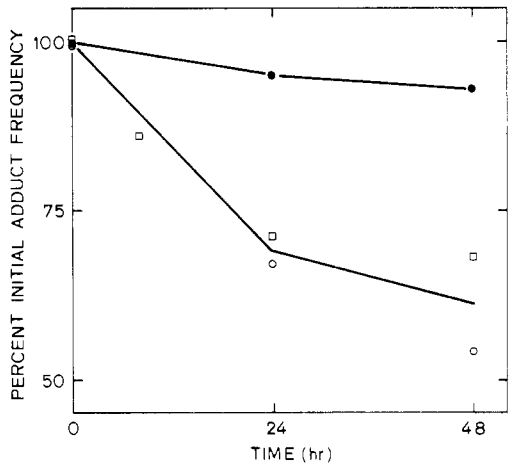

FIGURE 2: Time course of removal of bound radioactivity from α DNA and bulk DNA in cells treated with [^{3}H]HMT and UVA as noted in the text and incubated for the times shown. Solid symbols show results for α DNA and open symbols for bulk DNA. Squares and circles represent different experiments. The modification levels (in cpm of HMT/μg of DNA) were 136 for bulk (squares) and 255 for α and 228 for bulk (circles).

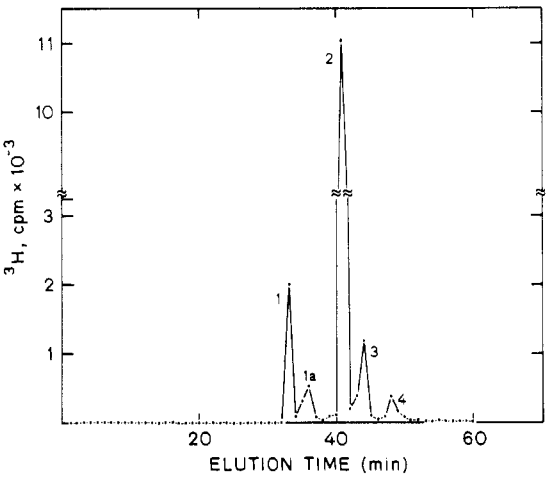

FIGURE 3: Analysis of HMT adducts in total DNA by HPLC, method A. BS-C-1 DNA was modified in vitro with [^{3}H]HMT as described in the text and analyzed as described under Experimental Procedures. The eluant was collected in 1-mL fractions for determination of radioactivity.

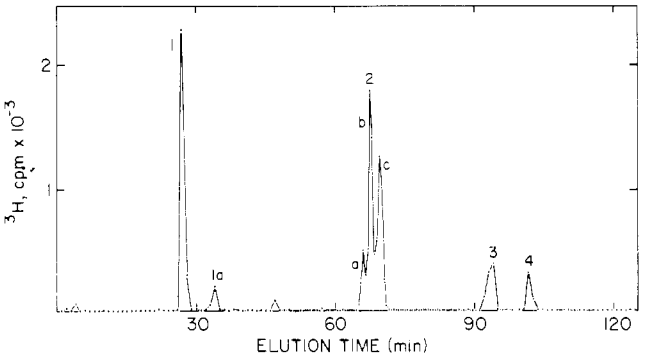

FIGURE 4: Analysis of HMT adducts in total DNA by HPLC, method B. A sample of the same DNA analyzed as shown in Figure 3 was analyzed as described under Experimental Procedures. The eluant was collected in 1-mL fractions before 50 min and after 85 min and in 0.5-mL fractions between 50 and 85 min.

(Kanne et al., 1982). Therefore, we assigned the peaks as listed in Table I.

Although the conditions used in this analysis gave highly reproducible, clean separation of most of the adducts, the two furan–T monoadducts were not resolved (Figure 3, peak 2). This was probably due, in part, to the fact that our detection is based upon collection of large fractions for scintillation counting rather than continuous optical monitoring. Although adduct frequencies obtained in vitro can be sufficient for detection by absorption measurements, biologically meaningful adduct frequencies are too low to be analyzed in this manner. We therefore modified the HPLC procedure to increase the resolution of the furan–T monoadducts so that their relative amounts in various samples could be compared (method B). Elution profiles obtained by methods A and B were similar except that the largest single peak of method A (peak 2) is resolved into three species by method B (Figure 4). Comparison of the relative amounts of the various components (Table I) indicates that the order of elution of the various species is the same for the two procedures. The assignments for components 1 and 2 were confirmed by preparing them by HPLC using method A and then reanalyzing them separately by using method B. This analysis also showed that component 1a is a breakdown product or alternate form of component 1 (the T–T cross-link). When purified component 1 was mock digested and stored frozen for several days, reanalysis showed that component 1a had increased to 45% of the total. We therefore summed these two components to determine cross-link frequences in our analysis. Component 1a was usually minor (<10% of peak 1).

