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Photoreversal-Dependent Release of Thymidine and Thymidine Monophosphate from Pyrimidine Dimer-Containing DNA Excision Fragments Isolated from Ultraviolet-Damaged Human Fibroblasts†

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ABSTRACT: To elucidate the enzymatic excision-repair process operative on cyclobutane-type pyrimidine photodimers in human dermal fibroblasts, we have examined excised dimer-containing material recovered in the trichloroacetic acid soluble fraction from far-ultraviolet-irradiated (254 nm, 40 J m^{-2}) and incubated (24 h) cell cultures. The excised DNA photoproducts were found in oligonucleotide fragments with an estimated mean chain length of ~3.7 bases. Exposure of these isolated excision fragments, labeled with [^{3}H]thymidine (dT), to a secondary, dimer-photoreversing fluence of far-UV (5.5 kJ m^{-2}) resulted in the release of free dT and thymidine monophosphate (TMP). Photorelease of these two radioactive species was measured by high-performance liquid chromatography, with TMP being detected as the increase in dT following bacterial alkaline phosphatase treatment. These data imply that the photoliberated dT and TMP moieties were attached to the excision fragments solely by the cyclobutane ring of the dimer. No evidence was obtained for the photoliberation of free thymine, thus corroborating a conclusion reached by others that the excision of dimers in human cells is not initiated by scission of an intradimer *N*-glycosyl bond. The sum of the tritium label recovered in dT plus TMP corresponded to approximately 40% of that disappearing from thymine-containing dimers on photoreversal, suggesting that in about 80% of the isolated excision fragments the dimer (i) is located at one end of the oligonucleotide and (ii) contains a break in its internal phosphodiester bond. These findings, coupled with our recent studies on xeroderma pigmentosum complementation group A and D cells [Paterson, M. C., Gentner, N. E., Middlestadt, M. V., & Weinfeld, M. (1984) *J. Cell. Physiol., Suppl. 3*, 45–62], lead us to propose cleavage of the intradimer phosphodiester linkage as the initial reaction in the multistep excision-repair process acting on pyrimidine dimers in the DNA of normal human cells.

Cyclobutyl dimers formed between adjacent intrastrand pyrimidines constitute the major photoproducts induced in the DNA of living cells by UV[1] radiation (Setlow, 1966). Studies of the metabolic fate of these lesions in both prokaryotes and eukaryotes have thus far established two principal processes for their restitution, enzymatic photoreactivation, and nucleotide excision repair. The former appears to be specific for pyrimidine dimers; this process is mediated by a DNA photolyase, an enzyme that binds to a dimer-containing site and, upon absorption of visible light energy, simply reverses in situ the cyclization reaction, thereby regenerating the two constitutive pyrimidines (Sutherland, 1978). Photolyase activity has been detected in a wide variety of organisms ranging from simple bacteria to complex mammals, and the enzyme has been

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[1] Abbreviations: BAP, bacterial alkaline phosphatase; C◇C, cyclobutane-type photodimer between cytosine and cytosine; C◇T, cyclobutyl cytosine–thymine photodimer; dT, thymidine; HPLC, high-performance liquid chromatography; OD_{260}, optical density measured at 260 nm; ODS, octadecylsilyl; PBS, phosphate-buffered saline; Py◇T, cyclobutyl pyrimidine (cytosine or thymine)–thymine photodimer; SAX, strong-anion exchange; TCA, trichloroacetic acid; TMP, thymidine monophosphate; Thy, thymine; T◇T, cyclobutyl thymine–thymine photodimer; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; U◇T, cyclobutyl uracil–thymine photodimer; U◇U, cyclobutyl uracil–uracil photodimer; UV, ultraviolet; XP, xeroderma pigmentosum.

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purified to homogeneity from *Streptomyces griseus* (Eker & Fichtinger-Schepman, 1975) and *Escherichia coli* (Sancar et al., 1984). In nucleotide excision repair, remedial action is achieved by the release of a dimer-containing single-strand fragment followed by replacement synthesis of a normal nucleotide sequence (Friedberg et al., 1981; Paterson et al., 1984). Excision of the dimer is accomplished by two flanking single-strand nicks (detailed below); a DNA polymerase then inserts correct nucleotides into the gap, utilizing the intact complementary strand for base-pairing instruction, and finally, the site is sealed by a DNA ligase.

Detailed analyses of the excision-repair process acting on dimers in in vitro bacterial systems have revealed interspecies differences, particularly in the early events. In *Micrococcus luteus* and T4 phage infected *E. coli*, for example, dimers are initially operated on by a DNA glycosylase that hydrolyzes the *N*-glycosyl bond on the 5′-pyrimidine member of the dimer pair prior to cleavage of the phosphodiester linkage on the 3′ side of the resultant baseless deoxyribose by an apurinic/apyrimidinic endonuclease (Haseltine et al., 1980; Demple & Linn, 1980; Radany and Friedberg, 1980; Nakabeppu & Sekiguchi, 1981). Subsequent cleavage of several nucleotides downstream from (i.e., on the 3′ side of) the dimer by an exonuclease leads to the release of an oligodeoxyribonucleotide fragment containing a dimerized thymine–thymidylate moiety at its 5′ end. In support of this two-step mechanism for strand incision first proposed by Haseltine and co-workers (Haseltine et al., 1980), the base Thy can be released from dimer-containing excision fragments isolated from *M. luteus* by exposure to a photoreversing fluence of UV light.

In contrast, no such photoreleasable Thy is present in the dimer-containing oligonucleotides excised during the course of repair of UV-damaged DNA in *E. coli* cells or cultured human fibroblasts (La Belle & Linn, 1982). This failure to detect photoliberation of Thy in excision fragments from *E. coli* is consistent with gene cloning experiments, indicating that dimer excision in this prokaryote is mediated by a multienzyme complex, termed UVRABC excinuclease, which concomitantly cuts the sugar–phosphate backbone at the eighth phosphodiester bond on the 5′ side of the photoproduct and the fourth bond on the 3′ side (Sancar & Rupp, 1983). The net result is release of a single-strand fragment that is apparently unmodified in the immediate proximity of the dimer.

The sequence of reactions initiating dimer removal in human cells has yet to be defined precisely. Evidence suggesting that the process may differ from that occurring in either *M. luteus* or *E. coli* arose from our earlier observation that enzymatic photoreactivation (*S. griseus* plus light) of DNA isolated from post-UV-incubated XP complementation group A or D cells led to the appearance of single-strand breaks (Paterson & MacFarlane, 1983; Paterson et al., 1984). These photoreactivation-induced breaks were not seen in DNA from similarly treated normal controls. This unforeseen finding suggested that during post-UV incubation of XP A or D cells the intradimer phosphodiester bond at such modified dimer-containing sites may be ruptured and that individual chains were then held together solely by the cyclobutane ring linking the two bases.

