On the Fidelity of Deoxyribonucleic Acid Synthesis Directed by Chromatin-Associated Deoxyribonucleic Acid Polymerase β†

Michael Fry,*,‡ Clyde W. Shearman, George M. Martin, and Lawrence A. Loeb*

ABSTRACT: Accuracy of poly[d(A-T)] synthesis catalyzed by chromatin-bound deoxyribonucleic acid (DNA) polymerase β was measured with several cell types. A new procedure was developed for the isolation of copied poly[d(A-T)] from chromatin DNA. This method involved in vitro copying of poly[d(A-T)] by native chromatin and subsequent selective fragmentation of chromatin by restriction nucleases, proteinase K, and heat denaturation. The fragmented natural DNA is then separated from the high molecular weight poly[d(A-T)] by gel filtration. The efficacy of DNA removal by this procedure was validated by cesium chloride gradient and nearest-neighbor analysis of the product of the reaction and by measurement of the fidelity of poly[d(A-T)] synthesis by *Escherichia coli* DNA Pol I contaminated with increasing amounts of DNA. Also, DNA polymerases dissociated from chromatin retain the same accuracy as that of native chromatin. Synthesis of poly[d(A-T)] by chromatin is catalyzed mainly by DNA polymerase-β. By use of the described technique, we find that the fidelity of this reaction is exceptionally low; approximately one dGTP was incorporated for every thousand complementary nucleotides polymerized.

Replication of DNA[1] should necessarily generate a faithful copy of the genome to maintain species identity. Rates of spontaneous mutations in dividing prokaryotic and eukaryotic cells suggest that stable misincorporation of a base during replication occurs at a frequency of 10^{-8}–10^{-11} per base pair synthesized (Drake, 1969). It is very difficult, however, to estimate the accuracy of repair of DNA in nondividing cells which do not propagate mutations. Most of the components of the replicative and repair machineries of animal cells, which confer accuracy in DNA synthesis, are not known. It is reasonable to assume, however, that part of the responsibility for maintaining accuracy of DNA synthesis lies with DNA polymerases. In prokaryotes, mutant DNA polymerase can cause mutations throughout the genome (Speyer, 1965). Hence, a first step in assessing the overall fidelity of DNA polymerization should be to measure the error rate of these enzymes. Three distinct types of DNA polymerases, designated DNA polymerases α, β, and γ, have been described in eukaryotic cells (Weissbach et al., 1975). Several lines of evidence suggest that DNA polymerase α is involved in DNA replication, whereas DNA polymerase β has been implicated in DNA repair (Loeb, 1974; Bollum, 1975; Weissbach, 1977; Sarngadharan et al., 1978). DNA polymerase γ seems to be similar to—and perhaps identical with—the polymerase found in isolated mitochondria (Bolden et al., 1977). The accuracy of in vitro copying of polynucleotides by purified mammalian DNA polymerases has been measured (Loeb, 1974; Salisbury et al., 1978; Seal et al., 1979; Linn et al., 1976; Krauss & Linn, 1980). These studies have yielded conflicting results in regard to both the number of different forms of DNA polymerases α and β that were isolated and the fidelity of DNA synthesis catalyzed by these enzymes. These discordant observations could be explained by loss during purification of certain forms of polymerases which possess inherently different fidelity. Alternatively, it is possible that the error rate of any polymerase may be dependent upon its interaction with other proteins which are removed during enzyme purification. Particularly with respect to physiological problems, it seems important to measure accuracy in crude preparations containing DNA polymerases which may better reflect the in vivo fidelity of DNA synthesis. An appropriate system for such measurements are chromatin-associated polymerases, which are capable of copying defined exogenous DNA templates (Kaftory & Fry, 1978; Kaftory et al., 1979; Weisman-Shomer et al., 1979). A major obstacle in assessing the fidelity of replication of defined synthetic polynucleotides by polymerases conjoined with chromatin is the presence of large amounts of genomic DNA within the chromatin. This endogenous DNA serves as a template for incorporation and thus invalidates measurements of incorporation of correct and incorrect bases into the defined exogenous polynucleotide.