From the published work of Straub et al. (1981), we expected the furan–T monoadducts to be resolved into only two components by method B. However, we consistently observed a third small peak (Figure 4, peak 2a), which comprised about 15% of the total radioactivity in peak 2. This small peak probably represents a breakdown product or alternate form of one of the other furan–T monoadducts and was treated as such in calculating the frequencies of the various adducts. Although we have not determined directly the identity of this species, its low frequency and the constancy of its size in

Table II: Analysis of HMT-DNA Photoadducts Generated in Vivo[a]

adduct	expt	^{3}H (cpm)		total ^{3}H (%)		adducts/base pair × 10^{-6}	
		α	bulk	α	bulk	α	bulk
T-T cross-links	A	524	1271	27	38	39[b]	53
	B	183	837	15	28	4.5	9.6
furan-T monoadducts	A	1077	1162	56	35	81	48
	B	821	1346	67	45	20	15
furan-C monoadducts	A	182	445	9	13	13	19
	B	185	494	15	17	4.6	5.9
pyrone monoadducts	A	147	445	8	13	11	19
	B	43	293	4	10	1.1	3.4

[a] Cells were treated with 0.5 μg/mL [^{3}H]HMT and 15 kJ/m^2 UVA (experiment A) or 0.2 μg/mL [^{3}H]HMT and 7.5 kJ/m^2 UVA (experiment B), and α DNA and bulk DNA were prepared and analyzed by HPLC using method B. [b] These were calculated in the same manner as for the values in Table I. Specific activities of HMT were 8.2 (experiment A) and 4.9 (experiment B) × 10^{15} cpm/mol. The levels of modification were (in cpm of HMT/μg of DNA) 1917 for α and 1819 for bulk in experiment A and 245 for α and 270 for bulk for experiment B.

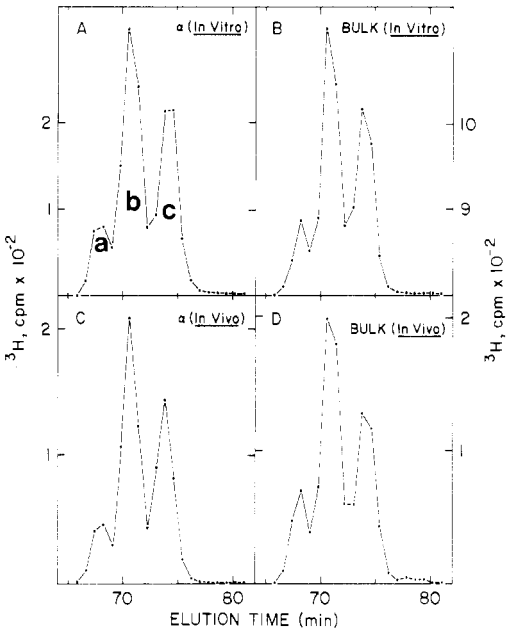

FIGURE 5: Comparative analysis of furan-T monoadducts formed in α DNA and bulk DNA. Regions of HPLC elution profiles containing furan-T monoadducts are shown plotted to make the central subcomponents (b) equal in height. (A) α DNA prepared from total DNA after modification in vitro. (B) Total DNA modified in vitro [from which the DNA shown in (A) was prepared]. (C and D) α DNA and bulk DNA, respectively, prepared from DNA modified in vivo. The complete profiles from which (B), (C), and (D) originate are shown in Figures 3, 4, and 6.