It occurred to us that these novel sites arising in XP A or D cells might reflect not an aberrant repair event but rather an accumulation of intermediates in a process that routinely takes place in normal cells in the course of excising dimers from UV-damaged DNA, i.e., that our inability to detect the accumulation of these sites in UV-treated normal cells might have been simply due to their rapid removal by ensuing reactions that take place in a fully functional repair process. We further reasoned that if (i) incision of the intradimer phosphodiester bond does occur in normal cells prior to the excision of a dimer-containing oligonucleotide and (ii) the dimer is situated at one end of the isolated excision fragment, then photoreversal of the excision products should result in the release of free dT or TMP. This paper details results of an experimental approach that strongly suggests that this is indeed the case. [Preliminary accounts of our findings have been reported elsewhere (Gentner et al., 1984; Paterson, 1986; Paterson et al., 1984, 1985; Weinfeld et al., 1984).]

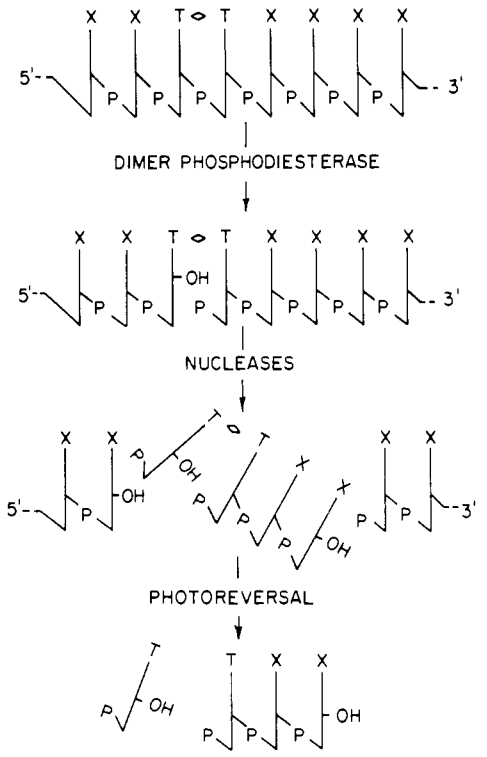

FIGURE 1: Rationale for investigation into early reactions in the nucleotide excision-repair process operative on UV-induced cyclobutyl pyrimidine dimers in the DNA of human fibroblasts. In our working model, excision of dimer-modified sites is achieved by enzymatic hydrolysis of the phosphodiester linkage between dimer-forming pyrimidines followed by classical strand incision and lesion excision events. The released dimer-containing fragments, which may subsequently be subjected to postexcision digestion in vivo, are then isolated in the TCA-soluble fraction of cell lysates and assayed for liberation of dT and TMP (shown here) upon photochemical cleavage of the cyclobutane ring joining the two pyrimidine bases.

Materials and Methods

General Experimental Plan. The overall experimental protocol followed here was essentially that described by La Belle and Linn (1982). In brief, cultured human fibroblasts, whose DNA had been prelabeled with [^{3}H]dT, were exposed to far-UV radiation and incubated for 24 h; the cell cultures were then lysed, and the dimer-containing excision fragments (soluble in 5% TCA) were isolated and analyzed by HPLC to determine if Thy, dT, or TMP was liberated after photochemically induced reversal of dimers (see Figure 1). This overall protocol was repeated on three different occasions with almost identical results. Data from a single experiment are shown here.

Fibroblast Strains and Their Cultivation. Our studies were conducted on the following two diploid fibroblast strains established from skin biopsies of human subjects: GM38 (9-year-old normal female) and XP6BE (19-year-old female with XP). The former strain was purchased from the NIGMS

Human Genetic Mutant Cell Repository (Camden, NJ) and the latter from the American Type Culture Collection (Rockville, MD). XP6BE, designated CRL 1157 (Cay Wen) by its supplier, has been assigned to complementation group D (Friedberg et al., 1979).

The strains were grown as monolayer cultures in Ham's F12 medium supplemented with 10% (v/v) fetal calf serum, 1 mM glutamine, 100 units of penicillin G mL^{-1}, and 100 μg of streptomycin sulfate mL^{-1} (henceforth referred to as growth medium). Cultures were routinely incubated at 37 °C in a humidified (75–85%) atmosphere of 5% CO_2–95% air. Cell samples were periodically verified to be free of *Mycoplasma* contamination by the [^{14}C]uracil/[^{3}H]uridine uptake assay of Schneider et al. (1974). Unless otherwise indicated, all cell culture supplies were obtained from Gibco/BRL, Inc. (Burlington, Ontario).

Radioactive Labeling of Cellular DNA. In the experiment to be detailed here, $\sim 10^6$ cells of the normal strain GM38 were seeded in each of 48 plastic Petri dishes (diameter 15 cm; Lux Scientific Corp., Newbury, CA) and incubated for 24 h to establish exponential growth. The cultures were incubated for an additional 72 h in Thy-free growth medium containing [^{3}H]dT (5 μCi/mL; stock sp act. 52.2 Ci/mmol; NEN Canada, Montreal, Quebec). The radioactive medium was then aspirated, after which each monolayer culture was washed twice with 20 mL of PBS (Dulbecco & Vogt, 1954). Upon addition of fresh nonradioactive growth medium, the cultures were incubated overnight to deplete endogenous nucleotide precursor pools of tritium label. XP6BE cells, which are known for their marked deficiency in dimer excision (Ehmann et al., 1978), were also plated out at $\sim 10^6$ per Petri dish in 16 dishes; these mutant fibroblasts were thereafter subjected to the same manipulations and treatments as the normal fibroblasts.

UV Irradiation and Subsequent Incubation of Cell Cultures. Upon discarding the postlabeling "chase" medium, the monlayer cultures were once again washed twice with PBS and were then drained thoroughly. The attached fibroblasts were immediately exposed in open dishes to a fluence of 40 J m^{-2} delivered by two 15-W germicidal (low-pressure mercury vapor) lamps (Model GE 15T8; General Electric, Toronto, Ontario), emitting 97% of their radiant energy at 254-nm wavelength. The incident fluence rate of the UV radiation source was 1.27 W m^{-2}, as calibrated by potassium ferrioxalate actinometry (Jagger, 1967). Five of the GM38 dishes were sham-exposed; these cultures served as unirradiated controls. Five of the 43 UV-treated GM38 dishes were stored immediately at 0 °C; these cultures served as irradiated, nonincubated controls. The remaining dishes were then replenished with fresh growth medium, and the UV-treated (or sham-treated) cultures were incubated for 24 h.