In this paper, we describe a novel protocol which enables measurements of the fidelity of copying of poly[d(A-T)] by chromatin-associated DNA polymerase β. This method involves both selective suppression of template activity of endogenous DNA and its removal from poly[d(A-T)] after chromatin-directed synthesis is completed. The described procedure may serve as a protocol applicable to measurement of the accuracy of copying of synthetic polynucleotides by any polymerase preparation which is heavily contaminated with genomic DNA. In the present paper we use this technique to show that DNA polymerase β associated with chromatin of several cell types is highly error prone.

Materials and Methods

Calf thymus DNA and micrococcal nuclease were obtained from Worthington Biochemical Corp. Restriction DNA endonucleases were supplied by Bethesda Research Laboratories and New England Biolabs. Proteinase K and hexokinase were purchased from Boehringer Mannheim. DNA polymerase I

† From the Department of Pathology and Joseph Gottstein Memorial Cancer Research Laboratory SM-30, University of Washington, Seattle, Washington 98195. *Received May 13, 1980.* Supported by grants from the National Institutes of Health (CA-24845 and AG-01751).

‡ On sabbatical leave from the Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel.

[1] Abbreviations used: DNA, deoxyribonucleic acid; MalNEt, *N*-ethylmaleimide; 2,3-ddTTP, 2,3-dideoxythymidine triphosphate; DE-52, diethylaminoethylcellulose-52; dNTP, equimolar mixture of dATP, dTTP, dCTP, and dGTP; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

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was purified from *Escherichia coli* by the method of Jovin et al. (1969), as supplemented by Springgate et al. (1973). Poly[d(A-T)] was prepared by the de novo reaction, using *E. coli* DNA polymerase I (Radding & Kornberg, 1962). The synthetic polynucleotide banded at a density of 1.6786 in a cesium chloride gradient, and its molecular weight was estimated to be 0.9×10^6. The purity of the heteropolymer was found to be 1 mol of dGMP for every 2×10^6 mol of polynucleotide synthesized (Agarwal et al., 1979). From competition experiments between dGTP and dATP, we estimate that if dCMP is present it is at a frequency of <1 in 2×10^5. Poly(dA)$_{100}$ was prepared with purified calf thymus terminal deoxynucleotidyl transferase (Chang et al., 1972). Unlabeled deoxyribonucleoside triphosphates were supplied by Calbiochem. Tritium labeled dGTP and [α-^{32}P]dTTP were the products of New England Nuclear. Purity of [^{3}H]dGTP was monitored by thin-layer chromatography as well as by showing a linear decrease in its incorporation upon dilution with unlabeled dGTP in fidelity assays (Battula & Loeb, 1975). Also, snake venom phosphodiesterase digestion of the product of the reaction shows that the tritiated label in the product is indeed dGMP (Agarwal et al., 1979). The nucleotide analogue 2,3-dideoxythymidine triphosphate (ddTTP) was obtained from P-L Biochemicals. Actinomycin D and *N*-ethylmaleimide (MalNEt) were purchased from Sigma Chemical Co. DEAE-cellulose (DE-52) was produced by Whatman, and Bio-Gel A-0.5 was supplied by Bio-Rad.

Animals and Cells. Mice, 6–12-month-old males, were the (C57BL/6 × BALB/c) F_1 hybrid strain of *Mus musculus* obtained from the Charles River (Cambridge, MA) aging colony. The mice were grown under specific pathogen-free conditions. Normal human lymphocytes were collected from blood donated by healthy human male volunteers of age 20–35. Lymphocytes were isolated 105 h after stimulation in vitro by phytohemagglutinin as previously described (Agarwal et al., 1978). Transformed lymphocytes (strain Molt-4) were obtained from the National Science Foundation, Atlanta, GA. HeLa cells grown in suspension culture to 7×10^5 cells/mL were a generous gift of Dr. M. Cooney, University of Washington. Exponentially grown L cells were donated by R. Perry and D. Kelley, The Institute for Cancer Research, Fox Chase, Philadelphia, PA.

