Proc. Natl. Acad. Sci. USA
Vol. 76, No. 2, pp. 814–818, February 1979
Cell Biology

Decreased fidelity of DNA polymerase activity during *N*-2-fluorenylacetamide hepatocarcinogenesis

(DNA nucleotidyltransferase/error-prone replication/chemical hepatocarcinogenesis)

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Communicated by Alex B. Novikoff, November 27, 1978

ABSTRACT **α and β DNA polymerases (DNA nucleotidyltransferase; deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) were isolated from nuclear and cytoplasmic fractions of rat livers exposed to a carcinogenic regimen with the hepatocarcinogen *N*-2-fluorenylacetamide and from 24-hr regenerating liver. The fidelity of polymerization of these enzymes was compared by determining the incorporation of noncomplementary deoxyribonucleoside triphosphates (misincorporation) on a poly(dA-dT)·poly(dA-dT) template, with $MnCl_2$ and $MgCl_2$ as divalent cations. Our initial studies indicate that the cytoplasmic α polymerases from carcinogen-exposed rat livers were strikingly error-prone whereas the nuclear and cytoplasmic β polymerases retained their fidelity throughout the feeding cycles. The misincorporation was significantly accentuated by $MnCl_2$ compared with that obtained with $MgCl_2$ as divalent cation. The products were sensitive to pancreatic DNase I digestion, indicating that the noncomplementary bases had been incorporated by the polymerization process. Nuclear α polymerase showed some degree of infidelity but less than that of cytoplasmic α polymerase.**

Of the alterations induced in target cells by carcinogenic agents, those that are obligatory for malignant evolution are unknown. However, it is generally accepted that the interaction of activated metabolites with nuclear DNA and the subsequent intervention of repair mechanisms might play a central role in the carcinogenic sequence. Defects in any of the numerous components of available repair pathways could result in significant aberrations in DNA structure and composition (1). Infidelity of DNA synthesis by mutagenized or altered DNA polymerases has been proposed as a mechanism for the accumulation of somatic mutations and subsequent alteration in cellular controls (2–4). The presence of DNA polymerases (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) that demonstrate infidelity has been reported in malignant cells (2, 5, 6) and a similar alteration has been induced by carcinogenic metallic ions (7, 8). The method for determining infidelity is based upon the incorporation of noncomplementary nucleotides into synthetic templates by DNA polymerase (5).

We have utilized intermittent exposure of the rat to the hepatocarcinogen *N*-2-fluorenylacetamide (FAA) to identify discrete phases of the carcinogenic process and this approach has permitted a detailed comparison of biologic and biochemical variables associated with reversible and irreversible (premalignant) alterations (9). For example, normal levels of repair endonucleases have been demonstrated throughout this carcinogenic regimen (10). The role of DNA synthesis during repair and the importance of DNA replication during cell division in the pathogenesis of the carcinogenic sequence (11) led us to examine the DNA polymerases during FAA-induced hepatocarcinogenesis.

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MATERIALS AND METHODS

Animals and Diets. Male Sprague–Dawley rats (Charles River Farms, North Wilmington, MA) were started on the carcinogenic regimen at 125-g body weight. This regimen and descriptions of the resultant hepatic alterations have been published in great detail (9, 10). FAA was mixed with a standard synthetic diet (no. 101, Bio-Serv, Frenchtown, NJ) at 0.06%. A feeding cycle consisted of 3 weeks of FAA followed by 1 week of regular meal; the four cycles of exposure were designated 1 × 3, 2 × 3, 3 × 3, and 4 × 3. At the termination of the 3 × 3 cycle, the livers were composed of small nodules which eventually disappeared without subsequent exposure to carcinogen. These livers did not give rise to malignancy. After 4 × 3, a subpopulation of nodules, often as large as 1 cm in diameter, was evident. These nodules, which persist, are considered a putative premalignant lesion; such livers eventually have a high incidence of hepatocellular carcinomas. Whole livers were pooled for analysis of polymerases at 3 × 3. Immediately at the end of 4 × 3, the premalignant nodules were dissected free from background liver and pooled. To examine the activity of normal DNA polymerases, age-matched rats that had been fed the basal diet underwent 70% hepatectomy and were sacrificed 24 hr later.

