Effect of DNA Polymerase Inhibitors on DNA Repair in Intact and Permeable Human Fibroblasts: Evidence That DNA Polymerases δ and β Are Involved in DNA Repair Synthesis Induced by *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine†

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ABSTRACT: The involvement of DNA polymerases α, β, and δ in DNA repair synthesis induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was investigated in human fibroblasts (HF). The effects of anti-(DNA polymerase α) monoclonal antibody, (p-*n*-butylphenyl)deoxyguanosine triphosphate (BuPdGTP), dideoxythymidine triphosphate (ddTTP), and aphidicolin on MNNG-induced DNA repair synthesis were investigated to dissect the roles of the different DNA polymerases. A subcellular system (permeable cells), in which DNA repair synthesis and DNA replication were differentiated by CsCl gradient centrifugation of BrdUMP density-labeled DNA, was used to examine the effects of the polymerase inhibitors. Another approach investigated the effects of several of these inhibitors on MNNG-induced DNA repair synthesis in intact cells by measuring the amount of [^{3}H]thymidine incorporated into repaired DNA as determined by autoradiography and quantitation with an automated video image analysis system. In permeable cells, MNNG-induced DNA repair synthesis was inhibited 56% by 50 μg of aphidicolin/mL, 6% by 10 μM BuPdGTP, 13% by anti-(DNA polymerase α) monoclonal antibodies, and 29% by ddTTP. In intact cells, MNNG-induced DNA repair synthesis was inhibited 57% by 50 μg of aphidicolin/mL and was not significantly inhibited by microinjecting anti-(DNA polymerase α) antibodies into HF nuclei. These results indicate that both DNA polymerases δ and β are involved in repairing DNA damage caused by MNNG.

The roles of three DNA polymerases (α, β, and γ) have been studied extensively in mammalian cells (Fry & Loeb, 1986). For example, polymerase α has been reported to be the major or sole DNA-replicative enzyme and has also been implicated in repair of DNA damage induced by UV (Dresler, 1984; Dresler & Lieberman, 1983; Dresler et al., 1982) and γ irradiation (Miller & Lui, 1982) and by alkylating agents (Miller & Chinault, 1982a,b). Polymerase β, the smallest of the polymerases (Fry & Loeb, 1986), has been proposed to be a repair enzyme (Fry & Loeb, 1986; Miller & Chinault, 1982a,b; Zimmerman et al., 1980; Hubscher et al., 1979; Castellot et al., 1979; Wilson et al., 1988; Zmudzka et al., 1988). Polymerase γ is localized in mitochondria and is responsible for mitochondrial DNA replication (Fry & Loeb, 1986; Zimmerman et al., 1980). In contrast, relatively little has been reported concerning the role of DNA polymerase δ, the most recently identified mammalian DNA polymerase (Byrnes et al., 1976; Goscin & Byrnes, 1982a,b; Lee et al., 1984). DNA polymerase δ is unique in that it has an intrinsic 3′→5′ exonuclease (Byrnes et al., 1976; Goscin & Byrnes, 1982a,b; Lee et al., 1984) that functions in a proofreading capacity to enhance the fidelity of DNA synthesis (Kunkel et al., 1987). Although human placental polymerases α and δ are immunologically distinct (Lee & Toomey, 1987), several studies suggest polymerases α and δ may be related. For example, a cryptic 3′→5′ exonuclease activity associated with *Drosophila* polymerase α can be unmasked when accessory proteins are removed (Cotterill et al., 1987). In addition, exonuclease activity may copurify with polymerase α activity (Hubscher, 1984; Chen et al., 1979; Vishwanatha et al., 1986). Studies from Bambara's laboratory (Crute et al., 1986; Wahl et al., 1986) have demonstrated two forms of polymerase δ isolated from calf thymus and proposed that δ_1 (with a weakly associated exonuclease activity) and δ_2 (with a tightly associated exonuclease activity) may be derived from a single polypeptide.