Preparation of Chromatin. Crude native chromatin was isolated from mouse liver as follows: Mice were sacrificed by cervical dislocation, and their livers were removed and immersed immediately in ice-cold 0.3 M sucrose and 4 mM $CaCl_2$. All subsequent steps were conducted in the cold. Livers of several animals were pooled and washed in 0.3 M sucrose and 4 mM $CaCl_2$. The tissues were finely minced and then homogenized by 50–80 strokes in a dounce homogenizer equipped with a B-type pestle. Nuclei were separated from cytoplasm and purified by centrifugation through a discontinuous sucrose gradient according to Lynch et al. (1975). The washed liver cell nuclei were hypotonically lysed, and their crude native chromatin was isolated as described by Knopf & Weissbach (1977). Nuclei were isolated from 1 to 3 g wet weight batches of human lymphocytes and L and HeLa cells, and their chromatin was extracted as previously described (Kaftory & Fry, 1978). Protein and DNA contents were routinely determined in the isolated chromatin according to the methods of Lowry et al. (1951) and Burton (1956), respectively. The protein/DNA in the various crude chromatin preparations ranged between 3.2 and 4.4. Chromatin (0.5–2.0 mg/mL) was stored at −80 °C in 1 mM Tris-HCl (pH 8.5), 1 mM dithiothreitol, 0.1 mM EDTA, and 40% glycerol. Under these storage conditions, chromatin-associated DNA polymerizing activity remained undiminished for at least 6 months.

Assay Conditions for DNA Synthesis. DNA synthesis activity of chromatin was assayed at 37 °C in a reaction mixture which contained, in a volume of 100 μL, 50 mM Tris-HCl buffer (pH 7.5), 1 mM dithiothreitol, 5 mM $MgCl_2$, glycerol to a final concentration of 20%, 10 mM phosphocreatine, 10 mg of creatine phosphokinase, 0.25 mM ATP, and 0.025 mM each of dATP, dGTP, dCTP, and [α-^{32}P]dTTP (sp act., 150–400 dpm/pmol). Chromatin was present at 100 μg as DNA/mL, and exogenous heat-denatured calf thymus DNA was added to saturation at 300 μg/mL. For monitoring of the synthesis of poly[d(A-T)], the reaction mixture was as the above except that dATP and [α-^{32}P]dTTP served as substrates and the synthetic heteropolymer was added to saturation at 275 μg/mL. Under these conditions, incorporation of labeled substrates into acid-preciptable material proceeded at a linear rate for at least 2 h. Activity of terminal deoxynucleotidyl transferase was assayed in a reaction mixture which contained, in a final volume of 25 μL, 200 mM potassium cacodylate buffer (pH 7.0), 1 mM $CoCl_2$, 10 mM mercaptoethanol, 1 mM [^{3}H]dGTP (sp act., 5000 dpm/pmol), exogenous poly(dA) (200 μg/mL), and chromatin (140 μg as DNA/mL). Incubation was at 37 °C for 60 min. The polymerization reaction was terminated by addition of perchloric acid and sodium pyrophosphates, and the acid-insoluble product was washed as previously described (Battula & Loeb, 1974). Counting efficiencies were 50% and 22% for ^{32}P and ^{3}H, respectively.

Assay Conditions for Measurement of Accuracy of Poly[d(A-T)] Copying. Fidelity of poly[d(A-T)] synthesis catalyzed by DNA polymerase I from *E. coli* was assayed under the conditions described by Agarwal et al. (1979). For assays of the fidelity of chromatin-directed synthesis of poly[d(A-T)], reaction mixtures at final volumes of 250 μL contained all the components detailed in the preceding paragraph and also 400 μM actinomycin D and the incorrect substrate [^{3}H]dGTP at a specific activity of 4×10^6–6×10^4 dpm/pmol. After termination of poly[d(A-T)] synthesis, the endogenous DNA of the chromatin was hydrolyzed and removed and the poly[d(A-T)] was isolated as detailed below. When chromatin-associated proteins were dissociated from DNA prior to the fidelity assay, the polymerization reaction was conducted in a final volume of 100 μL, with or without actinomycin D, and the product was washed as described by Battula & Loeb (1974).