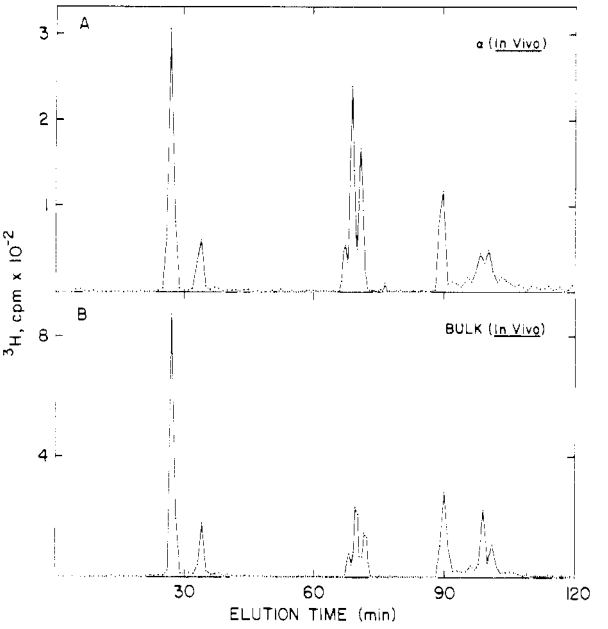

FIGURE 6: Analysis of HMT adducts in DNA modified in vivo. Cells were treated with 0.5 μg/mL HMT and 15 kJ/m^2 UVA, the DNA was isolated, and α DNA (panel A) and bulk DNA (panel B) were prepared and analyzed separately by HPLC, method B. Fractions were collected as described in the legend to Figure 4. This analysis corresponds to experiment A of Table II.

relation to the other components of peak 2 in all our experiments make its identity relatively unimportant for the conclusions we wish to draw.

Adduct Frequencies in α DNA and Bulk DNA Modified in Vitro. α DNA was prepared from a sample of the same DNA analyzed as shown in Figures 3 and 4, hydrolyzed, and analyzed by HPLC using method B (Table I). No significant differences were observed between α DNA and bulk DNA in the relative frequencies of all the adducts. Furthermore, the relative amounts of the two furan-T monoadducts were the same in α DNA and bulk DNA (Figure 5A,B).

Adduct Frequencies in α DNA and Bulk DNA Modified in Vivo. BS-C-1 cells were treated with [^{3}H]HMT and UVA, the DNA was purified, and α DNA and bulk DNA were isolated, digested, and analyzed by HPLC using method B. The amounts of the furan-T monoadducts relative to each other were identical in α DNA and bulk DNA (Figure 5C,D). For α DNA and bulk DNA, modified either in vitro or in vivo, the proportions of the three subfractions of the furan-T component were the following: a, 15%; b, 50%; c, 35%. The deficient repair of HMT damage in α DNA cannot therefore be due to a preferential formation in α DNA of one of the furan-T diastereomers over the other.

The complete analysis of the DNA modified in vivo (Figure 6 and Table II) did, however, show a difference between α DNA and bulk DNA. The frequency of cross-links in α DNA was significantly smaller than that in bulk DNA, with a corresponding increase in the frequency of furan-T monoadducts. The total adduct frequencies were similar for α and bulk; this is not immediately obvious from Figure 6 due to the fact that the profiles are plotted with the cross-link peaks at equal height. This result was obtained both with treatment conditions that nearly maximized the conversion of furan-T monoadducts to cross-links in the bulk DNA (Table II, experiment A) and those giving lower modification levels and cross-link conversion (experiment B). This result contrasts with that for DNA modified in vitro (Table I).

Removal of the Individual Adducts in Vivo. BS-C-1 cells were treated with 0.2 μg/mL ^{3}H-HMT and 3.75 kJ/m^2 UVA, and DNA was isolated either immediately after treatment or after the cells were incubated for 45 h. Both samples were

Table III: Removal of HMT Adducts by BS-C-1 Cells[a]

adduct	^{3}H (cpm)		total ^{3}H (%)		adducts/base pair × 10^{-6}		% removed
	initial	48 h	initial	48 h	initial	48 h	
T-T cross-links	155	136	3	5	0.4[b]	0.4	0
furan-T monoadducts							
sum	4333	2298	90	90	11	6.8	40
(a)[c]			(11)	(8)			
(b)			(52)	(51)			
(c)			(37)	(41)			
furan-C monoadducts	266	100	6	4	0.7	0.3	57
pyrone monoadducts	90	37	2	1	0.3	0.1	62

[a] Cells were treated with [^{3}H]HMT and UVA as described in the text, and α DNA and bulk DNA were prepared immediately or after a 48-h incubation and analyzed by HPLC using method B. [b] These were calculated in the same manner as for the values in Table I. The specific activity of the HMT was 8.4×10^{15} cpm/mol. The levels of modification were (in cpm of HMT/μg of DNA) 172 for the sample taken initially and 104 for the sample taken at 48 h. [c] In parentheses are given the relative amounts of the three components of the furan-T peak (as shown on Figure 5) as percent of the total furan-T peak.

