In Situ Enzymology of DNA Replication and Ultraviolet-Induced DNA Repair Synthesis in Permeable Human Cells†

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ABSTRACT: Using permeable diploid human fibroblasts, we have studied the deoxyribonucleoside triphosphate concentration dependences of ultraviolet- (UV-) induced DNA repair synthesis and semiconservative DNA replication. In both cell types (AG1518 and IMR-90) examined, the apparent K_m values for dCTP, dGTP, and dTTP for DNA replication were between 1.2 and 2.9 μM. For UV-induced DNA repair synthesis, the apparent K_m values were substantially lower, ranging from 0.11 to 0.44 μM for AG1518 cells and from 0.06 to 0.24 μM for IMR-90 cells. Control experiments established that these values were not significantly influenced by nucleotide degradation during the permeable cell incubations or by the presence of residual endogenous nucleotides within the permeable cells. Recent data implicate DNA polymerase δ in UV-induced repair synthesis and suggest that DNA polymerases α and δ are both involved in semiconservative replication. We measured K_m values for dGTP and dTTP for polymerases α and δ, for comparison with the values for replication and repair synthesis. K_m values for polymerase α were 2.0 μM for dGTP and 5.0 μM for dTTP. For polymerase δ, the K_m values were 2.0 μM for dGTP and 3.5 μM for dTTP. The deoxyribonucleotide K_m values for DNA polymerase δ are much greater than the K_m values for UV-induced repair synthesis, suggesting that when polymerase δ functions in DNA repair, its characteristics are altered substantially either by association with accessory proteins or by direct posttranslational modification. In contrast, the deoxyribonucleotide binding characteristics of the DNA replication machinery differ little from those of the isolated DNA polymerases. The K_m values for UV-induced repair synthesis are 5–80-fold lower than deoxyribonucleotide concentrations that have been reported for intact cultured diploid human fibroblasts. Thus, the "free" deoxyribonucleotide pools of the cell are probably adequate to support high rates of DNA repair synthesis in vivo, without the need for nucleotide compartmentation or "channeling". For replication, however, the K_m for dGTP is only slightly lower than the average cellular dGTP concentration that has been reported for exponentially growing human fibroblasts. This finding is consistent with the concept that nucleotide compartmentation is required for the attainment of high rates of DNA replication in vivo.

Substantial progress has been made in identifying the DNA polymerases involved in mammalian DNA replication and repair. Recent data indicate that DNA polymerase δ is involved in UV-induced[1] DNA repair synthesis (Dresler & Frattini, 1986, 1988; Dresler & Kimbro, 1987; Nishida et al., 1988) and implicate both DNA polymerase α and DNA polymerase δ in semiconservative DNA replication (Miller et al., 1985a,b; Kacmarek et al., 1986; Dresler & Frattini, 1986, 1988; Hammond et al., 1987; Dresler & Kimbro, 1987; Prelich et al., 1987a; Prelich & Stillman, 1988; Focher et al., 1988; Downey et al., 1988). The possibility that polymerase δ may be involved in both DNA replication and DNA repair synthesis is intriguing when one considers the dissimilarity of the products of these two processes. It seems likely that a single polymerase could function in both replication and repair only if its properties were significantly altered, either by direct posttranslational modification or by association with accessory proteins, prior to its participation in at least one of these processes. In situ modification of the characteristics of DNA polymerases is well documented in prokaryotic systems. The DNA replication complex of bacteriophage T4, for example, shows a dependence on dTTP concentration dramatically different from that of isolated T4 DNA polymerase; the K_m for dTTP of the replication complex ($\sim$40 μM) is much greater than that of the isolated polymerase (1–2 μM), and the replication complex shows maximal levels of DNA synthesis only at dTTP concentrations of 200 μM or above (Mathews & Sinha, 1982). This fact has considerable biologic importance because dTTP concentrations in T4-infected *Escherichia coli* are only about 65 μM, indicating that compartmentation or "channeling" of deoxyribonucleotides to sites of replication is necessary to achieve the high rates of T4 DNA replication seen in vivo (Mathews & Sinha, 1982).

Information about the enzymologic characteristics of DNA repair and replication systems in mammalian cells remains sketchy. K_m values either for a single dNTP or for all four dNTPs varied together have been measured for several subcellular replication systems (Friedman, 1974; Krokan et al., 1975; Berger & Johnson, 1976; Seki & Oda, 1977; Miller et al., 1978; Castellot et al., 1979; Reddy & Pardee, 1982; Wickremasinghe et al., 1983; van der Velden et al., 1984;

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[1] Abbreviations: UV, ultraviolet; dThd, thymidine; BrdUrd, 5-bromo-2′-deoxyuridine; BrdUTP, 5-bromo-2′-deoxyuridine 5′-triphosphate; BuPh-dGTP, N^2-(*p*-*n*-butylphenyl)-2′-deoxyguanosine 5′-triphosphate; ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate; dNTPs, 2′-deoxynucleoside 5′-triphosphates; dATP, 2′-deoxyadenosine 5′-triphosphate; dCTP, 2′-deoxycytidine 5′-triphosphate; dGTP, 2′-deoxyguanosine 5′-triphosphate; dTTP, thymidine 5′-triphosphate; K_m, Michaelis constant; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; K_i, inhibitor constant.