Removal of Endogenous DNA from Chromatin-Directed Fidelity Assays. The principle obstacle in measuring the accuracy of chromatin-directed synthesis of poly[d(A-T)] is the presence of large amounts of native DNA complexed within the chromatin. This DNA serves as template for incorporation of all four deoxynucleoside triphosphates, and thus its presence obscures measurements on the incorporation of incorrect substrates into exogenous poly[d(A-T)]. A procedure was developed to eliminate endogenous DNA and to isolate the poly[d(A-T)] product. After poly[d(A-T)] was copied in the fidelity reaction mixture by using native chromatin as an enzyme source, incorporation of the labeled substrates was terminated either by adding a 40–50-fold excess of unlabeled dNTP's or by the introduction of hexokinase and glucose (Kaftory & Fry, 1978). Chromatin DNA was subsequently subjected to hydrolysis at 37 °C by a series of specific restriction endonucleases each incapable of cleaving poly[d(A-T)] or poly[d(A-T)] containing misincorporated dGMP.

Chart I: Protocol for Measuring Fidelity of Poly[d(A–T)] Copying by Chromatin-Associated DNA Polymerases

(A) fidelity reaction
enzyme source: chromatin-associated DNA polymerase β
template-primer: poly[d(A–T)]
metal activator: Mg^{2+} (5 mM)
nucleotides: [α-^{32}P]dTTP, dATP, [^{3}H]dGTP (25 μM each)
inhibitor: actinomycin D (400 μM)
incubation: 2 h at 37 °C
termination: with 50-fold excess of unlabeled dNTP's at 0 °C

(B) hydrolysis and removal of DNA
*Hae*III (45 units/mL), 4 h, 37 °C
↓
*Hpa*II (24 units/mL)
*Hha*I (32 units/mL)
*Kpn*I (32 units/mL), 16 h, 37 °C
↓
NaCl to 60 mM
↓
*Hin*dIII (40 units/mL), 6 h, 37 °C
↓
proteinase K (6 mg/mL), 2 h, 37 °C
↓
heat denaturation (100 °C, 15 min)
↓
centrifugation (6000*g*, 5 min)
↓
gel filtration (Bio-Gel A-0.5m)
↓
collection of excluded volume

Typically, the reaction mixture was incubated for 4 h with 45 units/mL *Hae*III, followed by incubation with 32 units/mL *Kpn*I, 24 units/mL *Hpa*II, and 32 units/mL *Hha*I. The endonucleolytic reactions catalyzed by these enzymes were terminated by addition of 1 M NaCl to a final concentration of 60 mM. Salt at this concentration is required for maximum activity of *Hin*dIII, which was introduced at 40 units/mL for an additional 6-h period of incubation. In the next step, chromatin-associated proteins and the added restriction enzymes were hydrolyzed for 2 h at 37 °C by 6 mg/mL proteinase K. The size of the natural DNA was further reduced by denaturation at 100 °C for 15 min. After a brief cooling period which allowed poly[d(A-T)] to renature, the mixture was filtered through an 0.5 × 45 cm column of Bio-Gel A-0.5m. Equilibration and elution of the column were conducted with 50 mM Tris-HCl (pH 7.5), 25 mM EDTA, and 100 mM KCl. About 70% of the double-labeled poly [d(A-T)] was recovered in the excluded volume, whereas practically all the fragmented endogenous DNA as well as the unincorporated labeled substrates were retarded by the gel (see Results). The purity of the excluded poly[d(A-T)] was ascertained by separating it from any remaining DNA contaminant on neutral cesium chloride gradients. The ratio of [^{3}H]dGMP to [^{32}P]dTMP in the excluded macromolecular poly[d(A-T)] served as a measure of the accuracy of its synthesis by chromatin. We find that all the steps detailed above and shown schematically in Chart I are essential for adequate elimination of copied DNA from the system. However, attempts to introduce additional restriction nucleases with various specificities proved ineffective in further reducing contamination with copied DNA.