Several recent studies examining proliferating cell nuclear antigen (PCNA)[1]/cyclin have indirectly implicated DNA polymerase δ in eukaryotic DNA replication. PCNA/cyclin has been shown to be identical with the 36-kDa auxiliary protein of polymerase δ (Prelich et al., 1987a; Bravo et al., 1987). Both DNA polymerase δ activity (Prelich et al., 1987a; Bravo et al., 1987; Tan et al., 1986) and SV40 DNA replication are stimulated by PCNA/cyclin (Prelich et al., 1987a,b). In addition, PCNA/cyclin has also been implicated in DNA repair synthesis induced by UV irradiation (Celis & Madsen, 1986). Reconstitution studies by Nishidia et al. (1988) provided additional support for a role of polymerase δ in DNA repair synthesis in a subcellular system. They demonstrated that a soluble factor extracted from human fibroblasts restored UV-induced DNA repair synthesis activity and that this factor appeared to be DNA polymerase δ.

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[1] Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; ddTTP, 2′,3′-dideoxythymidine triphosphate; BuPdGTP, (butylphenyl)-deoxyguanosine triphosphate; HF, human fibroblast(s); BrdUTP, bromodeoxyuridine triphosphate; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen.

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Polymerase inhibitors have provided one of the few means of investigating the functions of mammalian DNA polymerases. Early studies using aphidicolin established that this drug inhibited DNA replication (Krokan et al., 1979; Pedrali-Noy & Spadari, 1979; Wist & Prydz, 1979; Yagura et al., 1982) and some types of DNA repair (Berger et al., 1979; Ciarrocchi et al., 1979; Hanaoka et al., 1979; Miller & Chinault, 1982a,b). Because aphidicolin was initially described as a specific inhibitor of polymerase α (Ikegami et al., 1978), it was concluded that polymerase α was solely responsible for DNA replication and at least partially involved in DNA repair synthesis. However, these conclusions were questionable after subsequent studies demonstrated that DNA polymerases α and δ exhibit similar sensitivities to aphidicolin (Goscin & Byrnes, 1982a,b; Hammond et al., 1987). The development of monoclonal antibodies specific for DNA polymerase α (Tanaka et al., 1982) and of (*p-n*-butylphenyl)deoxyguanosine triphosphate (BuPdGTP) (Khan et al., 1984; Wright & Dudycz, 1983), which is a more potent inhibitor of polymerase α than of polymerase δ (Hammond et al., 1987; Byrnes, 1985; Crute et al., 1986; Wahl et al., 1986; Lee et al., 1985), makes it possible to better dissect the roles of polymerases α and δ in DNA synthesis. Studies by several laboratories using these new polymerase inhibitors have supported an important role for polymerase δ in DNA replication in mammalian cells (Hammond et al., 1987; Dresler & Frattini, 1986). In addition, studies using BuPdGTP led Dresler et al. (1986) to conclude that polymerase δ is responsible for UV-induced DNA repair synthesis. We exploited these inhibitors to better define the potential role of polymerases α, β, and δ in MNNG-induced DNA repair synthesis in both intact and permeabilized human fibroblasts. This study reports, for the first time, the effect on DNA repair of anti-(DNA polymerase α) antibody (Tanaka et al., 1982) microinjected into intact human fibroblast nuclei. The results support a role for two DNA polymerases, δ and β, in MNNG-induced DNA repair synthesis.

Experimental Procedures

Reagents. [α-^{32}P]dCTP (3000 Ci/mmol) was from Amersham, and [*methyl*-^{3}H]thymidine (53 Ci/mmol) was from ICN. MNNG was from Aldrich. Purified rabbit bone marrow DNA polymerases α and δ were the generous gift of Dr. John Byrnes. Purified rat DNA polymerase β was the generous gift of Dr. Samuel Wilson, NIH. All other reagents were from Sigma.

Cell Culture. Normal human diploid fibroblasts (HF), derived from foreskin and designated CF-3 by the ATCC, were cultured in McCoy's 5A medium (Gibco) with 10% fetal calf serum (Flow Laboratories) and 10 μg of gentamicin/mL (Sigma). To obtain growth-arrested (G_1) cells for lysolecithin treatment, confluent cultures were placed in medium containing 0.1% fetal calf serum for 48 h. To obtain G_1 cells for autoradiography studies, cultures were plated onto grid glass cover slips (22 mm from Bellco) at a 1:4 split ratio from a confluent culture. After attaching in medium containing 10% serum, cells were maintained in medium containing 0.1% serum for 1 week, without medium change. To induce DNA repair synthesis, 0.1 mM MNNG was added to G_1 cells in medium containing 0.1% serum for 75 min before permeabilization or microinjection.