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Dresler, 1984; Jackson et al., 1986), for bleomycin-induced DNA repair synthesis in permeable baby hamster kidney cells (Castellot et al., 1979), and for UV-induced DNA repair synthesis in permeable human fibroblasts (Dresler, 1984). The significance of these data is limited, however, by the fact that few of the studies included controls to assure that the K_m values measured were not distorted by the presence of residual endogenous nucleotides or by deoxyribonucleotide degradation during the assay incubations. In addition, the kinetic complexity of DNA polymerase reactions (Detera et al., 1981) substantially reduces the utility of K_m values measured by simultaneously varying the concentrations of all four dNTPs.

In this paper, we report studies of the enzymology of DNA replication and UV-induced DNA repair synthesis in permeable human fibroblasts of two types (AG1518 and IMR-90). Our system for studying UV-induced DNA repair synthesis in permeable cells (Roberts & Lieberman, 1979; Dresler et al., 1982) has been extensively characterized. The permeable cells have been shown to perform all steps of DNA excision repair following UV damage, including the following: incision of damaged DNA (Dresler & Lieberman, 1983b), DNA repair patch synthesis (Dresler et al., 1982), ligation of completed repair patches (Hunting & Dresler, 1985), and the chromatin rearrangement that follows repair patch synthesis (Dresler et al., 1982). The length of the repair patches synthesized in UV-damaged permeable cells, approximately 30 nucleotides (Dresler, 1985), is essentially identical with that found in intact cells (Smith, 1978; Walker & Th'ng, 1982). The permeable fibroblast replication system was described briefly in a previous report (Dresler, 1984) and subsequently has been characterized more thoroughly (unpublished data). Replicative synthesis in permeable cells is both semiconservative and semidiscontinuous, as is replication in intact cells, and it primarily represents synthesis within replicons initiated before the cells are made permeable. Using these permeable cell systems, we have measured K_m values for dCTP, dGTP, and dTTP for DNA replication and UV-induced DNA repair synthesis. Control experiments exclude the possibility that either residual endogenous nucleotide pools or nucleotide degradation significantly influences the K_m values obtained. We have also measured K_m values for dGTP and dTTP for DNA polymerases α and δ, and we have compared these K_m values with the values measured for replication and repair to gain insight into the modifications of DNA polymerase function that are associated with their involvement in cellular DNA synthesis. In addition, comparison of the deoxyribonucleotide K_m values of DNA replication and repair synthesis with the dNTP concentrations that have been measured in intact diploid human fibroblasts (Snyder, 1984a) has suggested some conclusions about the mechanisms by which dNTPs are supplied to replication and repair synthesis sites in intact cells.

Experimental Procedures

Chemicals. Concentrations of nucleotide solutions were determined by UV absorbance. Purity of all nucleotides, determined by thin-layer chromatography on polyethyleneimine cellulose (Mathews, 1976), was greater than 95%.

Cell Culture. Human diploid fibroblasts (AG1518 and IMR-90; Institute for Medical Research) were passed into glass roller bottles, prelabeled with [^{14}C]dThd, and either used during exponential growth for studies of replication or grown to confluence and used for studies of repair synthesis (Dresler et al., 1982; Dresler & Lieberman, 1983a).

Preparation of Permeable Cells. Growth-phase or confluent cells were washed, collected, and made permeable in buffer A (10 mM Tris, pH 7.6 at 37 °C, 4 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, 3 mM dithiothreitol) as described (Dresler et al., 1982). The permeable cells were washed twice with buffer A at 4 °C and resuspended in buffer A at a concentration at $\sim 1 \times 10^7$ cells/mL. For studies of repair synthesis, the permeable confluent cell suspension was spread in a layer 1 mm thick in a plastic dish on ice and irradiated with 100 J/m^2 of UV (predominantly 254 nm) from a G15T8 lamp.

Measurement of DNA Synthesis in Permeable Cells. Portions of permeable cell suspension (0.05–0.1 mL) were mixed with 0.5 volume of reaction mix to give the following final concentrations: 40 mM Tris (pH 7.6 at 37 °C), 8 mM $MgCl_2$, 5 mM ATP, 167 mM sucrose, 2 mM dithiotheitol, 0.67 mM EDTA, either 15 mM KCl (for repair synthesis) or 75 mM KCl (for replication), and the indicated concentrations of dATP, dCTP, dGTP, and dTTP, with an α-^{32}P label in the indicated nucleotide. GTP, CTP, and UTP were not included in the replication reaction mixes used for these studies so that the data would reflect the effects of dNTP concentration solely on the elongation phase of DNA replication. Such data seem most appropriate for comparison with data for dNTP incorporation by isolated DNA polymerases. Samples were incubated at 37 °C for either 5 min (for measurement of replication) or 15 min (for measurement of repair synthesis). Reactions were stopped, and radioactivity was determined as described (Dresler et al., 1982; Dresler, 1984). Because permeable fibroblasts tend to clump together, the number of cells in each sample cannot be assumed to be constant. Therefore, for each sample, the ^{32}P radioactivity (which represents nucleotide incorporation during the permeable cell incubation) was divided by the ^{14}C radioactivity (which is proportional to the total DNA and, thus, to the total number of cells present) to give a value for specific nucleotide incorporation. Repair synthesis was calculated by taking the difference between specific nucleotide incorporation in corresponding damaged and undamaged samples. (Ratios of specific nucleotide incorporation in corresponding damaged and undamaged samples ranged from 5.1 to 29.7 in the experiments reported here.) Replication was measured as total specific nucleotide incorporation in undamaged growth-phase cells.