Analysis of the Product of the Reaction. Analysis of the nearest-neighbor frequencies in the product resulting from copying poly[d(A-T)] by chromatin-associated polymerases was performed according to Shearman & Loeb (1979).

Table I: Characterization of Chromatin-Associated DNA Polymerase Activity[a]

source of chromatin	exogenous templates	activity (pmol incorporated) no inhibitor	+4 mM MalNEt	+0.1 mM ddTTP
mouse liver	poly[d(A–T)]	159.1	148.5	2.2
	heat-denatured DNA	15.4	5.6	3.4
	poly(dA)$_{\overline{100}}$	<0.01		
PHA-stimulated normal human lymphocytes	poly[d(A–T)]	93.8	81.7	5.2
	heat-denatured DNA	58.0	5.5	46.3
	poly(dA)$_{\overline{100}}$	<0.01		
transformed human lymphocytes	poly[d(A–T)]	89.9	70.1	5.8
	heat-denatured DNA	293.4	0.0	196.3
	poly(dA)$_{\overline{100}}$	635.6		

[a] Chromatin at 100 μg as DNA/mL was incubated for 60 min at 37 °C in DNA polymerase assay mixture as given under Materials and Methods. Synthesis using poly(dA)$_{\overline{100}}$ was conducted under conditions for measurement of activity of terminal transferase (see Materials and Methods). A mixture containing natural DNA, p poly[d(A–T)], and polyd(A)$_{\overline{100}}$ had [α-^{32}P]dTTP + dNTP's, [α-^{32}P]dTTP + dATP, and [^{3}H]dGTP, respectively, as substrates. Specific activities of [α-^{32}P]dTTP and [^{3}H]dGTP were 500 and 1000 cpm/pmol, respectively. All templates were added to 300 μg/mL. MalNEt and 2,3-ddTTP were added prior to chromatin.

Neutral Cesium Chloride Density Gradient Centrifugation. Nucleic acids obtained in a processed fidelity assay were suspended in 50 mM Tris-HCl (pH 7.5), 25 mM EDTA, and 100 mM KCl, and cesium chloride was added to an initial density of 1.720 (refractive index, 1.4012). The solution was centrifuged for 70 h at 140000*g* at 20 °C in a 65 Ti rotor on a Beckman ultracentrifuge. About 30 fractions of 0.3 mL each were collected from the top of the tube, and acid-insoluble radioactivity was determined after adding 100 μg of calf thymus DNA (1 mg/mL) to each tube as a carrier.

Results

Activity of Chromatin-Associated DNA Polymerases. Eukaryotic DNA polymerases complexed in native chromatin are capable of efficient in vitro copying of exogenous DNA templates (Kaftory & Fry, 1978; Weisman-Shomer et al., 1979). Chromatin was isolated from different cell types, and the polymerases catalyzing the copying of exogenous templates were characterized (Table I). Copying of poly[d(A-T)] by all chromatin preparations is almost unaffected by 4 mM MalNEt, an inhibitor of DNA polymerases α annd γ, which does not suppress activity of DNA polymerase β (Weissbach, 1977; Krokan et al., 1979). Conversely, copying of this template is diminished by the nucleotide analogue 2,3-ddTTP which is known to inhibit DNA polymerases β and γ, but not DNA polymerase α (Edenberg et al., 1978; Krokan et al., 1979). It thus seems that chromatin-directed synthesis of poly[d(A-T)] is conducted primarily by DNA polymerase β. This conclusion is further sustained by observations on the copying of single-stranded DNA by chromatin. Table I indicates that chromatins from normal and transformed human lymphocytes contain considerable activity of DNA polymerase α, which is the enzyme responsible for the copying of added