Isolation of Excision Fragments. After completion of post-UV incubation, the cells in all 48 dishes were harvested by trypsinization, suspended in ice-chilled PBS containing Mg^{2+} and Ca^{2+} cations to inhibit the trypsin, and collected by centrifugation (1000*g* for 10 min at 4 °C). The pelleted cells were then resuspended in ice-cold PBS, pooled, and recentrifuged as above. After each pellet was taken up in 1–2 mL of 0.1 M Tris-HCl (pH 7.5) and an equal volume of ice-chilled 10% TCA was added, the resultant cell lysate was mixed thoroughly and held on ice for 15 min. The TCA-soluble and -insoluble fractions were separated by centrifugation (19000*g* for 10 min at 4 °C), after which the soluble fraction was extracted 5 times with 5 volumes of ice-cold diethyl ether to remove trace amounts of TCA. Finally, the resulting aqueous phase was taken to dryness, stored, and subsequently dissolved in water (1.3 mL) immediately before analysis of the dimer-containing excision fragments for photoreleasable products. The TCA-insoluble pellet containing bulk genomic DNA from the cell lysate was dissolved in 2 mL of 88% formic acid. Multiple (i.e., 10-, 20-, and 30-μL) aliquots were withdrawn, mixed with $\sim$10 mL of PCS cocktail (Amersham, Oakville, Ontario), and counted in a liquid scintillation system to determine the total TCA-insoluble radioactivity.

Analysis of Photoreleasable Products from Excision Fragments. A six-stage procedure utilizing reverse-phase HPLC was developed to monitor the liberation of Thy, dT, and TMP associated with photochemical reversal of excised dimers isolated from post-UV incubated normal (GM38) cells. The treatment protocol and rationale behind the performance of the various steps are outlined in Figure 2. The four basic treatments involved in carrying out the six-step procedure are now described in detail.

(*1*) *Photochemical Reversal of Excised Dimers.* An aliquot of the TCA-soluble material [$(0.5–1.0) \times 10^4$ cpm] was first diluted to 2 mL with 10% aqueous ethanol and then placed in an open Petri dish (diameter 35 mm). The solution was irradiated with 5.5 kJ m^{-2} germicidal light (incident fluence rate 3.7 W m^{-2}) to monomerize pyrimidine dimers, taken to dryness, and redissolved in an appropriate volume of water.

(*2*) *Bacterial Alkaline Phosphatase Digestion.* A sample of the acid-soluble fraction ($\sim 5 \times 10^3$ cpm) was incubated at 37 °C for 1 h in a 100-μL solution containing 10 mM $MgCl_2$, 0.5 mM TMP, and 0.5 unit of BAP (Sigma Chemical Co., St. Louis, MO); TMP was added as a UV marker to ensure that hydrolysis had gone to completion. This reaction was performed to convert TMP to its nucleoside and thereby measure the quantity of TMP in the sample, since TMP is obscured in the original extract by the dimer-containing excision fragments. At the completion of the digestion period, the reaction mixture was loaded directly onto the reverse-phase HPLC column.

(*3*) *Formic Acid Hydrolysis.* Samples of the TCA-soluble material, each containing $(0.5–1.0) \times 10^4$ cpm, were hydrolyzed in 1 mL of 88% formic acid in sealed thick-wall Pyrex tubes by heating at 180 °C for 45 min. This treatment, which converts DNA to free bases and acid-stable dimer photoproducts, results in the deamination of cytosine (in dimers) to uracil; hence, cytosine-containing dimers subsequently appeared as uracil-containing photoproducts in HPLC chromatograms (see below). The formic acid was removed by evaporation, whereupon the hydrolysate was redissolved in water before being applied to an HPLC column.

(*4*) *HPLC Analysis.* The instrumentation used for the reverse-phase chromatographic studies has been described previously (Cadet et al., 1983); the system was coupled to a Beckman Model 165 scanning detector (Beckman Instruments, Toronto, Ontario) for our work. The separation of the radioactive species in the variously treated TCA-soluble samples was performed on a Whatman Partisil-10 ODS-2 column (250 × 4.6 mm i.d.; Whatman Inc., Clifton, NJ), employing isocratic elution with 5 mM sodium potassium hydrogen phosphate buffer (pH 6.5), containing 1% methanol, at a flow rate of 0.5 mL/min. The first 40 fractions collected each contained 15 drops ($\sim$0.27 mL); thereafter, the number of drops per fraction was increased to 45.

The amount of TMP was independently measured by anion-exchange HPLC. This analysis was conducted on a Whatman Partisil 10 SAX/25 column (250 × 4.6 mm i.d.)

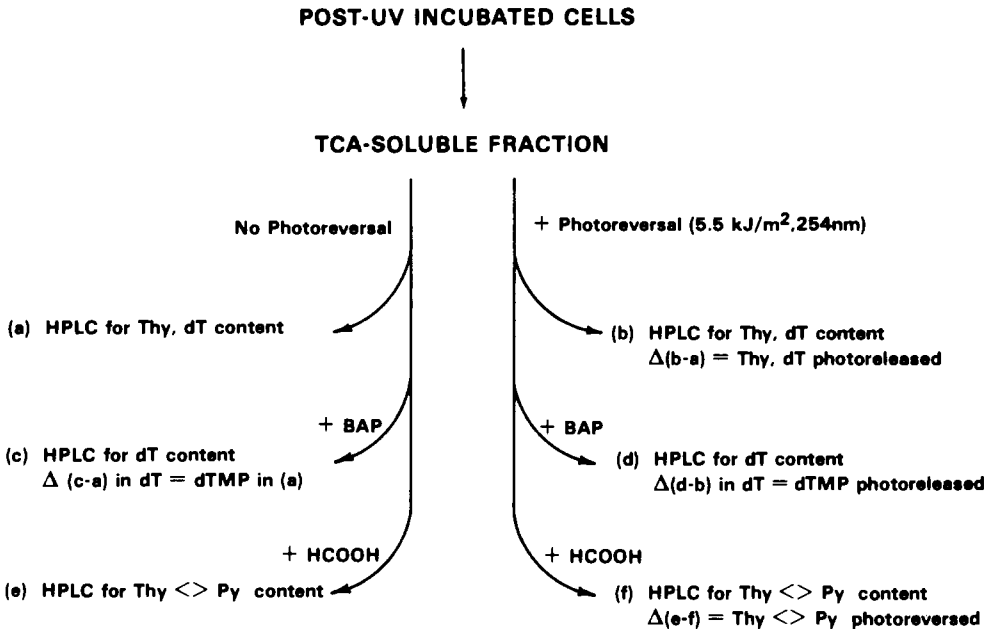

FIGURE 2: Six-stage protocol adopted to assay for dimer photoreversal-dependent release of Thy, dT, and TMP from TCA-soluble excision fragments isolated from far-UV-irradiated (40 J m^{-2}) and incubated (24 h) human dermal cells. The physical layout of the six steps denoted a–f is repeated in the reverse-phase HPLC chromatograms in Figure 3.