Hybridoma cells producing anti-(DNA polymerase α) antibodies SJK 132-20 or SJK 287-38 (Tanaka et al., 1982) were obtained from the ATCC and propagated in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Antibodies were purified from spent culture medium by protein A–Sepharose chromatography (Tanaka et al., 1982).

DNA Polymerase Assays. Purified DNA polymerases were incubated in 80 mM KCl, 5 mM $MgCl_2$, 35 mM Hepes (pH 7.2), 100 μg/mL DNase-activated DNA, 0.1 mM dATP, 0.1 mM [^{3}H]TTP, 25 μM dGTP, and 8 μM dCTP, with indicated concentrations of the inhibitors for 20 min at 37 °C. Incorporation of [^{3}H]TTP into DNA was determined as described (Hammond et al., 1987). DNA polymerases were preincubated with antibodies for 45 min at 0 °C before assays were begun. These conditions were used because they approximated the assay conditions used for in situ DNA synthesis, allowing easier comparison.

In Situ DNA Synthesis. To characterize DNA synthesis in situ, HF were trypsinized from 100-mm plates, washed with 150 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, and 35 mM Hepes (pH 7.2) (solution A) containing 0.2 mM phenylmethanesulfonyl fluoride, resuspended to 8×10^7 cells/mL in solution A, and permeabilized with 0.5 mg of lysolecithin/mL, final concentration, as described (Miller et al., 1978, 1985a,b). Methods for determining DNA repair in permeable cells have been described in detail (Miller & Chinault, 1982a,b). Briefly, following MNNG treatment, cells were permeabilized and 2×10^7 permeable cells/mL were incubated in solution A containing 1.25 mM ATP, 5.0 mM phosphoenolpyruvate, 0.1 mM dATP, 25 μM dGTP, 100 μM BrdUTP substituted for TTP, and 8 μM [α-^{32}P]dCTP (50 μCi/mL). Aliquots (400 μL) were incubated at 37 °C for 30 min in the presence or absence of inhibitors. Normal-density HF cell DNA was prepared by incubating exponential cells in DME medium containing [^{3}H]thymidine (0.1 μCi/mL) for 16 h. A known amount of normal-density [^{3}H]DNA was added to [^{32}P]DNA isolated from each permeable cell sample, and the DNA was then sheared, denatured, and centrifuged to equilibrium on CsCl gradients as described (Tseng & Goulian, 1975).

CsCl density gradients were utilized to separate BrdUMP-labeled DNA synthesized as the result of DNA repair (associated with normal-density DNA) from that synthesized due to replication (higher than normal-density DNA). Radiolabeled DNA synthesized in permeable cells that was associated with normal-density DNA was used to calculate DNA repair synthesis. The effects of various polymerase inhibitors on the amount of DNA repair synthesis were determined and expressed as percent inhibition of DNA repair synthesis relative to samples incubated without inhibitors.

Microinjection. Glass micropipets were prepared from capillary tubing (Omega Dot Tubing, 1.0-mm diameter, Redenck Haer and Co.) on a micropipet puller (Model P 80, Brown-Flaming type, Sutter Instruments). The tip diameters ranged from 0.2 to 0.3 μm. The capillary tubes were washed in 5 M HCl, sterile distilled water, and ethanol and baked at 200 °C overnight prior to pulling. Microinjection was carried out under direct visual control on a fixed stage of an inverted phase-contrast microscope (Nikon Diaphot, 200×). Movement of the micropipet was controlled by the Narishige NT-8 micromanipulator system. Anti-(DNA polymerase α) and control antibody solutions (5 mg/mL in phosphate-buffered saline) to be injected were loaded into the micropipet with a Hamilton 10-μL syringe (Model 101LT). The amount of injected sample was monitored by observing changes in the refractive index as fluid enters the cell. One hour after cell nuclei were microinjected, the glass cover slips were placed in medium containing 15 μCi/mL [^{3}H]dT for 60 min. Following rinses in medium and phosphate-buffered saline, cells were fixed in methanol–acetic acid (3:1), dried, dipped in

NTB2 emulsion (Kodak), and exposed in the dark for 2–4 weeks for autoradiography.