Table II: Effect of Actinomycin D on the Fidelity of DNA Synthesis with Poly[d(A–T)] and *E. coli* Pol I[a]

actinomycin D (μM)	incorporation dTMP (pmol)	incorporation dGMP (pmol)	error rate
0	265	0.0074	1/35 800
25	251	0.0067	1/37 200
50	263	0.0077	1/34 300
100	241	0.0067	1/36 100
250	259	0.0077	1/33 600
500	247	0.0072	1/34 500
1000	253	0.0077	1/32 700

[a] Assay conditions were as described under Materials and Methods. Incorporation in the absence of incubation was 1 pmol of dTMP and 0.0050 pmol of dGMP, and these values were subtracted from the values listed in the table. The error rate is defined as the ratio of noncomplementary nucleotide incorporated to total complementary nucleotide incorporated. Although in some experiments initial error rates of DNA Pol I were higher (1/20 000), actinomycin D failed to affect their level.

heat-denatured DNA (Kaftory & Fry, 1978; Weisman-Shomer et al., 1979). We also found that copying of the synthetic templates poly(dC) and poly(dT) is inhibited by MalNEt and not by 2,3-ddTTP (data not shown). However, despite the presence of DNA polymerase α in these chromatin preparations, it is predominantly DNA polymerase β which copies poly[d(A-T)]. Similar observations were made in chromatins from L and HeLa cells (results not shown). We further find that, whereas chromatins from normal lymphocytes and liver cells fail to elongate the primer poly(dA), this polynucleotide is efficiently extended by chromatin from an established cell line of lymphocytes known to contain terminal transferase (Srivastava & Minowada, 1973).

Effect of Actinomycin D on the Fidelity of Poly[d(A-T)] Synthesis. The goal of the present study is to measure the accuracy of poly[d(A-T)] synthesis catalyzed by chromatin-bound DNA polymerase. Fidelity of poly[d(A-T)] synthesis by DNA polymerase may be assessed by providing the enzyme with the correct substrates dATP and [α-^{32}P]dTTP at a low specific activity and with the erroneous nucleotide [^{3}H]dGTP at a very high specific activity. The ratio of ^{3}H to ^{32}P in the newly made polynucleotide yields the frequency of the incorporation of incorrect nucleotide (Battula & Loeb, 1974). However, if DNA is present in the polymerase preparation as in chromatin, it will serve as template for the incorporation of all four nucleotides. Thus, radioactive dGTP incorporated into DNA will obscure the infrequent dGTP misincorporation into poly[d(A-T)]. Actinomycin D, which binds to dG–dC base pair in double-stranded DNA (Goldberg & Friedman, 1971), reduced its template activity (results not shown). By contrast, neither the efficiency nor the fidelity of poly[d(A-T)] synthesis is affected by this drug. Actinomycin D at concentrations up to 1 mM does not affect the rate or accuracy of poly[d(A-T)] synthesis catalyzed by DNA polymerase I from *E. coli* (Table II). Table III shows that even minute amounts of exogenously introduced activated DNA, which serves as maximally efficient primer–template, greatly increase the apparent error rate of DNA polymerase I in copying poly[d(A-T)]. With 0.005 μg of activating DNA per assay, there is a 10-fold apparent reduction in fidelity. However, incorporation of dGMP into DNA can be completely inhibited by actinomycin D when the concentration of DNA is as great as 0.15 μg/assay. At higher concentrations of added activated DNA, actinomycin D reduces incorporation of dGMP but does not abolish it completely. Similar results were obtained with DNA polymerase α and β from human placenta and lymphocytes (data not shown). Actinomycin D can be instrumental, therefore, in inhibiting incorporation into DNA in extracts which contain small amounts of the natural DNA template. However, since chromatin replicative systems contain endogenous DNA at ~100 μg/mL, procedures additional to introduction of actinomycin D are required for complete elimination of the effects of DNA in the poly[d(A-T)] synthesizing system.