Table IV: Conversion of HMT Monoadducts to Cross-Links[a]

adduct	^{3}H (cpm)		total ^{3}H (%)		adducts/base pair × 10^{-6}	
	−[d]	+	−	+	−	+
T-T cross-links	155	353	3	15	0.4[b]	1.9
furan-T monoadducts						
sum	4333	1863	90	77	11	9.6
(a)[c]			(11)	(12)		
(b)			(52)	(51)		
(c)			(37)	(37)		
furan-C monoadducts	266	118	6	5	0.7	0.6
pyrone monoadducts	90	94	2	4	0.3	0.5

[a] Cells were treated as described in the text, and total DNA was analyzed by HPLC using method B. [b] These were calculated in the same manner as for the values in Table I. The specific activity of the HMT was 8.4×10^{15} cpm/mol. The levels of modification were 170 cpm of HMT/μg of DNA for both the DNA samples. [c] In parentheses are given the relative amounts of the furan-T subcomponents (as shown on Figure 5), as percent of the total furan-T peak. [d] (−), without reirradiation; (+), with reirradiation.

analyzed by HPLC using method B (Table III).

The vast majority (90%) of the adducts formed in BS-C-1 cells under the conditions used in this experiment were furan–T monoadducts (Table III). No significant differences in the relative proportions of the subcomponents of the furan–T peak were observed when DNA isolated immediately after treatment was compared to DNA isolated from cells after 48 h of repair. Therefore, the different furan–T monoadducts are removed equally well from the DNA. The minor adducts, the furan–C and pyrone monoadducts, apparently were proficiently removed in these cells. Although there was apparently no repair of the T–T cross-links, the initial cross-link frequency in this experiment was too low to make such a calculation for the species meaningful. In fact, we have recently found that cross-links are removed from DNA in confluent normal fibroblasts at least as well as the furan–T monoadducts (M. E. Zolan, D. Okumoto, and C. A. Smith, unpublished data).

Conversion of Monoadducts to Cross-Links. We examined the possibility that one of the furan–T monoadducts was a favored substrate for the formation of interstrand cross-links in vivo. BS-C-1 cells were treated with 0.2 μg/mL [^{3}H]HMT and 3.75 kJ/m^2 UVA. Some of the cells were lysed immediately, while others were washed several times with phosphate-buffered saline to remove unbound HMT and then reirradiated with 7.5 kJ/m^2 UVA to convert some of the monoadducts to cross-links. DNA was prepared from both samples and analyzed by HPLC using method B. The washing procedure was effective since the specific activity of DNA modification (cpm/μg of DNA) did not increase in the reirradiated sample (Table IV). Kanne et al. (1982) reported that essentially all the cross-links formed in vitro are derived from the furan–T monoadducts, and our results show that this is also true in vivo (Table IV). There was apparently no bias in the formation of cross-links from the different furan–T monoadducts; the relative proportions of the furan–T subcomponents were the same in the sample with 3% cross-links as in the sample with 14.5% cross-links.

Discussion

Our previous study (Zolan et al., 1982a) showed that stationary phase African green monkey cells in culture did not remove the photoadducts of AMT from α DNA as efficiently as they did from the bulk of the DNA. This was also detected as a reduced amount of repair synthesis in α DNA after cells were treated with this agent, or with angelicin, a furocoumarin which makes only monoadducts under the conditions we used. The result with angelicin made it unlikely that the deficient repair of α DNA was related to formation of interstrand cross-links. However, since a number of different furocoumarin monoadducts form, it appeared plausible that some type(s) of monoadduct, whose formation was somehow favored in α, might be relatively refractory to removal. To examine this possibility, we studied formation and repair of the adducts of another furocoumarin, HMT, which have been well characterized (Straub et al., 1981; Kanne et al., 1982).

The response of the excision repair system to photoproducts of HMT was similar to that reported for AMT and angelicin; although the overall adduct frequencies in α DNA and bulk DNA were similar, repair synthesis in and removal of adducts from α DNA were only about 20% of those from the bulk (Figures 1 and 2). It should be noted that although the HMT concentration used for the repair replication experiments was over a hundredfold greater than that used with the [^{3}H]HMT, the repair deficiencies were similar.