Autoradiographic Analysis. Following development with D-19 (Kodak), cells were viewed under an Olympus BH2 microscope. Autoradiographic analysis was quantitated by using an automated video image analysis system, consisting of an Ikegami video camera, AT&T image capture board, and PC limited 286 computer. Areas within individual nuclei were outlined with a mouse. With this video image analysis system, the size of silver grains was slightly enlarged, and when two or more grains touch, they were automatically scored as one particle. For this reason, the total cross-sectional areas of silver grains within the nuclear areas outlined by the mouse were calculated from a captured video image. This value was then divided by the cross-sectional area of a single silver grain (determined by outlining individual silver grains with the mouse) to determine more accurately the number of silver grains. Because it was not possible to exactly outline the perimeter of nuclei with the mouse, areas within nuclei were outlined; data are expressed as number of silver grains/10^3 μm^2 nuclear area measured. Measurements indicated that the nuclei in this study were approximately 320 μm^2, while the average nuclear area outlined using the mouse was 208 μm^2. Nuclei contained 10–60 silver grains, depending on the specific treatment and on the length of time of exposure to emulsion.

Results

DNA excision repair synthesis was examined in growth-arrested (G_1) HF cells treated with 100 μM MNNG, an alkylating agent that methylates DNA bases at specific sites (Roberts, 1978). Methylated bases are removed by DNA glycosylases, and resulting apurinic sites are repaired by an excision repair mechanism, which is detected by measuring incorporation of deoxynucleotides into DNA. A small amount of DNA synthesis occurred in untreated (control), growth-arrested HF cells, and CsCl gradient analysis of BrdUMP-labeled DNA was undertaken to determine if this synthesis was due to DNA replication or DNA repair. Figure 1A shows that the DNA synthesized in permeable cells in the absence of DNA damaging agents was higher than normal density, indicating this DNA synthesis was replicative in nature. This replicative synthesis can be attributed to a small number of cells in the G_1 population (<4%) that were not growth arrested (Miller & Chinault, 1982b). When G_1 HF cells were treated with MNNG to induce repair synthesis (Figure 1B), >80% of the DNA synthesized in permeable cells was associated with normal-density DNA. The association of newly synthesized DNA with normal-density DNA is diagnostic of DNA repair synthesis (Miller & Chinault, 1982b; Ross & Moses, 1976). MNNG has been shown to both induce DNA repair synthesis and inhibit DNA replication (Droy et al., 1988). Hence, analysis of radioactive DNA that comigrated with normal-density DNA was utilized to assess the specific effects of various inhibitors on DNA repair synthesis (Figure 1C). Typical values for the amounts of DNA synthesized in permeable HF cells, expressed as picomoles of dCTP incorporated per 5×10^5 cells per 30 minutes, were 0.176 for G_1 cells and 0.325 for G_1 cells treated with MNNG. Identical results were obtained when DNA synthesized in permeable cells was radiolabeled with [^{3}H]dATP and normal-density DNA was labeled with [^{14}C]thymidine.

A comparison of the effects of several well-characterized inhibitors of DNA polymerases on purified preparations of DNA polymerases α, β, and δ and on MNNG-induced DNA repair synthesis in permeable cells is presented in Table I. One hundred micromolar ddTTP had little effect on the activity