Table I: Yield of Excised Thymine-Containing Dimers Recovered from the TCA-Soluble Fraction of Post-UV Incubated Human Fibroblasts

strain	UV fluence (J m^{-2})	incubation time (h)	[^{3}H]Thy (cpm $dish^{-1}$) acid soluble, [A]	[^{3}H]Thy (cpm $dish^{-1}$) acid insoluble (×10^{-6}), [B]	% Thy acid soluble, [A/(A + B)] = [C]	% Thy dimerized in acid-soluble fraction T◇T, [D]	% Thy dimerized in acid-soluble fraction U◇T,[a] [E]	% of total Thy in excised dimers T◇T, [C × D] = [F]	% of total Thy in excised dimers U◇T, [C × E] = [G]	dimers excised per cell (×10^{-4})[b] T◇T, [F × H × 0.5]	dimers excised per cell (×10^{-4})[b] U◇T, [G × H]
GM38	0	24	84	2.97	0.0028	0.0	0.0	0.0	0.0	0	0
GM38	40	24	2189	2.66	0.0823	25.2	23.8	0.0207	0.0196	33	63
XP6BE	40	24	773	4.03	0.0192	3.2	3.1	0.0006	0.0006	1	2

[a] Note that U◇T dimers are secondary photoproducts resulting from the deamination of C◇T dimers during formic acid hydrolysis of excision fragments. [b] Calculations were based on the following considerations: (i) In an asynchronous population, each human diploid fibroblast has 6×10^{-12} g—that is, 3.6×10^{12} daltons—of genomic DNA (Sober, 1968). (ii) Assuming the average molecular weight of a mononucleotide to be 327 daltons, there are 1.10×10^{10} nucleotides per genome. (iii) In human DNA, the fraction of nucleotides containing thymine is 0.29 (Sober, 1968); therefore, the number of thymine residues per genome equals 3.19×10^9 [H]. (iv) The percentage of radiolabel is divided by two in order to compute T◇T as a percentage of total pyrimidines. No correction is necessary for U◇T, since only the thymine can be labeled. (v) All of the excised dimers remain intact inside the cells and are recovered in the acid-soluble material.

with a Waters Associates (Mississauga, Ontario) liquid chromatograph system (an M6000A pump in tandem with an M45 pump, an M720 system controller, an M730 data module, and a U6K injector) attached to a Beckman Model 165 scanning detector. The elution gradient profile used was the following: 99% buffer A (1 mM potassium phosphate containing 10% ethanol, pH 6.6) and 1% buffer B (0.4 M potassium phosphate containing 10% ethanol, pH 6.6) for 5 min followed by a linear gradient from 1 to 100% buffer B over a period of 30 min. The 100% buffer B flow was then maintained for an additional 10 min, after which collection was stopped and the column was returned to starting conditions. The flow rate was 0.8 mL/min, and 0.4-mL fractions were collected throughout.

All HPLC buffers were prepared from double-distilled water filtered through Millipore type HA filters (pore size 0.45 μm; Millipore, Milford, MA).

Retention times of the various radioactive species under study—that is, T◇T, U◇T, Thy, dT, and TMP—were determined by measuring absorbance at 220 and 260 nm of authentic standards kindly supplied by Dr. J. Cadet (Grenoble, France) before counting tritium label.

In all cases, fractions were collected directly into scintillation vials carried in a Model 1320 Bio-Rad fraction collector (Bio-Rad Laboratories, Richmond, CA). In those fractions containing high salt (i.e., from SAX-HPLC), sufficient water (0.5 mL) was added to allow complete dissolution in the scintillation cocktail. After the addition of 10 mL of PCS cocktail, the radioactivity content of the fractions was determined in a liquid scintillation system.

Results

La Belle and Linn (1982) reported that most (if not all) of the cyclobutyl pyrimidine dimers released from the DNA of UV-irradiated human fibroblasts by the excision-repair machinery are retained within short oligonucleotides inside the cells for extended periods (≥24 h); accordingly, the vast majority of these excision products are readily recoverable in the TCA-soluble fraction of post-UV-incubated cultures. As a prelude to our primary aim in this study—the examination of dimer-containing excision fragments for dimer photoreversal-dependent liberation of dT or TMP—we first measured the amount of Py◇T dimers present in the acid-soluble material from normal (GM38) cell cultures that had been exposed to 40 J m^{-2} germicidal light and then incubated for 24 h. The protocol adopted was almost identical with that described by La Belle and Linn (1982). Our results, summarized in Table I, indicate that ~0.04% of the total label was recovered in the

Table II: Reverse-Phase HPLC of TCA-Soluble Material from Post-UV (40 J m^{-2}) Incubated (24 h) Normal Human (GM38) Fibroblasts for Release of Thy, dT, and TMP upon Photochemical Reversal of Pyrimidine Dimers in Excision Fragments

	treatment of acid-soluble material[b]			free radioactive DNA constituents (% of total acid-soluble counts)			excised dimers (% of total acid-soluble counts)	
step[a]	photoreversal	BAP	HCOOH	Thy	dT	TMP	T◇T	U◇T[c]
a	-	-		12.3	2.3			
b	+	-		9.1	5.3			
c	-	+			2.8	(0.5)		
d	+	+			11.2	(5.9)		
e	-		+	35.8			25.2	23.8
f	+		+	58.2			14.6	13.4

[a] Step in the six-stage procedure for characterization of excision fragments, as shown in Figure 2. [b] Minus, sham-treated; plus, treated; no entry, not done. [c] Derived from C◇T dimers by deamination.

form of TCA-soluble Thy-containing dimers [i.e., T◇T (0.02%) and C◇T (0.02%)]. In contrast, no such radioactivity was detected in the acid-soluble material from the unirradiated control cultures—that is, GM38 cells sham-irradiated prior to incubation in parallel with the irradiated cells.