Separation of Nuclear and Cytoplasmic Fractions. The postmitochondrial cytoplasmic (30,000 × *g* supernatant) or nuclear fractions of 70–100 g of rat liver were separated according to the procedure of Lynch *et al.* (12), except that the buffers contained either 0.3 or 2.3 M sucrose in 2.5 mM $MgCl_2$/0.5 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride (PMSF). The purified nuclei were then extracted with 2.0 M NaCl in 0.01 M Tris·HCl, pH 8.0/0.5 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride (TDP buffer) for 4 hr. After dialysis against 13 vol of TDP buffer overnight, the soluble nuclear proteins, which contain 95% of all nuclear DNA polymerases, were separated from the DNA–histone precipitates by centrifugation (13).

Purification of DNA Polymerases from Cytoplasmic and Nuclear Fractions. The postmitochondrial cytoplasmic fractions and the nuclear proteins were precipitated by adjusting to 70% ammonium sulfate saturation and dialyzing against 0.01 M Tris·HCl, pH 8.0/10% (wt/vol) glycerol/0.1 mM EDTA/1 mM dithiothreitol (TGED buffer). α and β DNA polymerases were then purified by a modification of the procedure of Chan and Srivastava (14). These enzymes were initially purified by chromatography on DEAE-cellulose (DE-23) and then were separated by 0.2–0.9 M NaCl linear gradient elution of a phosphocellulose (P11) chromatography column in TGED buffer. α DNA polymerase was generally eluted by 0.23–0.37 M NaCl and β DNA polymerase was eluted by 0.4–0.5 M NaCl.

Abbreviation: FAA, *N*-2-fluorenylacetamide.

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The activity peaks were pooled separately and concentrated in an Amicon standard cell using a PM10 filter. In some of the experiments, α DNA polymerase was further purified by chromatography on hydroxylapatite; β DNA polymerase was further purified by Sephadex G-100 chromatography. Specific activities of nuclear α and β DNA polymerase after chromatography on hydroxylapatite and Sephadex G-100 were 1065 and 1777 units/mg of protein, respectively; the activities of the cytoplasmic α and β polymerases were approximately 270 and 1050 units/mg of protein, respectively. The purified α DNA polymerase was characterized by a low pH (pH 7.0) and low salt (0.025 M NaCl) requirement for optimal activity as well as its extreme sensitivity to *N*-ethylmaleimide inhibition. The purified β DNA polymerase required an alkaline pH (pH 8.6) and high salt concentration (0.15–0.2 M NaCl) for optimal activity and was resistant to *N*-ethylmaleimide inhibition.

Misincorporation (Infidelity) Assay. To measure the fidelity with which poly(dA-dT)·poly(dA-dT) was copied by DNA polymerase, the incorporations of the correct nucleotides (dATP and dTTP) and incorrect nucleotide (dGTP) were compared in separate assays, using the same enzyme preparation, by a modification of the procedure of Springgate and Loeb (5). For determination of total synthesis, the reaction mixture in a final volume of 0.1 ml contained: 10 mM Tris·HCl (pH 7.4); 0.2 mM $MnCl_2$ or 2 mM $MgCl_2$; 20 mM dithiothreitol; 20 μM dATP, dTTP, and dGTP; 1 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]dTTP (specific activity, 583 cpm/pmol); 0.1 A_{260} unit of poly(dA-dT)·poly(dA-dT); and 0.1 M KCl present with $MnCl_2$. Assays were initiated by adding 20–40 μl of enzyme. For measurement of incorrect incorporation, identical reaction mixtures were used except that they contained 20 μM unlabeled dATP and dTTP and [^{3}H]dGTP (specific activity, 3810 cpm/pmol; 10 μCi/assay).

In some of the studies, the misincorporation assay was performed under conditions of limited substrate according to a modification of the procedure of Linn *et al.* (4). [^{3}H]dTTP, when present, was at a specific activity of 34 cpm/fmol; [^{3}H]dGTP was present at a specific activity of 94 cpm/fmol. The reaction mixtures were incubated at 37°C for 1 hr, and then 20 μl of unlabeled carrier dGTP (2.5 mg/ml) was added. Samples were then precipitated by trichloroacetic acid and processed as described (15). Determinations were done in duplicate or triplicate and the results given are the means after subtraction of controls without added poly(dA-dT)·poly(dA-dT) template. The latter procedure was utilized to eliminate the possibility that contaminating DNA in enzyme preparations was participating in the assay.