Determination of Apparent K_m. For determinations of apparent K_m, either replication or UV-induced repair synthesis was measured in permeable cells incubated with a number of reaction mixes containing different concentrations of the dNTP being studied (which was labeled with ^{32}P, unless otherwise indicated). The other three dNTPs were present at 100 μM, unless otherwise indicated. For each experiment, duplicate assays were performed at each nucleotide concentration and the results were averaged. The apparent K_m and V_{max} for each experiment were determined by fitting to these data a curve of the form described by the Michaelis–Menten equation:

$$v = V_{max}/(1 + K_m/S)$$

where S = the concentration of the varied dNTP, v = the measured replication or repair synthesis activity at that dNTP concentration, V_{max} = the maximal activity at infinite concentration of the varied dNTP, and K_m = the apparent Michaelis constant for the varied dNTP. Curve fitting was performed with the multiple regression FIT FUNCTION procedure of the RS/1 software package (Bolt, Beranek, and Newman) run on a Digital Equipment Co. VAX 11/780 computer.

Determination of the Stability of Deoxyribonucleoside Triphosphates under Permeable Cell Reaction Conditions. Replication reactions were prepared as described above with permeable growth-phase AG1518 cells and either 5 μM [α-^{32}P]dGTP, 50 μM dATP, 50 μM dCTP, and 50 μM dTTP

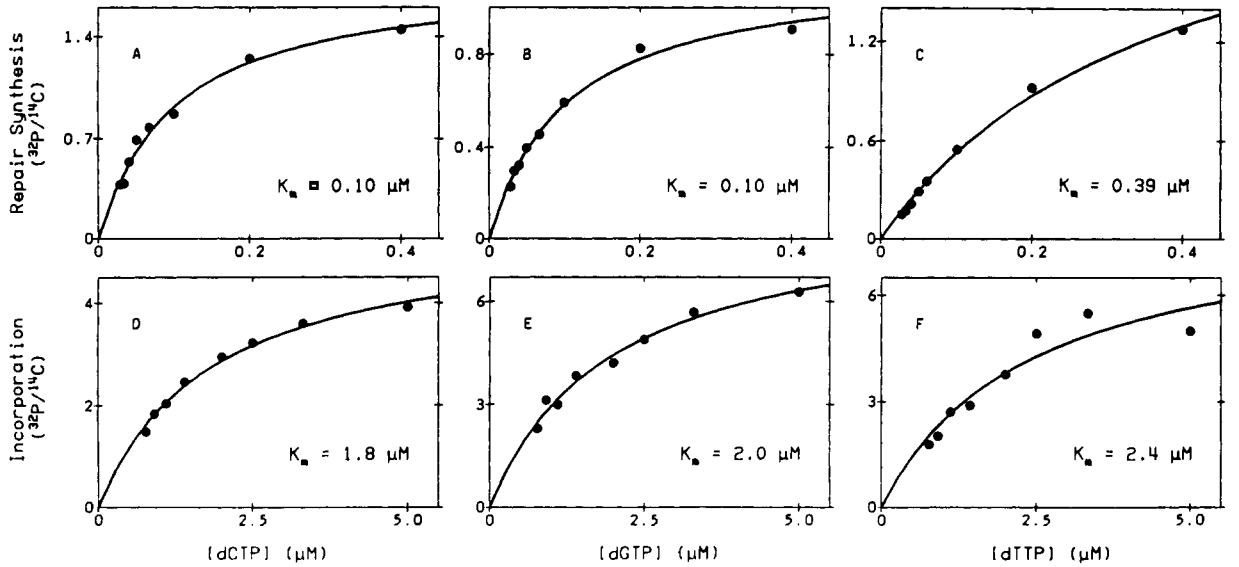

FIGURE 1: Determination of apparent K_m values for dCTP, dGTP, and dTTP for DNA replication and UV-induced DNA repair synthesis. Replication and repair synthesis were determined in permeable AG1518 fibroblasts incubated with reaction mixes containing the indicated concentrations of the dNTP being studied (which was labeled with ^{32}P) with the other three dNTPs at 100 μM. Each point is the average of two determinations. The apparent K_m was determined by fitting to the data a curve of the form described by the Michaelis–Menten equation.

Table I: Apparent K_m Values for Semiconservative DNA Replication and UV-Induced DNA Repair Synthesis[a]

		K_m (μM)			
		replication		repair synthesis	
cell type	nucleotide	std conditions	^{32}P in another dNTP	std conditions	^{32}P in another dNTP
AG1518	dCTP	1.8 (±0.5)	1.3 (dGTP)	0.11 (±0.03)	ND[b]
	dGTP	1.7 (±0.2)	1.2 (dCTP)	0.11 (±0.03)	ND
	dTTP	2.9 (±1.1)	2.1 (dCTP)	0.44 (±0.09)	0.48 (dCTP)
IMR-90	dCTP	1.7 (±0.2)	1.1 (dGTP)	0.06 (±0.01)	ND
	dGTP	1.2 (±0.2)	1.2 (dCTP)	0.07 (±0.03)	ND
	dTTP	2.7 (±1.1)	2.2 (dCTP)	0.24 (±0.05)	ND

[a] Apparent K_m values were determined in permeable human fibroblasts of the indicated type as described under Experimental Procedures. Values for standard conditions are the means of either three or four determinations, with the standard deviations in parentheses. Apparent K_m values were also measured (one determination each) with the ^{32}P label in the dNTP indicated in parentheses, which was held constant at 5 μM. [b] ND, not determined.

or 5 μM [α-^{32}P]dTTP, 50 μM dATP, 50 μM dCTP, and 50 μM dGTP. A repair synthesis reaction mix was prepared with permeable confluent AG1518 cells, 3 μM [α-^{32}P]dTTP, 3 μM dATP, 3 μM dCTP, and 3 μM dGTP. The reaction mixes were placed at 37 °C, and samples were removed at the indicated times. The fraction of the ^{32}P label in deoxyribonucleoside monophosphate, diphosphate, and triphosphate at each time point was determined by thin-layer chromatography (Mathews, 1976; Dresler, 1984).