By measuring the dimer content in the TCA-insoluble fraction of cell lysates, we determined that the yield of Py◇T dimers induced in the DNA of GM38 fibroblasts by 40 J m^{-2} of 254-nm radiation corresponded to 0.197% of the total Thy bases; in contrast, the amount of acid-insoluble radioactivity present in Thy-containing dimers from cultures incubated for 24 h after receiving the same UV treatment was equal to 0.154%, implying that ~20% of the dimers initially introduced (i.e., 0.043% of the total label) was released from cellular DNA during the incubation period (Cadet et al., 1983; our unpublished data). In general, these observations demonstrated that GM38 cells sustained and excised UV damage in amounts comparable to the values previously reported for normal human fibroblasts (Ehmann et al., 1978; Regan et al., 1968; Setlow et al., 1969). The results of these independent investigations in which dimer excision was monitored by the disappearance of radioactive Thy-containing dimers from acid-insoluble DNA in postirradiation-incubated cultures are in excellent agreement with the outcome of the complementary study discussed above wherein dimer removal was measured by the postirradiation incubation-dependent appearance of acid-soluble Py◇T counts in cellular lysates. Taken together, these data signify that our protocol permits quantitative recovery of released dimers, as expected from the findings of La Belle and Linn (1982).

Assuming that the conditions set out in footnote *b* to Table I were to hold, it can be estimated that each normal (GM38) cell excised, on average, ~33 × 10^4 T◇T dimers and ~63 × 10^4 C◇T dimers during postirradiation incubation. Hence, approximately twice as many C◇T dimers accumulated as did T◇T dimers in the 24-h incubation period, an observation consistent with the findings of Niggli and Cerutti (1983) and of Gentner and co-workers (Gentner et al., 1981) in a preliminary account from our own laboratory.

The ability of XP6BE cells to excise T◇T and C◇T dimers is also shown in Table I. It is evident that this representative XP group D strain is severely impaired in effecting the removal of either dimer species, implying a common excision-repair process for at least these two types of pyrimidine dimers. Once again, our findings are in accord with those in the literature [see, e.g., Ehmann et al. (1978)].

Having demonstrated that our protocol allowed essentially complete recovery of dimers excised by human cells in the course of restoring their UV-damaged DNA to normal structure and function, we then performed the six-stage procedure outlined in Figure 2 in order to assay for the liberation of dT or TMP from the isolated excision fragments concomitant with the photochemical monomerization of Py◇T dimers. The resulting reverse-phase HPLC chromatograms corresponding to each of the six steps are shown in Figure 3, and the results are summarized in Tables II and III.

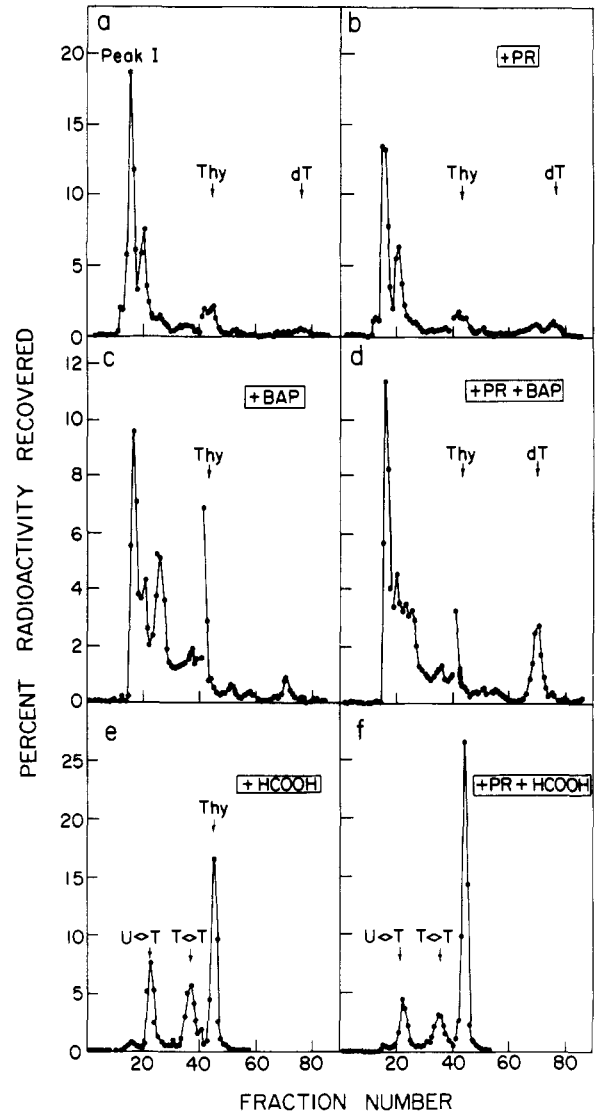

FIGURE 3: Reverse-phase HPLC elution profiles from an ODS-2 column of tritium radioactivity contained in TCA-soluble material from postirradiation-incubated human fibroblasts, according to the six-stage protocol outlined in Figure 2. Chromatographic elution and fractionation conditions were as described under Materials and Methods. The first 40 fractions contained 15 drops each; thereafter, 45-drop fractions were collected. The retention times depicted for Thy, dT, U◇T, and T◇T were obtained from the absorbance of cochromatographed authentic UV markers. PR, photoreversal.

Table III: Quantitative Relationship between Thymine Derivatives Released and Pyrimidine Dimers Reversed upon Photochemical Treatment of Excision Fragments from Post-UV (40 J m^{-2}) Incubated (24 h) Human (GM38) Cells[a]

photoreleased material (% of total acid-soluble counts)			photoreversed dimers (% of total acid-soluble counts)	
Thy	dT	TMP	T◇T	U◇T[b]
−3.2 (9.1–12.3)	3.0 (5.3–2.3)	5.4 (5.9–0.5)	10.6 (25.2–14.6)	10.4 (23.8–13.4)

[a] Data derived from Table II. [b] Derived from C◇T dimers by deamination.