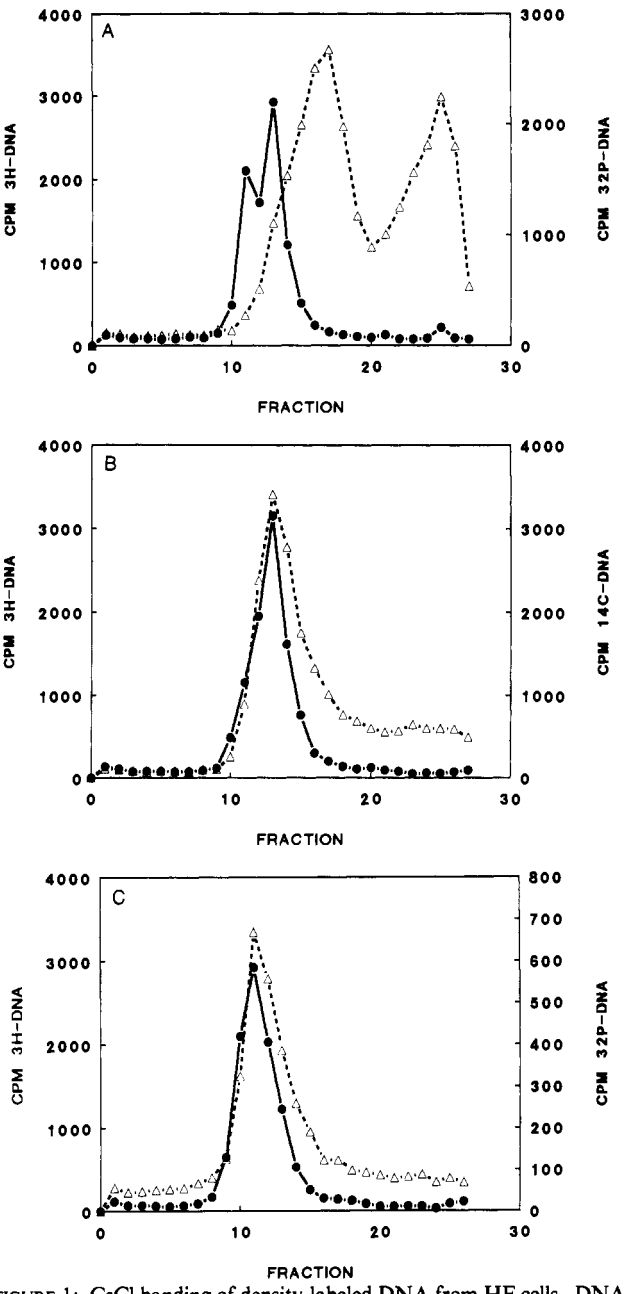

FIGURE 1: CsCl banding of density-labeled DNA from HF cells. DNA synthesized in situ was density labeled with BrdUTP and radiolabeled with [^{32}P]dCTP. After incubation at 37 °C for 30 min, the cells were lysed and DNA was isolated, sheared, denatured, and centrifuged to equilibrium on CsCl gradients as described under Experimental Procedures. Centrifugation was in a Ti 75 rotor at 45 000 rpm for 48 h at 25 °C. The gradients were fractionated from the top (fraction 1) and acid precipitated on GF/A filters (Miller & Chinault, 1982a,b). (A) G_1 cells; (B) MNNG-treated G_1 cells; (C) MNNG-treated G_1 cells incubated with 50 μg/mL aphidicolin. (Δ) [^{32}P]dCMP- and BrdUMP-labeled DNA; (●) [^{3}H]dTMP-labeled normal-density DNA. CsCl density was linear between fraction 4 (1.620–1.630 g/mL) and fraction 24 (1.794–1.821 g/mL).

of purified polymerases α or δ but inhibited DNA polymerase β >95%, confirming previous reports (Scovassi et al., 1980). MNNG-induced DNA repair synthesis in permeabilized cells was inhibited 29% by ddTTP, suggesting that DNA polymerase β has a significant role in MNNG-induced DNA repair. Aphidicolin, a potent inhibitor of both polymerases α and δ, inhibited MNNG-induced DNA repair synthesis 56%, indicating that polymerase α and/or δ has a major role in this type of repair synthesis. Repair synthesis induced by MNNG, however, was resistant to two polymerase α inhibitors, 10 μM BuPdGTP, and saturating concentrations of monoclonal an-

Table I: Effects of Inhibitors on MNNG-Induced DNA Repair and on DNA Polymerases[a]

	% inhibition			
		DNA polymerase		
	MNNG repair	α	β	δ
100 μM ddTTP	29	<5	95	<5
50 μg/mL aphidicolin	56	85	NE	80
100 μg of SJK 132-20	13	>95	NE	NE
10 μM BuPdGTP	6	>95	NE	32
300 μM BuPdGTP	36	100	NE	86