Preparation and Assay of DNA Polymerases. DNA polymerase δ was prepared from calf thymus as described (Crute et al., 1986). The enzyme was shown to have an intrinsic 3′-5′-exonuclease and to be strongly inhibited by aphidicolin, weakly inhibited by BuPh-dGTP, and resistant to the anti-polymerase α antibody SJK 287-38. K_m values for dGTP and dTTP were determined as described above in a reaction mix containing 100 mM Tris (pH 7.6 at 37 °C), 15 mM $MgCl_2$, 100 μg/mL activated DNA (Fisher et al., 1979), 3.3 μg/mL calf thymus histone (Sigma), 10% glycerol, and 10 mM dithiothreitol, with the α-^{32}P labeled in the varied dNTP and the nonvaried dNTPs at 100 μM. Calf thymus histone was added to permit assay of polymerase δ with activated DNA as template–primer (Wahl et al., 1986). Reactions were incubated at 37 °C for 10 min, and incorporation was determined as described above.

DNA polymerase α was isolated from the HeLa human tumor cell line (Fisher & Korn, 1977) and, as expected, was highly sensitive to aphidicolin, to butylphenyl-dGTP, and to the anti-polymerase α antibody SJK 287-38 and only weakly inhibited by dideoxythymidine triphosphate. To determine K_m values, polymerase α was assayed by using activated calf thymus DNA as template–primer as described (Dresler & Kimbro, 1987), with an α-^{32}P label in the varied dNTP and the nonvaried dNTPs at 100 μM.

Results

Deoxyribonucleotide Concentration Dependences for DNA Replication and UV-Induced DNA Repair Synthesis. Apparent K_m values for dNTPs can be determined in permeable cells by measuring DNA replication or UV-induced DNA repair synthesis at a number of concentrations of a given dNTP and, with a multiple regression procedure, fitting to the data a curve of the form described by the Michaelis–Menten equation. Curves showing good fits to the data are routinely obtained (Figure 1). By fitting curves directly to the primary data, we avoid the inappropriate weighting of points at the low end of the dNTP concentration range, which occurs when one fits lines to data plotted in the double-reciprocal (Lineweaver–Burk) manner. We have made multiple determinations of apparent K_m values for dCTP, dGTP, and dTTP for both replication and UV-induced repair synthesis in permeable AG1518 and IMR-90 human fibroblasts (Table I). [K_m values for dATP were not determined because the 5 mM ATP present in the assays introduces sufficient dATP, 0.5–1.0 μM

(data not shown), that such measurements would be unreliable.] For both cell types, the apparent K_m values for UV-induced DNA repair synthesis were less than 0.5 μM, while the K_m values for replicative DNA synthesis were between 1 and 3 μM. In addition, for both cell types, the apparent K_m values for dTTP of UV-induced repair synthesis were significantly greater than the corresponding K_m values for dCTP and dGTP ($p < 0.01$ in all cases). For replication, the apparent K_m's for dTTP were also greater than the K_m's for dCTP and dGTP, but the differences were not statistically significant.

Striking features of the data in Table I are the very low K_m values obtained for UV-induced repair synthesis. We considered the possibility that these low K_m values might be an artifact, perhaps resulting from a difference in the compositions of the standard reaction mixes used for assaying repair and replication. The single difference between the standard repair synthesis and replication reaction mixes used for the K_m determinations in Table I was the presence of 15 mM KCl in the repair assays and 75 mM KCl in the replication assays. As a control, we measured K_m values for replication in the presence of 15 mM KCl and found that they did not differ significantly from those obtained at 75 mM KCl (data not shown).

We also investigated the possibility that K_m values measured in our permeable cell systems might be distorted by the presence in the permeable cells of significant levels of residual endogenous dNTPs. This possibility is significant because when the dNTP varied in a K_m determination also contains the radioactive label used for measuring incorporation, the determined K_m will be equal to the true K_m plus the concentration of endogenous dNTP (Segel, 1975). In the presence of significant levels of endogenous dNTP, an artifactually high apparent K_m would be measured. The effect of endogenous nucleotides can be circumvented by placing the radioactive label used to follow DNA synthesis in one of the three dNTPs that is held constant. This approach was used to measure the K_m values of replication for dCTP, dGTP, and dTTP and the K_m of UV-induced repair synthesis for dTTP. (Because the K_m values for dCTP and dTTP for repair synthesis were so low, we did not investigate the possibility that they were artifactually elevated by endogenous dNTPs.) The results (Table I) indicate that our K_m values are at most only slightly altered by the presence of endogenous dNTPs.