Other Methods. Hydrolysis of the DNA product was performed by pancreatic DNase I digestion as described by Linn *et al.* (4). α and β DNA polymerase were assayed according to Chan *et al.* (15) by using activated calf thymus DNA and four deoxynucleoside triphosphates. One unit of enzyme incorporates 1 nmol of [^{3}H]dTMP into trichloroacetic acid-insoluble material after incubation at 37°C for 1 hr.

RESULTS

Fidelity of DNA Polymerase Purified from Normal Regenerating Rat Liver and FAA-Exposed Rat Liver at Substrate Levels > K_m (20 μM). Cytoplasmic α and β DNA polymerase fractions from FAA-treated and control liver were purified through ammonium sulfate precipitation, DEAE-cellulose (DE-23), and phosphocellulose chromatography. The α polymerase fractions were further purified through hydroxylapatite chromatography, and the β polymerase fractions were passed through a Sephadex G-100 gel filtration column. Fidelity (misincorporation) assays were performed with substrate levels > K_m (20 μM) for dATP, dTTP, and dGTP as described by Springgate and Loeb (5). Due to the limited availability of enzyme, 0.2 mM $MnCl_2$ was used as the divalent cation as described by Linn *et al.* (4) in the majority of experiments. The correct (complementary) incorporation and the incorrect (noncomplementary) incorporation are shown in Table 1. The misincorporation assay was determined to be linear within 1 hr of incubation and required dATP and dTTP as well as divalent cations.

To justify statistical analysis of the observed differences in misincorporation, additional tissue samples were analyzed; the results from various samples are shown in Table 2. High error rates were observed with cytoplasmic α polymerase from FAA-exposed livers.

Fidelity of DNA Polymerase Purified from Normal Regenerating Rat Liver and FAA-Exposed Rat Liver at Limited Substrate Concentration. To confirm the differential rate of misincorporation and the specificity of the cytoplasmic α polymerase fraction, additional tissues were processed. Nuclear as well as cytoplasmic α and β DNA polymerase were purified simultaneously through ammonium sulfate precipitation, DEAE-cellulose (DE-23), and phosphocellulose chromatography. Misincorporation assays were repeated with 0.2 mM $MnCl_2$ and at limited substrate concentration (0.03–0.3 μM) as described by Linn *et al.* (4). From Table 3, showing the results of a single experiment for [^{3}H]dTMP and [^{3}H]dGMP, it is apparent that substrate concentration does not significantly affect the differences in fidelity as determined with the different cytoplasmic enzymes. The absolute error rates of nuclear α polymerase were not as high as those seen with the cytoplasmic α polymerase fraction, although they were relatively increased compared with normal liver.

Nuclear β DNA polymerase from normal and from FAA-treated rat liver had low error rates. With cytoplasmic β DNA

Table 1. Incorporation of complementary (correct) and noncomplementary (incorrect) nucleotides by cytoplasmic DNA polymerases purified from normal and FAA-exposed rat liver copying poly(dA-dT)·poly(dA-dT)

	Correct incorporation ([^{3}H]dTMP), cpm				Incorrect incorporation ([^{3}H]dGMP), cpm				
Polymerase	+poly-(dA-dT)	−poly-(dA-dT)	Net	pmol	+poly-(dA-dT)	−poly-(dA-dT)	Net	pmol	Error rate*
Normal α	16,051	231	15,820	54.27	635	595	40	0.0105	1/5, 168
3×3 α	13,329	401	12,928	44.35	6315	789	5526	1.4504	1/31
4×3 α	7,784	479	7,305	25.06	609	514	95	0.0250	1/1,002
Normal β	149,975	290	149,685	513.50	790	630	160	0.0420	1/12,226
3×3 β	82,982	326	82,656	283.55	609	537	72	0.0189	1/15,002

Assay was performed in the presence of 0.2 mM $MnCl_2$ and at substrate levels > K_m (20 μM). Background values (zero time incubation) ranged from 200 to 400 cpm for dTMP and 300 to 600 for dGMP.

* Error rate = dGMP/(dTMP + dAMP).