[a] MNNG-induced DNA repair synthesis was assayed in permeable growth-arrested HF cells in the presence of the indicated inhibitors, as described under Experimental Procedures. Inhibition values reflect the amount of radioactivity associated with normal-density DNA in cells assayed in the presence of inhibitor, relative to the amount associated with normal-density DNA in cells assayed in the absence of inhibitor. Values shown are the average for two or three experiments; the range was ± <10%. Purified polymerases were assayed under conditions similar to DNA repair in order to more accurately compare the results. NE = no effect. Each value indicated above is the average of triplicate determinations; the range of the triplicates was <7%.

tibody SJK 132-20 (Table I) or SJK-287-38 (not shown). Saturating concentrations of antibodies are defined as those concentrations that abolish polymerase α activity extracted from 8×10^6 cells and that produce maximal inhibition of DNA replication in permeable HF and CV-1 cells (Hammond et al., 1987; Miller et al., 1985a; Kaczmarek et al., 1986). In contrast, MNNG-induced repair synthesis was inhibited 36% by 300 μM BuPdGTP. This concentration of BuPdGTP inhibited completely polymerase α activity and reduced by 85% polymerase δ activity.

The effect of aphidicolin and of anti-(DNA polymerase α) antibody on MNNG-induced DNA repair synthesis in intact HF cells was then determined. Autoradiographic analysis demonstrated that <2% of the cells were in S phase (heavily labeled with [^{3}H]dT), with or without MNNG treatment. Following MNNG treatment, essentially all nuclei in G_1 cells contained >10 silver grains/nucleus. Aphidicolin reduced the silver grain density over nuclei by 57%, relative to controls receiving the DMSO solvent, indicating that an aphidicolin-sensitive DNA polymerase is at least partially involved in repairing MNNG-induced DNA damage in intact cells. These results compare favorably with the observation that aphidicolin inhibits 56% of the MNNG-induced DNA repair synthesis in permeable cells (Table I). By contrast, microinjection of intact cells with antibody SJK 287-38 had little effect on the density of silver grains, relative to cells injected with phosphate-buffered saline (Table II). Microinjection of an antibody directed against rat pyruvate kinase also had little effect on the silver grain density (Table II). The ineffectiveness of anti-(DNA polymerase α) antibodies in inhibiting MNNG-induced DNA repair synthesis in intact cells correlates well with the results reported above using the same antibodies in permeabilized cells. To confirm that the anti-(DNA polymerase α) antibodies were capable of inhibiting polymerase α in intact HF cells, exponentially growing cells were microinjected with SJK 287-38, pulsed with [^{3}H]dT, and processed for autoradiography as described under Experimental Procedures. Video image analysis of the autoradiographic slides revealed that DNA replication was inhibited approximately 55% (Table II) after the microinjection of SJK 287-38, relative to microinjection of a control antibody. This result confirms our previous report that anti-(DNA polymerase α) antibodies inhibit DNA replication when microinjected into cell nuclei (Kaczmarek et al., 1986) and shows that microinjected antibodies can exert specific effects within cell nuclei.

Table II: Effects of Microinjected Antibodies and Aphidicolin on MNNG-Induced DNA Repair Synthesis in Intact Cells

experiment	n	no. of grains/10^3 μm^2	±SE	% inhibn
repair 1				
control (DMSO)	72	177	±7	
aphidicolin (50 μg/mL)	72	75	±4	57
repair 2				
control	75	177	±6	
PK	73	149	±6	16
repair 3				
control	75	220	±6	
287-38	98	193	±7	13
repair 4				
control	77	134	±8	
287-38	75	148	±7	0
replication				
control	76	256	±6	
287-38	72	117	±10	55

[a] Growth-arrested or exponential cultures of HF cells were used for MNNG-induced DNA repair studies or replication studies, respectively. Following treatment with MNNG (repair studies only) and [^{3}H]-dT, autoradiography was performed to determine the effects of the indicated compounds on DNA repair or DNA replication. The samples were analyzed as described to quantitate the density of silver grains in nuclei. Repair 1–repair 4 are the results of four different experiments; n is the number of nuclei analyzed; silver grain density is expressed as the number of silver grains/10^3 μm^2 nuclear area; SE is standard error. Percent inhibition is relative to control samples; controls for repair 1 received DMSO in culture medium; all other controls were microinjected with PBS. PK is antibody directed against rat liver pyruvate kinase, and 287-38 is antibody (Tanaka et al., 1982) directed against KB cell DNA polymerase α.