A second phenomenon that could produce an artifactually high K_m is rapid destruction of the varied dNTP during the permeable cell reaction. We have previously shown (Dresler, 1984) that dCTP is stable under permeable cell replication assay conditions. Further studies indicate that dGTP (Figure 2A) and dTTP (Figure 2B) also remain undegraded during the course of the replication assay and that dTTP is degraded only slightly during the permeable cell repair synthesis assay (Figure 2C). Thus, the high K_m values for dCTP, dGTP, and dTTP seen for replicative synthesis and the high K_m for dTTP for UV-induced repair synthesis cannot be ascribed to rapid destruction of those dNTPs during the permeable cell assays. We conclude that the K_m values in Table I represent valid measures of the enzymologic characteristics of DNA replication and UV-induced DNA repair synthesis in diploid human fibroblasts.

Deoxyribonucleotide Concentration Dependences for DNA Polymerases α and δ. Both UV-induced DNA repair synthesis (Berger et al., 1979; Ciarrocchi et al., 1979; Hanaoka et al., 1979; Waters, 1981; Snyder & Regan, 1981, 1982; Collins et al., 1982; Dresler et al., 1982; Miller & Chinault, 1982a,b; Dresler & Lieberman, 1983a; Tyrrell, 1983; Dresler, 1984)

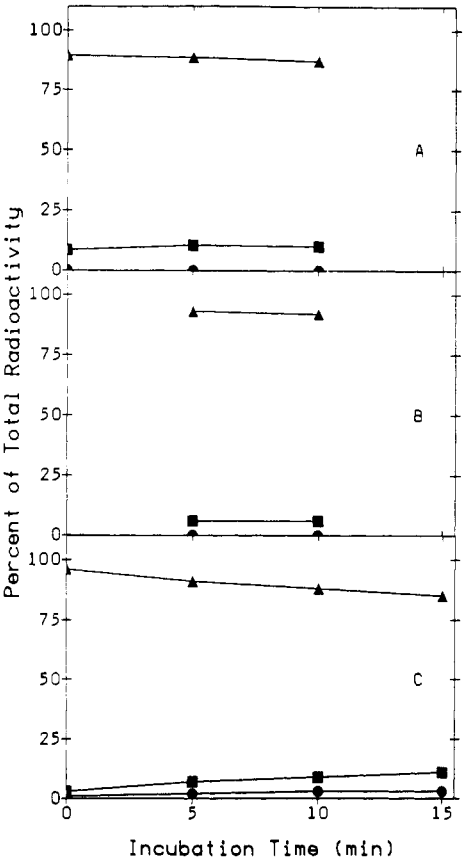

FIGURE 2: Stability of dNTPs under permeable cell reaction conditions. Replication reactions were prepared with permeable growth-phase AG1518 cells and (A) 5 μM [α-^{32}P]dGTP, 50 μM dATP, 50 μM dCTP, and 50 μM dTTP or (B) 5 μM [α-^{32}P]dTTP, 50 μM dATP, 50 μM dCTP, and 5 μM dGTP. (C) A repair synthesis reaction was prepared with permeable confluent AG1518 cells, 3 μM [α-^{32}P]dTTP, 3 μM dATP, 3 μM dCTP, and 3 μM dGTP. The reaction mixes were placed at 37 °C and sampled at the indicated times. The percentages of the total ^{32}P label present as deoxyribonucleoside triphosphate (▲), diphosphate (■), and monophosphate (●) at each time point were determined by thin-layer chromatography.

Table II: Apparent K_m Values for DNA Polymerases α and δ[a]

nucleotide	apparent K_m (μM)	
	polymerase α	polymerase δ
dGTP	2.0 (±0.8)	2.0 (±0.4)
dTTP	5.0 (±1.1)	3.5 (±0.9)

[a] Apparent K_m values were determined as described under Experimental Procedures. Values are the means of either three or four determinations, with standard deviations in parentheses.

and semiconservative DNA replication (Krokan et al., 1979; Longiaru et al., 1979; Oguro et al., 1980; Kwant & van der Vliet, 1980; Huberman, 1981) are inhibited by aphidicolin and thus are mediated by one or both of the aphidicolin-sensitive DNA polymerases, α and/or δ (Huberman, 1981; Lee et al., 1981). As mentioned in the introduction, recent data from permeable cell systems specifically implicate DNA polymerase δ in UV-induced DNA repair synthesis and suggest that polymerases α and δ may both participate in DNA replication. Involvement of polymerase δ in replication is also supported by the finding that PCNA/cyclin, a protein that is synthesized mainly in S-phase of the cell cycle (Celis et al., 1987) and acts as a specific accessory factor for polymerase δ (Prelich et al., 1987b; Bravo et al., 1987), is required for replication in an SV40 model system (Prelich et al., 1987a; Prelich & Stillman, 1988). We have measured K_m values for dGTP and dTTP for polymerases α and δ (Table II), for comparison with the

corresponding values for replication and repair synthesis. The K_m values for polymerases α and δ are similar and, for both nucleotides, are slightly greater than the K_m values for permeable cell replication.