Table 2. Incorporation of complementary and noncomplementary nucleotides by cytoplasmic DNA polymerases purified from normal and FAA-exposed rat liver copying poly(dA-dT)·poly(dA-dT)

Polymerase	Incorporation, pmol dTMP + dAMP	Incorporation, pmol dGMP	dGMP/ dTMP + dAMP error rate	Average error rate	Error rate ± SEM × 10^5
Normal α	4.29	0.0016	1/2681		
(n = 5)	53.49	0.0490	1/1091		
	11.50	0.0120	1/958	1/1845	54.18 ± 41.19
	54.20	0.0105	1/5162		
	49.30	0.0090	1/5477		
3 × 3 α	44.30	1.6640	1/26.6		
(n = 3)	95.28	2.6000	1/36.6	1/43.7	2284.00 ± 1742.30
	38.70	0.1400	1/276.4		
4 × 3 nodule α	24.20	0.0250	1/968		
(n = 4)	75.17	0.0660	1/1139	1/920.8	108.60 ± 34.74
	2.38	0.0020	1/1190		
	45.88	0.0730	1/628		

Assay was performed in the presence of 0.2 mM $MnCl_2$ and at substrate levels > K_m (20 μM). Results presented are the average after subtraction of control without template.

polymerase, the absolute amounts of dGMP incorporation were so low as to preclude valid statistical analysis of observed differences.

Thus, high error rates were observed with cytoplasmic α polymerase isolated from FAA-exposed livers but the nuclear and cytoplasmic β polymerases retained their fidelity throughout the feeding cycles. Nuclear α polymerase showed some degree of infidelity but less than that of cytoplasmic α polymerase.

Fidelity of DNA Polymerase with $MgCl_2$ as Divalent Cation. It has been shown that $MnCl_2$ can induce a higher error rate both in mutated T4-induced DNA polymerase and in normal enzyme (16). Therefore, we measured the misincorporation rate of normal and FAA-treated rat liver DNA polymerase by substituting 2 mM $MgCl_2$ for 0.2 mM $MnCl_2$ as the divalent cation (Table 4). Under these conditions, the error rate of cytoplasmic α polymerase from regenerating liver was much lower. This is consistent with the results of Salisbury *et al.* (17) showing that α polymerase from regenerating rat liver appears to be faithful when $MgCl_2$ is used as the divalent cation.

Effect of Pancreatic DNase I on Misincorporation. To determine if the noncomplementary nucleotides were incorporated into the polynucleotides and were not due to their nonspecific binding, the products of synthesis were subjected to pancreatic DNase I digestion as described by Linn *et al.* (4). Misincorporation assays were set up in duplicate with normal or 3 × 3 cytoplasmic α polymerase and poly(dA-dT)·poly(dA-dT) as template. [^{3}H]dTTP was used as the complementary nucleotide and [^{3}H]dGTP as the noncomplementary nucleotide. Incorporation was allowed to proceed for 1 hr at 37°C.

At the end of incubation, $MgCl_2$ was added to 7.5 mM and 40 μg of pancreatic DNase I was added to one set of the tubes. The tubes were incubated at 37°C for another 15 min. The ^{3}H-labeled nucleotide incorporation in the DNase I treated and untreated reactions was then determined. Table 5 shows that the misincorporation is sensitive to pancreatic DNase I digestion. In the absence of DNase I and in the presence of poly(dA-dT)·poly(dA-dT) template, a significant amount of dGMP was incorporated by 3 × 3 rat liver. The net misincorporation (702 cpm) was obtained by subtracting the background counts [i.e., in the absence of poly(dA-dT)·poly(dA-dT)]. The net incorporation of [^{3}H]dGMP was sensitive to pancreatic DNase I because in the presence of DNase I the dGMP incorporation fell to background (535 cpm). This experiment supports the

Table 3. Incorporation of complementary and noncomplementary nucleotides by DNA polymerases from normal and FAA-exposed rat liver using poly(dA-dT)·poly(dA-dT) as template