Discussion

In attempting to dissect the roles of DNA polymerases α, β, and δ in MNNG-induced DNA repair synthesis, it was important to investigate the effects of different DNA polymerase inhibitors in both intact and permeable cells. All aspects of normal DNA repair should occur in intact cells; however, many DNA polymerase inhibitors, including nucleoside triphosphates and antibodies, do not readily penetrate the plasma membrane. In addition, while many nucleoside precursors of the nucleotide inhibitors do enter intact cells, some may not be phosphorylated to triphosphates (Krokan et al., 1979). Although microinjection or electroporation techniques allow the introduction of nonpenetrating compounds into intact, viable cells, it is difficult or impossible to determine the active concentration of these inhibitors inside the cell or nucleus. Therefore, only specific inhibitors can provide useful information. In the present study, only the effects of aphidicolin (penetrates intact cells and inhibits only DNA polymerases α and δ) and of microinjected monoclonal antibodies (specifically inhibit polymerase α) were investigated for their effects on MNNG-induced DNA repair synthesis in intact cells. The results (Table II) suggest that an aphidicolin-sensitive DNA polymerase, resistant to anti-(DNA polymerase α) antibodies, was responsible for approximately 60% of the MNNG-induced DNA repair synthesis. Presumably, this repair synthesis represents DNA polymerase δ. The introduction of monoclonal anti-(DNA polymerase δ) antibodies (Lee & Toomey, 1987) into cells should help clarify this presumption.

The advantage of studying the effects of DNA polymerase inhibitors on DNA repair synthesis in subcellular preparations is that nuclei are exposed to known concentrations of the inhibitors, and many inhibitors (including nucleotides) are freely permeable to the nucleus. The primary disadvantage of studying DNA repair in subcellular systems is that the

degree to which normal DNA repair processes operate is not known. Comparing results with intact cells and subcellular systems provides additional confidence in the results obtained. In lysolecithin-permeabilized cells, MNNG-induced DNA repair synthesis was inhibited approximately 30% by a concentration of ddTTP that nearly abolishes polymerase β activity but only minimally decreases (<5%) polymerase α and δ activity (Table I). Aphidicolin inhibited MNNG-induced DNA repair synthesis 56% (Table I), in good agreement with results in intact cells (Table II). Antibody SJK-132-20 and 10 μM BuPdGTP are potent inhibitors of polymerase α, but these inhibitors reduced MNNG-induced DNA repair synthesis only 6–13% in permeable cells (Table I). The lack of inhibition of MNNG-induced DNA repair in permeable cells by anti-(DNA polymerase α) antibodies also agrees well with results in intact cells (Table II). The studies in permeable cells suggest that polymerases δ and β are responsible for approximately 60% and 30%, respectively, of the DNA repair synthesis induced by MNNG; however, DNA polymerase δ is implicated, in part, by default. The availability of a DNA polymerase δ specific inhibitor(s) would provide more definitive support for its role in DNA repair synthesis.

Although DNA polymerase inhibitors provide useful tools for investigating the roles of DNA polymerases, the ability to extrapolate the effects of inhibitors on purified DNA polymerases to in situ or in vivo effects of the inhibitors may be limited. For example, while 300 μM BuPdGTP abolished polymerase α activity and reduced polymerase δ activity 86%, MNNG-induced DNA repair synthesis was decreased only 36% by 300 μM BuPdGTP (Table I). The reason 300 μM BuPdGTP did not reduce MNNG-induced DNA repair synthesis as much as did aphidicolin is not obvious. However, the extent of BuPdGTP inhibition of polymerases α and δ can be influenced by the DNA template (Hammond et al., 1987; Byrnes, 1985; Lee & Toomey, 1987; Khan et al., 1985). It may be difficult to extrapolate the specificity of BuPdGTP inhibition for purified polymerase α or δ assayed in vitro on synthetic template–primers to the inhibition of these polymerases acting at a replication fork. On the other hand, inhibition of DNA synthesis by anti-(DNA polymerase α) antibodies (Miller et al., 1985a,b; Kaczmarek et al., 1986; Hammond et al., 1987) may be a better indication of the role of DNA polymerase α than is the inhibition of DNA synthesis by 10 μM BuPdGTP. These antibodies are specific for polymerase α, and inhibition of polymerase α by these antibodies is independent of the DNA template. DNA replication is reduced by anti-(DNA polymerase α) monoclonal antibodies in permeable cells (Hammond et al., 1987; Miller et al., 1985a) and in intact cells microinjected with these antibodies [Table II and Kaczmarek et al. (1986)]. This inhibition appears to reflect a specific inhibition of polymerase α by the antibody: there is a dose-dependent relationship between antibody concentration and inhibition of replication (Miller et al., 1985a; Kaczmarek et al., 1986); another DNA-directed process, RNA synthesis, is not altered (Miller et al., 1985a); and antibodies that bind polymerase α, but do not inhibit activity (Tanaka et al., 1982), only minimally reduce replication (15%) (Miller et al., 1985a).