Direct HPLC analysis of a sample of the acid-soluble material in the GM38 lysate resulted in the chromatographic profile illustrated in Figure 3a. One large peak, denoted peak I, and a second smaller one are evident at early retention times. Both contain predominantly Py◇T oligonucleotides, as deduced from the observation that much less TCA-soluble radioactivity in this region of the chromatogram was recovered from nonirradiated cultures (Table I) or from UV-irradiated, nonincubated cultures (data not shown). An appreciable quantity of free Thy (i.e., 12.3% of the total acid-soluble counts) and a smaller amount of dT (2.3%) are also observed (see Table II). (Note: The appearance of Thy as an apparent doublet in certain panels in Figure 3, such as a and c, is an artifact that arose during gradient fractionation due to the tripling in volume size at fraction 41 onward.) While the chromatogram in Figure 3a provides a good measure of the initial amount of dT in the acid-soluble fraction, any free TMP initially present in this sample as isolated is masked by peak I material. A second sample of the acid-soluble material was therefore digested with BAP to convert TMP to its deoxyribonucleoside (Figure 3c). The net increase in dT following this treatment, which equals 0.5% of the total TCA-soluble label (Table II), thus represents the quantity of free TMP present in the acid-soluble fraction from GM38 cells after 24 h of post-UV incubation. The levels of dT and TMP directly liberated by dimer photoreversal were determined by (i) exposing two additional samples of the TCA-soluble fraction to 5.5 kJ m^{-2} germicidal light, (ii) incubating one sample with BAP, and (iii) separating the radioactive species in both samples by HPLC. Comparative examination of the resulting chromatograms in Figure 3b,d with their nonphotoreversed counterparts in Figure 3a,c indicates that 3.0 and 5.4% of the total acid-soluble radioactivity was released as dT and TMP molecules, respectively, by the photoreversing fluence of 254-nm radiation (Tables II and III).

In contrast to what was found for dT and TMP, exposure of excision fragments to the photoreversing fluence of 254-nm light did not result in an increased level of free Thy (Figure 3a,b and Table III). This result confirms the earlier conclusion of La Belle and Linn (1982). [Note: As expected, BAP digestion by itself did not produce any measurable free base (unpublished results); hence, the bulk of the material that comigrated with Thy after BAP treatment alone or after photoreversal and BAP treatments together (panels c and d of Figure 3, respectively) must represent short oligonucleotides made more hydrophobic by either treatment; this is presumably a consequence of release of a highly hydrophilic moiety, such as inorganic phosphate or TMP.]

Finally, we measured the extent to which the Py◇T dimers in the excision fragments were photoreversed under our experimental conditions in order to determine the quantitative relationship between dT and TMP residues released and Thy-containing dimers monomerized by the photoreversing radiation. This was accomplished by subjecting an aliquot of the acid-soluble material to photoreversing light (5.5 kJ m^{-2}, 254 nm) while holding a second aliquot in the dark and then determining the dimer content of both aliquots by HPLC

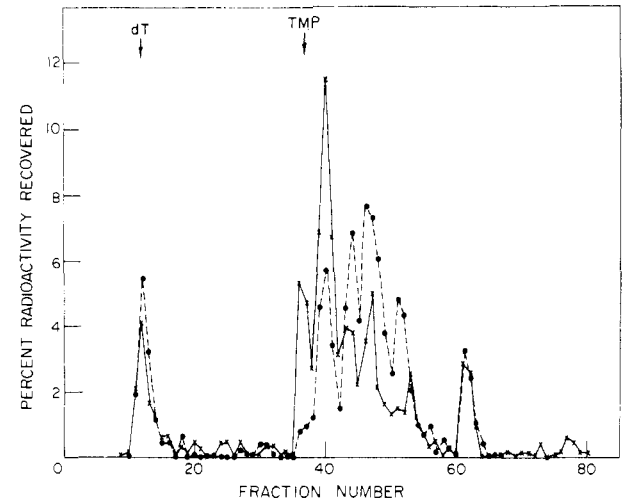

FIGURE 4: SAX-HPLC elution profiles of ^3H radioactivity in peak I dimer-containing excision fragments unexposed (●) and exposed (×) to dimer-photoreversing UV treatment. The peak I material was obtained by HPLC separation on an ODS-2 column (see Figure 3a and text). Consult Materials and Methods for details concerning chromatographic conditions. Both dT and TMP peaks were identified with authentic markers.

analysis of formic acid hydrolysates according to the method of Cadet and co-workers (Cadet et al., 1983). The resulting chromatograms are presented in Figure 3e,f. As given in Table II, 49% of the ^3H-labeled Thy resided in cyclobutyl dimers, equally distributed between T◇T and C◇T. This finding is in close agreement with that reported by La Belle and Linn (1982) for excision fragments. After photoreversing irradiation, the portion of the total acid-soluble radioactivity in dimers was reduced to 28%. Hence, the quantity of radioactivity photochemically converted from dimers to monomers was equal to 21%, with both dimer types being photoreversed at equal efficiency. Assuming that (i) the cellular DNA was uniformally labeled with tritiated Thy, (ii) dimers were located at one end of the isolated excision products, and (iii) the phosphodiester bond between the two dimer-forming pyrimidines was hydrolyzed, then each photochemically reversed T◇T or C◇T dimer should have liberated, on average, half of its radioactivity as dT/TMP. It thus follows that a maximum of 50% of the counts in photoreversed Py◇T dimers—that is, ~10.5% of the total acid-soluble label—would be released as dT and/or TMP. The actual amount of radioactive dT plus TMP recovered (8.4%; Table III) corresponded to ~80% of this theoretical maximum, and we therefore estimate that some 80% of the Py◇T dimers may have their intradimer phosphodiester bond hydrolyzed and additionally be located at one terminus of the isolated excision products.

Confirmation that TMP is released by photoreversing radiation was obtained by employing an independent system, namely, SAX-HPLC. The chromatographic behavior of the largest subfraction of TCA-soluble material (peak I obtained from the ODS-2 column; see Figure 3a) on a SAX-HPLC column is shown in Figure 4. (This subfraction represents

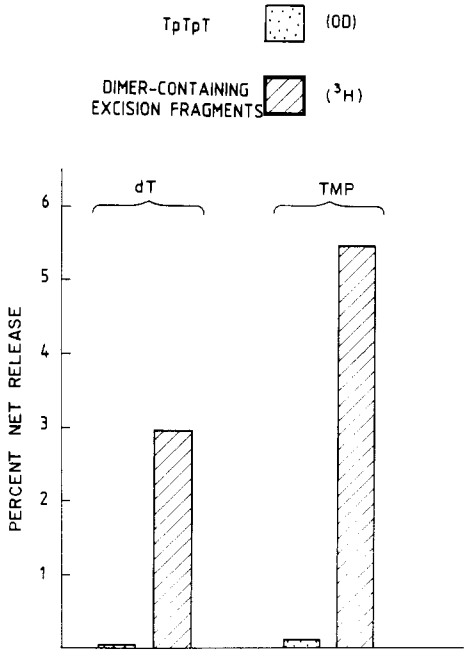

FIGURE 5: Comparison of photoreleasable dT and TMP from oligomer TpTpT and from Py◇T-containing excision fragments labeled with [^{3}H]dT. For TpTpT, monomer release is expressed as a percentage of total OD_{260} and, therefore, as a percentage of total Thy. In the case of the dimer-containing excision fragments, radioactivity in released monomers is plotted as a percentage of input radioactivity and, consequently, also as a percentage of total Thy.