Polymerase*	Incorporation, fmol dTMP + dAMP	Incorporation, fmol dGMP	dGMP/ dTMP + dAMP error rate	Average error rate (n)	Error rate ± SEM × 10^5
Normal Cpα	4,770	1.70	1/2,806	1/2,727 (4)	36.66 ± 12.05
3 × 3 Cpα	649	12.10	1/53.6	1/57.2 (3)	1747.00 ± 1292.00
4 × 3 Cpα	734	4.32	1/170	1/242.4 (3)	412.50 ± 156.80
Normal Npα	1,670	0.42	1/3,976	1/3,891 (3)	25.70 ± 1.36
3 × 3 Npα	1,680	0.42	1/4,000	1/3,294 (2)	30.35 ± 7.56
4 × 3 Npα	1,150	0.53	1/2,170	1/1,517 (2)	65.90 ± 28.10
Normal Npβ	87,400	13.70	1/6,380	1/6,872 (3)	14.55 ± 4.46
3 × 3 Npβ	41,420	5.80	1/7,141	1/5,899 (3)	16.95 ± 6.72
4 × 3 Npβ	27,280	4.50	1/6,062	1/5,485 (3)	18.23 ± 5.88
Normal Cpβ	87,000	2.69	1/32,370	1/20,161 (3)	4.96 ± 4.13
3 × 3 Cpβ	34,880	3.50	1/9,966	1/9,804 (3)	10.27 ± 1.11
4 × 3 Cpβ	24,370	0.80	1/30,462	1/29,411 (3)	3.40 ± 1.83

Assays were performed in the presence of 0.2 mM $MnCl_2$ with limited substrate concentration (0.03–0.3 μM). Results presented are the average after subtraction of control without template.

* Cpα, cytoplasmic α polymerase; Npα, nuclear α polymerase; Npβ, nuclear β polymerase; Cpβ, cytoplasmic β polymerase.

Table 4. Incorporation of complementary and noncomplementary nucleotides by cytoplasmic DNA polymerases purified from normal and FAA-exposed rat liver copying poly(dA-dT)·poly(dA-dT)

Polymerase	Incorporation, net cpm dTMP + dAMP	Incorporation, net cpm dGMP	dGMP/ dTMP + dAMP error rate
Normal α	4,801 (16.47)	4 (0.0010)	1/16,470
	7,159 (24.56)	4 (0.0010)	1/24,560
	13,406 (45.99)	21 (0.0056)	1/8,213
3 × 3 α	9,736 (33.40)	32 (0.0084)	1/3,976
4 × 3 α	12,459 (42.74)	31 (0.0080)	1/5,343
Normal β	173,792 (596.20)	120 (0.0316)	1/18,867
3 × 3 β	129,776 (445.20)	72 (0.0190)	1/23,431

Assay conducted in the presence of 2 mM $MgCl_2$ and at a substrate level > K_m (20 μM). Results presented are the average after subtraction of controls without template. Values in parentheses are the net incorporation expressed as pmol.

contention that dGMP is incorporated into the product synthesized by the enzyme rather than being nonspecifically bound.

Terminal transferase activity (data not shown) was not detectable in either regenerating or treated livers (14).

DISCUSSION

The accumulation of aggregate errors in DNA has been proposed as a basis for aging (4) and for carcinogenesis and tumor progression (1, 2, 3, 18). One mechanism for the introduction of such errors would be the decreased fidelity of DNA polymerases during repair activity or cell division. Support for these hypotheses has been derived mainly from experiments in which polydeoxynucleotide templates were utilized and incorporation of noncomplementary bases was determined *in vitro*. By using this methodology, altered fidelity of isolated DNA polymerases has been demonstrated with enzyme obtained from viruses (19, 20), from aging cell lines (4) or human leukemia cells (5), and as a result of interaction with carcinogenic metals (7). It has also been suggested that decreased fidelity of DNA polymerases during chemical carcinogenesis could result from interaction with the carcinogen or its activated metabolites or from gene effects induced by these agents with resultant mutagenized enzymes. To date, no experimental evidence has been available to support this hypothesis.