Registry No. MNNG, 70-25-7; DNA polymerase, 9012-90-2.

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Enthalpic and Entropic Contributions to Actin Stability: Calorimetry, Circular Dichroism, and Fluorescence Study and Effects of Calcium†

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ABSTRACT: The ΔH associated with the thermal unfolding of G-actin has been determined by differential scanning calorimetry (DSC) to be 142 ± 5 kcal/mol, with the T_m (melting temperature) at 57.2 ± 0.5 °C, at pH 8.0 (heating rate 0.5 K/min). The transition is broad and cannot be treated as a single transition that mimics a two-state process, suggesting the existence of domains. Deconvolution is done to fit it into two quasi-independent two-state transitions. For F-actin, the transition is more cooperative, with a cooperative ratio (the ratio of van't Hoff enthalpy and calorimetric enthalpy) of 1.4, indicating intermonomer interaction. The ΔH of the thermal unfolding of F-actin is 162 ± 10 kcal/mol with a T_m at 67.0 ± 0.5 °C. A state of G-actin similar to that of the heat-denatured form, designated D-actin, is obtained by removing tightly bound Ca^{2+} with EGTA. The DSC-detectable cooperative transition is completely lost when the free calcium concentration of the medium is 1×10^{-11} M or lower, using a Ca^{2+}/EGTA buffer system. However, circular dichroism (CD) shows that the helix content of actin, 32% in the G-form, is only partially reduced to 19% in this apo form. The CD spectrum and the helix content of the calcium-depleted actin are almost identical with those of the heat-denatured D form. This loss of 40% of the native helical content is irreversible in both cases. The remaining 60% of the native helical content cannot be further eliminated by heating to 95 °C. A complete and reversible unfolding of the D-actin can be obtained by 5 M guanidinium chloride or 8 M urea. The heat denaturation as well as chemical denaturant unfolding have also been followed by the intrinsic fluorescence of tryptophans. A red shift of the emission maximum from 325 to 335 nm is observed with heat and EGTA denaturation. Completely unfolded actin has an emission maximum at 345 nm. The accessibility of hydrophobic binding sites upon heat and EGTA denaturation is detected by ANS (anilinonaphthalenesulfonate) binding; the total number of binding sites increases by about 5-fold upon denaturation. These findings suggest a two-step pathway for the complete unfolding of G-actin, $N \rightarrow D \rightleftharpoons U$, where N, D, and U denote the native, a denatured but compact, and the completely unfolded states, respectively. The first irreversible step is characterized by a large enthalpic change and involves the dissociation of the high-affinity Ca^{2+}. This transition involves melting of two independent domains. The second reversible step is purely entropic and cannot be induced by heating. The structural characteristics of D-actin are intermediate between N- and U-actin. ΔG, ΔH, and ΔS of each conformational transition are given.

The folding–unfolding transition of small globular proteins is highly cooperative and approaches the two-state process [for a recent review, see Privalov and Gill (1988)]. For proteins with larger molecular weights or complex structures, the presence of independent cooperative domains can be detected or deduced from the analysis of calorimetric data (Privalov, 1982; Sturtevant, 1987). Recent studies of the thermal stability of proteins (Baldwin, 1986; Privalov & Gill, 1988) in-

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