the majority of the dimer-containing material; because of its pronounced hydrophilic character, it was considered to be the component most likely responible for TMP release.) In the absence of photoreversing irradiation, application of the peak I subfraction to the SAX column yielded a chromatogram characterized by five major peaks of material with mobilities similar to those of short oligonucleotides but essentially devoid of free TMP. Following photoreversal treatment, however, the level of radioactivity coeluting with a UV marker of TMP increased to approximately 9% of the total label initially present in peak I or about 5% of the total acid-soluble radioactivity. Concomitantly, there was a shift toward shorter retention times in the distribution of oligonucleotide counts, an observation consistent with the loss of a charged moiety from parental species that were previously more tightly retained. This photoreversal-dependent change in the chromatographic profile strongly supports our conclusion, reached from the reverse-phase HPLC analyses depicted in Figure 3, that TMP is photoreleasable from excision fragments isolated from postirradiation-incubated normal human cells.

We recognized the possibility existed that the large fluence of UV light required for dimer photoreversal might itself generate significant amounts of free dT and TMP. That this is not the case was suggested by the results of the following control experiments: (i) the recovery of negligible amounts of dT or TMP when solutions of either oligomer TpTpT (Figure 5) or pTpTpT (data not shown) were treated with 5.5 kJ m^{-2} germicidal light in vitro; and (ii) the inability to detect significant quantities of photoreleasable dT or TMP after radiolabeled cellular DNA, either double or single stranded, was subjected to the same fluence of photoreversing radiation (data not shown).

Discussion

Our results confirm and extend the previous studies of La Belle and Linn (1982) showing that Thy is not released on photoreversal of pyrimidine dimers in excision fragments isolated from UV-irradiated and incubated human dermal fibroblasts. More importantly, we have demonstrated the liberation of free dT and TMP in association with photochemical reversal of these same isolated Py◇T dimers. These excision products are released by the cellular excision-repair machinery in the course of ameliorating the deleterious effects of UV damage to DNA. Our inability to obtain evidence for release of free Thy upon photochemical monomerization of Py◇T dimers in the isolated excision fragments argues that cleavage of *N*-glycosyl bonds of the dimerized pyrimidines is not an intermediate step in the dimer excision-repair process operative in human cells; this observation is also consistent with the apparent absence of a dimer-DNA glycosylase activity in human cell free extracts (Lindahl, 1982). Rather, the detection of photoreleasable dT and TMP in the Py◇T-containing excision segments [but not in similarly irradiated model oligonucleotides of comparable size (Figure 5)] is consistent with the notion that the phosphodiester linkage between the dimer-forming pyrimidines has been cut prior to cell lysis and thus may be an early reaction in the process by which human cells excise UV-induced cyclobutyl pyrimidine dimers from their DNA.

The ion-exchange HPLC chromatogram in Figure 4 indicates that the excision fragments isolated from postirradiation-incubated human cells are heterogeneous in size. Nevertheless, an approximate mean length can be calculated from the radiolabel in Py◇T dimers relative to that in Thy monomers. This has been done for the HPLC analysis of HCOOH-hydrolyzed TCA-soluble material depicted in Figure 3e. In making this calculation, we have assumed (i) that each fragment contains only one dimer and (ii) that the base composition of the isolated oligonucleotides (exclusive of the dimerized bases) is the same as that for bulk genomic DNA. If these two assumptions hold, the ratio of Thy bases to cyclobutyl pyrimidine dimers in the excision products is then given by the equation:

$$(\%\text{ counts in Thy}) - (\%\text{ counts in Thy + dT + TMP before hydrolysis})/(\%\text{ counts in C}\diamond\text{T}) + 0.5(\%\text{ counts in T}\diamond\text{T}) + X(\%\text{ counts in C}\diamond\text{T})$$

where percent counts in the indicated radioactive species is expressed as a percentage of the total label in the hydrolyzed TCA-soluble material and X (a correction factor for the content of unlabeled C◇C dimers) is equal to C◇C/C◇T in excised oligonucleotides. Using our unpublished observation that C◇C/C◇T is 0.24 and obtaining the other values from Table II, the equation then becomes

$$\frac{35.8\% - 15.2\%}{23.8\% + 0.5(25.2\%) + 0.24(23.8\%)} = 0.49$$

If we take Thy as 0.29 of the bases in human DNA (Sober, 1968), we compute the mean number of remaining nondimerized bases in the isolated excision fragments to be approximately 1.7 (i.e., 0.49/0.29). And hence, the mean average length of the fragments, including the dimer, is about 3.7 bases. The correct number may be somewhat lower if dimers are preferentially formed within pyrimidine-rich regions of the genome, a possibility for which there is some evidence [see, e.g., Birnboim & Paterson (1978)].

Our results have led us to estimate that in approximately 80% of the Py◇T-containing excision fragments isolated from postirradiation-incubated human fibroblasts the dimer (i) is located at one end of the fragment and (ii) possesses a broken internal phosphodiester bond. The actual percentage of excision fragments containing such modified dimer sites may be

slightly lower if, as seems likely, a small proportion of the isolated oligonucleotides contains adjacent monomeric pyrimidines that become dimerized by the photoreversing radiation treatment. This, in turn, would result in an underestimation of the actual number of original intracellularly excised dimers that had been monomerized in vitro by the secondary irradiation. The observed shortfall in dT/TMP molecules photoreleased relative to dimers photoreversed, irrespective of the precise amount, can be readily explained if, for example, the first aforementioned condition—namely, that the dimer lies at one terminus of the isolated oligonucleotide—does not hold for every fragment. Photochemical reversal of a dimer with at least one flanking nucleotide on both sides would simply produce two shorter pieces, each at least dimeric in size. Given the calculated average length of the isolated excision fragments (~3.7 bases), it is not unreasonable that approximately 20% might be at least tetrameric and possibly contain an "internal" dimer.