Table 5. Effect of pancreatic DNase I on incorporation of noncomplementary nucleotides (measured as cpm) into poly(dA-dT)·poly(dA-dT) by cytoplasmic α DNA polymerase from normal and FAA-exposed rat liver

Condition	Normal dTMP	Normal dGMP	3 × 3 dTMP	3 × 3 dGMP
0 incubation:				
+ Template; − DNase I	268	418	250	416
− Template; − DNase I	218	370	219	398
1-hr incubation:				
+ Template; − DNase I	15,050 (50.83)	605 (0.0262)	10,657 (35.44)	1237 (0.184)
+ Template; + DNase I	268	460	308	446
− Template; − DNase I	231	505	325	535
− Template; + DNase I	237	480	296	489

Values in parentheses are the net incorporation expressed in pmol after subtraction of control without template.

The most striking result of the current study was the demonstration of the rapid and persistent loss of fidelity of cytoplasmic DNA polymerase of rat hepatocytes upon exposure to the carcinogen FAA. This infidelity was accentuated when $MnCl_2$ was used as the divalent cation. Mn^{2+} has been shown to cause an increased rate of misincorporation of mutated T4 DNA polymerase (16) and an α DNA polymerase from aging human fibroblasts (4). The physiological significance of the $MnCl_2$-induced increase in error rate is at present unknown. However, it has been suggested that Mn^{2+} is mutagenic (7, 8). Although the decrease in fidelity could also be demonstrated with Mg^{2+} as the divalent cation, the number of counts above background was relatively low. The results therefore should only be viewed as supportive evidence for the data with Mn^{2+}. The increase in misincorporation by α polymerase from FAA-exposed liver could be demonstrated with phosphocellulose- or hydroxylapatite-purified enzyme preparations under conditions such that the substrates were both limited and > K_m.

Previously, no tangible suggestion was available to explain the nature of events that occurred in the lag period between the cessation of carcinogen exposure and the appearance of malignancy. These carcinogen-induced hepatic nodules are characterized by a persistently increased rate of cell replication even after cessation of carcinogen exposure (21). It appears possible, therefore, that one aspect of progression of the malignant process during this period is the continuing accumulation of abnormalities in DNA.

The selectivity of the process is emphasized by the apparent resistance of cytoplasmic or nuclear β DNA polymerase to similar alteration. Although unsupported by adequate experimental data, it has been suggested that β DNA polymerase is a component of the excision-repair system of mammalian cells (22). Its resistance to the effects of FAA is similar to that of repair endonucleases (10) and might be related to their constitutive nature and the importance of this pathway in the repair of spontaneous DNA damage. It is interesting that a number of previous reports also emphasized the retention of the fidelity of β DNA polymerase (23, 24).

The basis of the infidelity of α DNA polymerase was not determined by these experiments. Although it is possible that covalent binding of carcinogen was responsible for this alteration, it remains to be determined if altered DNA polymerase function persists after cessation of carcinogen exposure. At least two mechanisms might result in persistently aberrant enzyme activity. Carcinogen-bound enzyme could produce sufficient alteration of DNA to result in a mutagenized enzyme, or the synthesis of abnormal enzyme might result directly from carcinogen-induced-DNA alteration. Lastly, transient or persistent aberration could result from the synthesis of an error-prone enzyme species hypothesized in the SOS pathway.

The degree to which the altered fidelity of polymerase will

effect a permanent alteration in hepatocyte DNA is unknown, as is the conceptual problem of how such alterations result in a malignant phenotype. The system for DNA replication and repair is far more complex than the intervention of this single enzyme. However, this demonstration of infidelity fulfills the expectations of several schema for carcinogenic progression (2, 3, 18).

One additional finding suggests the possible presence of multiple species of α DNA polymerase resulting from exposure to FAA. Although a reproducible trend toward increasing infidelity of nuclear α polymerase was detected after exposure to FAA, this was not statistically significant when compared to normal values and was far less than that for cytoplasmic α polymerase. Because this disparity was most striking during exposure to FAA, it is possible that this distribution is the result of a differential solubility of the complex between carcinogen and polymerase and, therefore, an artifact of cell fractionation. However, the possibility of multiple enzyme species must be considered. Multiple forms of cytoplasmic α polymerase have been reported in calf thymus (25) and myeloma cells (26, 27), and modified forms of α polymerase have been detected in leukemia cells (28) and other cell types (29–31). Further characterization is required before the individuality of these species is ascertained or their relationship to carcinogenesis is determined.

The technical assistance of T. Goldflam, M. Thomas, and R. Weatherford is greatly appreciated. This work was supported by Grant CA-20657 from the National Institutes of Health.

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