The most attractive candidate for an adduct refractory to repair was one of the two diastereomers of the furan–T monoadduct, since these are the major products formed. Therefore, we developed an HPLC procedure that provided

increased resolution of these two adducts so that we could examine their relative proportions in various samples. We found that the relative proportions of these two major adducts were the same in α DNA and bulk DNA, whether modification was in vitro or in vivo (Figure 5). In addition, each of the two diastereomers appears to be converted to cross-links at the same rate. This was shown directly in bulk DNA in vivo (Table IV) and can also be inferred from the fact that the two furan–T diastereomers were in the same relative proportions in all the DNA samples analyzed, regardless of the overall monoadduct:cross-link ratios. We also found that each of the furan–T monoadducts was removed from the bulk of the DNA at the same rate (Table III). In addition, the minor monoadducts were shown to constitute about the same proportions of total bound psoralen in α DNA and bulk DNA, both in vitro and in vivo. Therefore, the deficient repair of furocoumarin adducts from α DNA cannot be due to an increased formation in α DNA of some major adduct that is intrinsically refractory to repair. This is also supported by our previous finding that repair of α DNA in cells treated with *N*-acetoxyacetylaminofluorene is also inefficient (Zolan et al., 1982a) and our more recent demonstration that removal of the primary adducts of activated aflatoxin B_1 is markedly deficient in α DNA (Leadon et al., 1983). For these compounds, in contrast to the furocoumarins, a single major adduct species is formed, and the mechanism of adduct formation does not require intercalation into the DNA.

Our measurements of the frequencies of the various adducts of HMT did reveal one striking difference between α DNA and bulk DNA in vivo. At the same overall level of modification, the frequency of cross-links in α DNA was considerably less then that in the bulk DNA. This was demonstrated at two different levels of total modification and cross-link frequency (Table II). The major cross-link species are formed by further photoaddition of the unreacted pyrone end of a furan–T monoadduct to a thymine in the opposite strand. Therefore, although monoadducts form with equal frequency in α DNA and bulk DNA, the process of conversion of monoadducts to cross-links is somehow inhibited in α DNA in vivo. This was not the case when DNA was treated in vitro; the monoadduct:cross-link ratios for α and bulk DNA were the same (Table I).

These results imply that α sequences have an unusual chromatin conformation in these cells under the conditions of our experiments. It is possible that some property of this putative special chromatin structure is responsible for the deficient recognition and/or repair of certain types of chemical damage in α DNA. For example, such a chromatin structure might restrict access of certain cellular repair enzymes to damage in α DNA.

Our data (Table II) show that there is not a barrier to the formation of cross-links in α but that the rate of conversion of the furan–T monoadducts to cross-links is lower than for the monoadducts in the bulk of the DNA. We have not carried out an experiment in which cells are reirradiated after removal of unbound psoralen to test whether the maximum frequency of cross-links attainable in α DNA is different from that in bulk DNA.

It seems most likely that the altered rate of cross-link formation is due to some increased constraint in the DNA in α-chromatin. Johnston et al. (1981) reported that after the formation of a monoadduct in DNA by reaction at its furan end, a delay of about 1 μs is necessary before cross-link formation can take place, suggesting that a reorientation of the unreacted pyrone end of the psoralen with respect to the unreacted DNA strand must occur for proper juxtaposition of the sites for the second photoaddition. Peckler et al. (1982) have recently determined the crystal structure of the furan–T monoadduct formed by 8-methoxypsoralen. Model-building studies based on this structure indicated that, besides unwinding the DNA, a cross-link introduces a kink that may approach 70°. The DNA participiating in furocoumarin photoaddition in chromatin is known to be the internucleosomal, linker DNA (Hanson et al., 1976; Cech & Pardue, 1977; Zolan et al., 1982b). A more condensed chromatin structure might reduce the flexibility of the linker DNA, thus lowering the quantum yield for cross-link formation. This type of structure might be expected for α-chromatin, since this DNA is not transcribed.

The character of this putative altered chromatin structure is rather subtle since it does not prevent cross-link formation, does not alter the rates of the initial photobinding, and does not appreciably limit access of the linker DNA to small enzymes like staphylococcal nuclease, DNase II, or *Eco*RI (Fittler & Zachau, 1979; Brown et al., 1979; Singer, 1979). In work to be presented elsewhere, we show that a number of adducts whose repair is thought to be initiated by small glycosylases or AP endonucleases are repaired proficiently in α, suggesting that if the chromatin structure limits access to repair enzymes, it does so only for the system that recognizes bulky adducts, and which may be a large enzyme complex. As discussed previously, proficient repair in α of pyrimidine dimers may occur because introduction of these lesions in nucleosome core DNA may alter the chromatin structure (Zolan et al., 1982a).