On the basis of the results described here, it is of interest to point out the usefulness of utilizing, where technically feasible, lesion-containing excision fragments to study the metabolic fate of any one of the multitude of chemically disparate DNA defects subject to excision repair. First, when attempting to quantitate low levels of repair in particular cell types, as exemplified by severely defective XP group D cells (Table I), the experimentalist is able to monitor, as a function of posttreatment incubation time, an increase in the number of fragments starting from background levels; this is much preferable to the alternative conventional method in which excision of a given lesion is determined by the time-dependent reduction in its incidence in genomic DNA, an approach that requires subtraction of one large number from another. Second, and more important, the excision products represent a marked enrichment (some 1000-fold or more) of a specific defect relative to its normal (unmodified) counterpart in DNA; as a general rule, this, in turn, greatly eases the rigor with which a separation or detection system must deal with modified compared to normal moieties.

At present, we are unable to determine whether the Py◇T-containing excision fragments from postirradiation-incubated GM38 fibroblasts remain unaltered in size from the time of their enzymatic release from genomic DNA until their extraction from cultures at 24 h after UV exposure. It is entirely possible that the oligonucleotides assayed by us are first subjected to postexcision degradation by nonspecific endogenous nucleases. Indeed, exonuclease activities operative on single-stranded DNA have been identified in various human sources (Doniger & Grossman, 1976; Friedberg et al., 1977). Such activities may routinely catalyze the progressive removal of nucleotides from one or both ends of the excision fragments until becoming blocked on encountering the aberrant structure of the oligonucleotides in the immediate vicinity of the dimer. The average size of the repair patch inserted in the gap arising from removal of a dimer is now believed to be in the order of 20–25 nucleotides [derived from Figure 5 in Smith & Okumoto (1984)]; thus, the size of the excised dimer-containing excision products could conceivably be considerably larger than that (~3.7 bases) observed at the time of their eventual isolation. At any rate, if such postexcision nucleolytic digestion does take place within human cells, the process must occur within minutes (and not hours) after the excision event because the average lengths of the Py◇T-containing fragments from cultures incubated for 6 and 12 h after UV irradiation are within 20% of that found for the 24-h postirradiation cultures (unpublished observations). Likewise, our data do not exclude the possibility that the apparent cleavage of the intradimer phosphodiester bond in excision fragments may occur as a result of postexcision exonucleolytic activity. We consider this improbable, however. First and foremost, there is our evidence, alluded to at the outset, that the excision-repair process may malfunction after this intradimer incision step has occurred in XP group A and D cells (Paterson et al., 1985). Second, this phosphodiester linkage between dimerized pyrimidines appears to be refractory to all general "salvage" exonucleases so far tested (Setlow et al., 1964; M. Liuzzi and M. Weinfeld, unpublished data).

Instead, the findings presented here, in combination with our observations on XP A and D cells (Paterson et al., 1984, 1985), prompt us to postulate that breakage of the phosphodiester linkage between dimer-forming pyrimidines constitutes the initial step in the nucleotide excision-repair pathway operating on these UV photoproducts in human cells (see Figure 1). We further propose that the purpose of performing this step may be to produce a localized conformational change at the dimer-containing site such that the site is now recognizable by a generalized "bulky lesion-repair complex" possibly analogous to the UVRABC excinuclease complex in *E. coli* (Sancar & Rupp, 1983). Hence, according to our model, incision of the intradimer phosphodiester bond is then followed by classical strand incision/lesion excision/repair resynthesis/strand ligation reactions [for a detailed description of the various ramifications of the model, see Paterson et al. (1985)]. This model for dimer repair in human cells differs from that advanced for *M. luteus* and phage T4 infected *E. coli* in that the *N*-glycosyl bond on the 5′-pyrimidine of the dimer remains intact. It should be emphasized, however, that in both models the *same* phosphodiester linkage is incised: in human cells this is proposed as the first step, whereas in the two prokaryotic systems depyrimidination of the deoxyribose moiety on the 5′ side of this bond precedes the "internal incision" reaction. An attempt is now being made to identify and characterize the putative pyrimidine dimer-DNA phosphodiesterase that presumably initiates dimer removal in human cells.

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Registry No. C◇C, 10457-04-2; C◇T, 10457-03-1; dT, 50-89-5; TMP, 365-07-1; Thy, 65-71-4; T◇T, 14122-25-9; U◇T, 10457-05-3; U◇U, 4840-71-5.

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Characteristics and Variations of B-Type DNA Conformations in Solution: A Quantitative Analysis of Raman Band Intensities of Eight DNAs†

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ABSTRACT: Raman spectra were obtained from four bacterial DNAs varying in GC content and four periodic DNA polymers in 0.1 M NaCl at 25 °C. A curve fitting procedure was employed to quantify and compare Raman band characteristics (peak location, height, and width) from 400 to 1600 cm^{-1}. This procedure enabled us to determine the minimum number of Raman bands in regions with overlapping peaks. Quantitative comparison of the Raman bands of the eight DNAs provided several new results. All of the DNAs examined required bands near 809 (±7) and 835 (±5) cm^{-1} to accurately reproduce the experimental spectra. Since bands at these frequencies are associated with A-family and B-family conformations, respectively, this result indicates that all DNAs in solution have a mixture of conformations on the time scale of the Raman scattering process. Band characteristics in the 800–850-cm^{-1} region exhibited some dependence on CG content and base pair sequence. As previously noted by Thomas and Peticolas [Thomas, G. A., & Peticolas, W. L. (1983) *J. Am. Chem. Soc. 105*, 993], the poly[d(A)]·poly[d(T)] spectra were qualitatively distinct in this region. The A-family band is clearly observed at 816 cm^{-1}. The intensity of this band and that of the B-family band at 841 cm^{-1} were similar, however, to intensities in the natural DNA spectra. Three bands at 811, 823, and 841 cm^{-1} were required to reproduce the 800–850-cm^{-1} region of the poly[d(A-T)]·poly[d(A-T)] spectra. This may indicate the presence of three backbone conformations in this DNA polymer. Analysis of intensity vs. GC content for 42 Raman bands confirmed previous assignments of base and backbone vibrations and provided additional information on a number of bands.

Several investigations have shown that the structure of duplex DNA is influenced by its base pair sequence and environment. X-ray diffraction studies on DNA crystals have demonstrated three classes of DNA structures, A, B, and Z (Rich et al., 1981; Dickerson et al., 1982; Shakked et al., 1983). Small localized alterations were observed within each type or family of DNA structure (Wang et al., 1980; Drew

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