Discussion

Our permeable cell systems have proven to be useful for studying the enzymology of DNA replication and UV-induced DNA repair synthesis in human cells. For both replication and repair, the dependence of dNTP incorporation on dNTP concentration conforms to the Michaelis–Menten equation (Figure 1), allowing one to determine K_m values for individual substrate nucleotides. Control experiments indicate that the K_m values measured in permeable cells are not significantly affected either by the presence of residual endogenous nucleotides or by dNTP degradation during the permeable cell reactions. Deoxyribonucleotide K_m values measured in diploid human fibroblasts of two types (AG1518 from neonatal foreskin and IMR-90 from fetal lung) were very similar (Table I), suggesting that the values represent general characteristics of DNA replication and repair, at least in normal fibroblasts.[2]

As mentioned in the introduction, comparison of the K_m values for cellular DNA replication and repair synthesis with the K_m values for the DNA polymerases involved in these processes may help us to identify ways in which the polymerases are modified to perform specific tasks within the cell. The K_m values for repair synthesis provide a dramatic example of this. Although DNA polymerase δ apparently mediates UV-induced DNA repair synthesis in human cells (Dresler & Frattini, 1986, 1988; Dresler & Kimbro, 1987; Nishida et al., 1988), the K_m values for UV-induced repair synthesis are 8–33-fold lower than the corresponding K_m values for isolated polymerase δ (compare Table I with Table II). A possible explanation for this disparity is that, in the cell, polymerase δ associates with other proteins to form a repair synthesis complex with characteristics substantially different from those of the polymerase itself. Although the differences between the K_m values of polymerase δ and of repair synthesis are sizable, they are no greater than the 20–40-fold increase in the K_m for dTTP of bacteriophage T4 DNA polymerase that occurs when it associates with accessory proteins to form the T4 DNA replication complex (Mathews & Sinha, 1982). It is, therefore, reasonable to suggest that modification of polymerase δ by accessory proteins could produce the K_m values observed for UV-induced repair synthesis. The one known accessory protein for polymerase δ, PCNA/cyclin (Bravo et al., 1987; Prelich et al., 1987b), which markedly increases the processivity of the polymerase (Prelich et al., 1987b), is synthesized mainly in S-phase of the cell cycle (Celis et al., 1987) and is required for replication in an SV40 model system (Prelich et al., 1987a; Prelich & Stillman, 1988), suggesting that it may be involved in cellular DNA replication. Following UV irradiation, however, a dramatic rearrangement of PCNA/cyclin takes place in the nuclei of non-S-phase human cells, suggesting that the protein may also be involved in repair (Celis & Madsen, 1986). Even if PCNA/cyclin is involved in DNA repair, its likely involvement in DNA replication, which has high deoxyribonucleotide K_m values, indicates that it is not responsible for the low K_m values of UV-induced repair synthesis.

A second possible explanation for the observed differences between the K_m values for repair synthesis and DNA polymerase δ is that a fraction of the polymerase δ in the cell undergoes posttranslational modification, generating an enzyme species that functions specifically in DNA repair. Although possible heterogeneity of polymerase δ in human cells has not been studied, two forms of polymerase δ, which apparently differ from one another as the result of proteolytic cleavage between the polymerase and exonuclease domains of the enzyme, have been isolated from calf thymus (Crute et al., 1986). Final elucidation of the biochemical basis of the low deoxyribonucleotide K_m values of repair synthesis awaits isolation and/or reconstitution in vitro of the repair synthesis machinery.

The K_m values for replicative DNA synthesis, in marked contrast to the values for UV-induced repair synthesis, are only slightly less than the K_m values for DNA polymerases α and δ (compare Table I with Table II), the enzymes that are candidates for involvement in DNA replication. Several previous studies indicate that the replicative DNA polymerase(s) in mammalian cells function(s) within multiprotein complexes like those found in prokaryotic cells (Hubscher et al., 1982; Yagura et al., 1982; Wickremasinghe et al., 1982, 1983; Noguchi et al., 1983; Ottiger & Hubscher, 1984; Ottinger et al., 1987). Our data suggest that the formation of such complexes in human fibroblasts alters only slightly the dNTP binding characteristics of the polymerase(s).

Our deoxyribonucleotide K_m data can also be used to gain insight into the mechanisms by which substrate dNTPs are supplied to sites of replication and repair. Previous studies have shown that UV-induced DNA repair synthesis in diploid human fibroblasts utilizes dATP and dGTP synthesized by "salvage" pathways from exogenous deoxyguanosine and deoxadenosine as efficiently as it utilizes dATP and dGTP synthesized de novo (Snyder, 1984b). This result suggests that dNTPs required for repair synthesis may be derived directly from "free" cellular dNTP pools, without specialized compartmentation or channeling. To test this hypothesis, we compared the deoxyribonucleotide K_m values of UV-induced repair synthesis with the deoxyribonucleotide concentrations that are likely to be found in intact cells. Taking the dNTP pool sizes for cultured diploid human fibroblasts measured by Snyder (1984a), assuming a mean cell volume of 2 pL (Williams & Friedberg, 1982), and assuming that dNTPs are evenly distributed throughout the cell, one obtains the following dNTP concentrations: for unsynchronized, exponentially growing cells, dCTP 5.7 μM, dGTP 3.2 μM, dTTP 24.7 μM; for S-phase cells (synchronized by serum starvation), dCTP 10.0 μM, dGTP 4.0 μM, dTTP 40.8 μM; for confluent (growth-arrested) cells, dCTP 4.8 μM, dGTP 2.6 μM, dTTP 2.2 μM. The deoxyribonucleotide K_m values for UV-induced repair synthesis are 29–103-fold lower than the corresponding growing cell dNTP concentrations and 5–80-fold lower than the corresponding dNTP concentrations for confluent cells. In either growing or growth-arrested cells, high rates of DNA repair could be supported by free cellular deoxyribonucleotide pools without the need for compartmentation or channeling to produce higher local dNTP concentrations at sites of repair synthesis.