Comparisons of α to "bulk" DNA should not be taken to imply that bulk DNA is homogeneous with respect to chromatin structure or repair. Many different chromatin conformations undoubtedly exist and may affect repair of DNA to different extents. The 20–30% repair in α DNA we do observe may reflect transition of one kind of structure to another near the ends of the α arrays or to whole arrays of α in different structures.

To date, we have confined our studies to confluent cultures, in which the cells are largely quiescent and probably in a G_0-like state. The amount of normal DNA synthesis we observe in the unirradiated cells is much less than 20–30% of the synthesis that would take place in exponentially growing cells; thus, the repair that does take place in α DNA cannot be attributed to the presence of cycling cells in the population. However, chromatin structure must undergo changes before or during DNA replication and mitosis, and α DNA may be more efficiently repaired in other phases of the cell cycle.

This study adds another example to the increasing number of instances in which furocoumarins have provided sensitive probes for nucleic acid conformation in living cells (Hanson et al., 1976; Hearst, 1981; Sinden et al., 1980, 1983; Wiesehahn et al., 1977).

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Stimulation of Yeast RNA Polymerase II Transcription by Critical Values of Supercoiling†

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ABSTRACT: RNA chains of discrete length were obtained in vitro by yeast RNA polymerase II directed transcription of a supercoiled plasmid. On the basis of the amount and the molecular weight of the RNA chains synthesized in the absence of reinitiation events, the number of actively transcribing RNA polymerase molecules has been calculated. A stimulation of transcriptional activity was found to be related to the torsional strength of negative supercoiling of the template. The DNA unwinding angle measured in the complexes formed with the enzyme in the presence of three ribonucleoside triphosphates equals 485 ± 30°, marking a melting effect of 14 base pairs per bound enzyme molecule.

Supercoiling of the DNA template is now considered an essential requirement for the in vitro transcription by purified eukaryotic RNA polymerase II. In previous works (Ballario et al., 1981; Pedone et al., 1982), we have studied the in vitro expression of cloned yeast sequences such as the 2-μm DNA and the yeast transposable element Ty1. These sequences are known to be in vivo under the control of RNA polymerase II. We have shown that yeast RNA polymerase II prefers supercoiled templates to relaxed and linear ones and that a modulation of in vitro transcriptional activity is related to the degree of supercoiling of the template.

As reported by Lescure (Lescure, 1983), when the rNTP[1] concentration is lowered to submillimolar values, purified yeast RNA polymerase II can initiate and terminate transcription at specific points on a supercoiled template. We have adopted this protocol for a cloned fragment of the yeast transposable element Ty1 and obtained a reproducible pattern of RNA chains synthesized in vitro by yeast RNA polymerase II. The RNA synthesis was also performed in the presence of heparin to prevent reinitiation events so as to correlate the number of RNA chains produced with that of active RNA polymerase molecules.

To assess the effect of the degree of supercoiling of the DNA template on transcription, we have prepared an allomorphic series of the cloned circular DNA template at increasing values of negative superhelical densities. Yeast RNA polymerase II transcribes linear or relaxed circular molecules poorly; on the contrary, we observed a stimulation of transcription of supercoiled templates mainly at values of supercoiling close to those of the native conformation.

An attempt to measure the DNA unwinding angle induced by RNA polymerase on the template was made by following the strategy reported by Gamper & Hearst (1982) for the prokaryotic *Escherichia coli* RNA polymerase. Binary complexes between DNA and yeast RNA polymerase are not as stable as those with the prokaryotic enzyme, but the addition of three rNTPs enabled us to measure the extent of DNA unwinding induced by the enzyme.

Materials and Methods

Template. The subclone p30-δ (generous gift from P. Philippsen) was derived from the original yeast clone TyD15 (Cameron et al., 1979) and contains 750 base pairs of yeast insert, including 300 base pairs of one of the direct repeats of yeast transposable element Ty1. The insert is cloned in pBR322 between *Bgl*II and *Sal*I restriction sites. Native

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EB, ethidium bromide; PEI-cellulose, poly(ethylenimine)-cellulose; rNTP, ribonucleoside triphosphate; scDNA, supercoiled DNA; EDTA, ethylenediaminetetraacetic acid.

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