[2] Castellot et al. (1979), using permeable baby hamster kidney cells, have measured a K_m for dNTPs for bleomycin-induced DNA repair synthesis of approximately 160 μM. This K_m is much greater than the values we have measured for UV-induced repair synthesis. It should be recognized, when comparing these values, that the damage produced by bleomycin (predominately strand breaks) differs markedly from that produced by UV [predominately pyrimidine dimers; see Friedberg (1985)]. Also, the DNA repair synthesis studied by Castellot et al. (1979) was mediated primarily by DNA polymerase β, while UV-induced DNA repair synthesis is mediated primarily by polymerase δ (Dresler & Frattini, 1986, 1988; Dresler & Kimbro, 1987; Nishida et al., 1988). It is not surprising, therefore, that the K_m values of these two DNA repair systems are different.

Table III: Apparent K_i Values of DNA Replication and UV-Induced DNA Repair Synthesis

inhibitor	apparent K_i (μM)	
	replication	repair synthesis
aphidicolin[a]	0.1	0.2
BuPh-dGTP[b]	3	3
ddTTP[c]	40	25

[a] Values from Dresler (1984). [b] Values from Dresler and Frattini (1988). [c] Values from Dresler and Kimbro (1987).

Our studies have also revealed that the K_m for dTTP for UV-induced repair synthesis is about 4-fold greater than the corresponding K_m's for dCTP and dGTP. The differences are statistically significant ($p < 0.01$) for both cell types examined. (Replication also shows higher K_m's for dTTP than for dCTP and dGTP, but none of the differences were statistically significant.) This situation might be an indication that dTTP is not the preferred thymidine nucleotide substrate for repair synthesis and that, in the intact cell, some of the distal steps of dTTP synthesis take place in association with the putative repair synthesis complex. Consistent with this suggestion is the finding of Keyse and Tyrrell (1985) that thymidine is used more efficiently than dTTP as a substrate for UV-induced repair synthesis in a permeable cell system identical with the one used here.

The K_m values for DNA replication lie much closer than the K_m values of repair to the dNTP concentrations calculated to be present in growing human fibroblasts. In fact, the calculated dGTP concentration for S-phase cells exceeds the K_m for dGTP of replication by only 2–3-fold. This result suggests that, in intact human fibroblasts, compartmentation of deoxyribonucleotides (at least of dGTP) may be necessary to achieve maximal rates of replicative DNA synthesis. The plausibility of this suggestion is supported by the finding that multiple functionally distinct dNTP pools exist in growing human fibroblasts. Synder (1984b) demonstrated this using cells treated with hydroxyurea, an inhibitor of ribonucleotide reductase that, by blocking de novo deoxyribonucleotide synthesis, inhibits DNA replication. Addition of deoxyribonucleosides to the culture medium of these cells restored dNTP levels to normal through the action of the "salvage" biosynthetic pathways, but failed to relieve the inhibition of replication. Apparently, dNTPs synthesized by the salvage mechanisms could not be efficiently used for DNA replication. Studies in a variety of other eukaryotic systems also have led to conclusions that dNTP pools in growing cells are compartmented [reviewed in Mathews and Slabaugh (1986)].

We presume that the very low K_m values of UV-induced DNA repair synthesis provide some selective advantage to the cell. As mentioned above, low K_m values allow repair synthesis to proceed rapidly even in situations where cellular dNTP pools are small. The relative resistance of UV-induced repair synthesis in intact cells to inhibition by hydroxyurea (Cleaver, 1969; Snyder, 1984b) is an example of this phenomenon. Because dramatic decreases in specific dNTP pools are induced in some cell types by DNA damage (Newman & Miller, 1983), low deoxyribonucleotide K_m values for repair synthesis may be advantageous even in growing cells that would, in the unperturbed state, have large dNTP pools. Another advantage is suggested by the fact that the K_m values of UV-induced repair synthesis for dGTP and TTP are substantially lower than those of replication (Table I), but the K_i values of replication and repair synthesis for the competitive nucleotide analogues BuPh-dGTP and ddTTP are very similar (Table III). The very low K_m/K_i ratios of the repair synthesis machinery suggest that it has a greater ability than the replication apparatus to discriminate against altered substrate nucleotides. DNA damaging agents can directly damage free cellular dNTPs (Topal, 1985), indicating that such discriminative ability would be of biologic value. Because DNA replication is inhibited following DNA damage (Cleaver, 1978) but repair synthesis proceeds at a rapid rate, it would be particularly advantageous for repair synthesis systems to be able to distinguish effectively between normal and altered substrate dNTPs. DNA excision repair in human cells is essentially error-free (Maher et al., 1979; Glover et al., 1979; Konze-Thomas et al., 1982; Maher et al., 1982), indicating that repair patch synthesis in vivo is a high-fidelity process, as our data suggest. It should be noted that the fidelity of any form of DNA synthesis is the net result of many factors, including the relative affinities of correct and incorrect substrate dNTPs for the DNA polymerase involved, the relative rates of incorporation into DNA of correct and incorrect substrate dNTPs that have bound to the polymerase, and the rates of exonucleolytic proofreading of incorrectly incorporated nucleotides (Loeb & Kunkel, 1982). Thus, the fact that the K_m/K_i ratios for DNA replication are higher than those for DNA repair synthesis does not necessarily indicate that the overall fidelity of replication is lower than that of repair synthesis. The data simply indicate that with respect to one type of mutagenic challenge, the discriminative ability of repair synthesis appears to exceed that of replication.

The very low K_m values of repair synthesis also have a potentially negative consequence. As Fersht (1984) has noted, a low K_m value tends to drop an enzyme–substrate complex into a thermodynamic "pit", increasing the activation energy for the reaction and decreasing the reaction rate. In fact, the rate at which repair patch synthesis proceeds is quite slow. Estimates of the time required for completion of a patch ($\sim$30 nucleotides in length) range from 3 to 10 min (Erixon & Ahnstrom, 1979; Hunting et al., 1985). Slow synthesis of repair patches may be the price paid for a repair system that effectively discriminates against damaged substrate nucleotides.

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Ribosomal Binding and Dipeptide Formation by Misacylated tRNA^{Phe}'s†

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ABSTRACT: Eight structurally modified peptidyl-tRNA^{Phe}'s were employed to study P-site binding and peptide bond formation in a cell-free system involving *Escherichia coli* ribosomes programmed with poly(uridylic acid). It was found that the two analogues (*N*-acetyl-D-phenylalanyl-tRNA^{Phe} and *N*-acetyl-D-tyrosyl-tRNA^{Phe}) containing D-amino acids functioned poorly as donors in the peptidyltransferase reaction and that two *N*-acetyl-L-phenylalanyl-tRNA^{Phe}'s differing from the prototype substrate in that they contained 2′- or 3′-deoxyadenosine at the 3′-terminus failed to form dipeptide at all when L-phenylalanyl-tRNA^{Phe} was the acceptor tRNA. Interestingly, all four of these peptidyl-tRNA's bound to ribosomes to about the same extent as tRNA's that functioned normally as donors in the peptidyltransferase reaction, at least in the absence of competing peptidyl-tRNA species. Two peptidyl-tRNA's lacking an amino group were also tested. In comparison with *N*-acetyl-L-phenylalanyl-tRNA^{Phe} it was found that *trans*-cinnamyl-tRNA^{Phe} and 3-phenylpropionyl-tRNA^{Phe}'s formed dipeptides to the extent of 53 and 80%, respectively, when L-phenylalanyl-tRNA^{Phe} was used as the acceptor tRNA. *N*-Acetyl-β-phenylalanyl-tRNA^{Phe} was found to be the most efficient donor substrate studied. Both isomers transferred *N*-acetyl-β-phenylalanine to L-phenylalanyl-tRNA^{Phe}; the nature of the dipeptides formed in each case was verified by HPLC in comparison with authentic synthetic samples. Further, the rate and extent of peptide bond formation in each case exceeded that observed with the control tRNA, *N*-acetyl-L-phenylalanyl-tRNA^{Phe}.

Peptide bond formation is one of several partial reactions that jointly constitute protein biosynthesis. The peptide bond forming process is catalyzed by peptidyltransferase; in bacteria this activity is an integral part of the 50S ribosomal subunit (Allen & Zamecnik, 1962; Nathans, 1964; Traut & Monro, 1964; Monro, 1967; Harris & Symons, 1973a,b; Symons et al., 1979). Although several studies have attempted to define the structural and spatial parameters requisite for effective participation of aminoacyl- and peptidyl-tRNA's in the peptidyltransferase reaction, most of these have relied on the use of aminoacylated oligonucleotides structurally related to the 3′-terminus of aminoacyl-tRNA (Monro & Marker, 1967; Monro et al., 1968; Mercer & Symons, 1972; Hecht, 1977; Quiggle et al., 1981) or on aminoacyl-tRNA analogues accessible via aminoacyl-tRNA synthetase catalyzed misacylations (Hecht et al., 1974; Chinali et al., 1974; Hecht, 1977; Alford & Hecht, 1978; Pezzuto & Hecht, 1980; Wagner & Sprinzl, 1983).

Recently, we have described a technique ("chemical aminoacylation") in which T4 RNA ligase was employed to couple 2′(3′)-O-acylated pCpA derivatives to tRNA-C_{OH}'s,[1] i.e., tRNA's lacking the 3′-terminal pCpA. The success of the ligation reaction was not a function of the nature of the *O*-acyl group; accordingly, a structurally diverse collection of misacylated tRNA's was prepared (Heckler et al., 1984a,b) and shown to function in ribosome-mediated peptide bond formation (Heckler et al., 1983; Roesser et al., 1986). Described herein are experiments that explore in more detail the structural requirements for ribosomal binding by peptidyl-tRNA analogues, as well as acyl group transfer from the bound species. Key findings include the ability of poly(U)-programmed *Escherichia coli* ribosomes to bind *R* and *S* isomers of *N*-acetylaminoacyl-tRNA's containing α-amino acids and the transfer of simple acyl groups from their 2′(3′)-*O*-acyl-tRNA derivatives to L-phenylalanyl-tRNA^{Phe}. Also studied was *N*-acetyl-β-L-phenylalanyl-tRNA^{Phe}, which was shown to participate in dipeptide formation more quickly and to a greater extent than *N*-acetyl-L-phenylalanyl-tRNA^{Phe}. Remarkably, both enantiomers of β-phenylalanine functioned equally as well in dipeptide formation; the structures of the derived *N*-acetyl-β-phenylalanyl-L-phenylalanines were verified

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[1] Abbreviations: tRNA-C_{OH}, tRNA missing the 3′-terminal cytidine and adenosine moieties; BD-cellulose, benzoylated (diethylaminoethyl)-cellulose; Pipes, 1,4-piperazinediethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-pressure liquid chromatography; DEAE-cellulose, (diethylaminoethyl)cellulose.

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