Table III: Effect of Actinomycin D on the Amount of Activated DNA Tolerable in Normal Fidelity Assay[a]

activated DNA (μg/mL)	no actinomycin D dGMP (pmol)	no actinomycin D apparent error rate	500 μM actinomycin D dGMP (pmol)	500 μM actinomycin D apparent error rate
0	0.0075	1/40 000	0.0078	1/38 500
0.1	0.0812	1/3 690	0.0073	1/41 100
1.0	0.6746	1/440	0.0081	1/37 000
3.0	3.864	1/80	0.0085	1/35 300
10	7.339	1/40	0.137	1/21 900
30	30.56	1/10	0.0419	1/7 160
100	53.45	1/5	0.1362	1/2 200

[a] Assay conditions were as described in the legend to Table II. The incorporation of [^{3}H]dGTP in assays without DNA templates was <0.005 pmol; it was not affected by actinomycin D.

Removal of DNA from Poly[d(A-T)] Fidelity Assay Systems. A general procedure was developed for measurement of accuracy of poly[d(A-T)] synthesis in systems heavily contaminated with DNA. This method involves selective inhibition of natural DNA as a template and its subsequent separation from poly[d(A-T)]. The procedure was described in detail under Methods and is depicted schematically in Chart I. Poly[d(A-T)] is copied by a DNA-containing polymerizing system in the presence of actinomycin D to preferentially inhibit the template activity of DNA. After incorporation is terminated, the system is exposed to a series of restriction endonucleases which cleave DNA but not poly[d(A-T)]. To further reduce the size of protein–DNA complexes, we hydrolyzed the chromatin-associated proteins by proteinase K, denatured the DNA by heat, and removed aggregrates by centrifugation. Upon gel filtration, high molecular weight poly[d(A-T)] was excluded from the Bio-Gel A-0.5 column, whereas DNA fragments and free labeled nucleotides were retarded. Mixtures of poly[d(A-T)] and native DNA were copied by *E. coli* DNA Pol I under fidelity assay conditions to assess the potency of this method. After 15 min period of polymerization, incorporation of labeled nucleotides was terminated by heat and the addition of a 50-fold excess of nonradioactive dNTP's. The reaction mixture was subjected to the procedure depicted in Chart I, and ^{3}H/^{32}P ratios were determined in material excluded from the molecular sieve (Table IV). The fidelity of *E. coli* Pol I in this experiment was 1/26 000, and this accuracy was not diminished significantly by the addition of up to 200 μg of native DNA/mL. To examine the applicability of this procedure to chromatin-directed replication of poly[d(A-T)], we conducted the following experiment. Mouse liver chromatin at a concentration of 100 μg as DNA/mL was used as an enzyme source in fidelity assays. In designated reactions, actinomycin D was omitted, and after conclusion of the polymerization reaction, resulting acid-precipitable matieral was extensively washed as previously described (Battula & Loeb, 1974). In a second parallel experiment, actinomycin D was present in the reaction mixture, and the nucleic acids were separated on Bio-Gel A-0.5m without being treated by restriction nucleases and proteinase K. A third group of assays was fully processed as described in Chart I. The nucleic acids obtained in all three

Table IV: Accuracy of *E. coli* Polymerase I after Elimination of Added Native DNA by Digestion with Restriction Nucleases and by Gel Filtration[a]

native calf thymus DNA (μg/mL)	[^{3}H]dGMP incorporation (pmol)	[^{3}H]dTTP incorporation (pmol)	frequency of misincorporation (dGMP/dATP + dTTP)
none	0.0406	535.8	1/26 394
100	0.0499	533.8	1/22 196
200	0.0559	624.0	1/22 330

[a] Poly[d(A–T)] was copied by DNA polymerase I for 15 min at 37 °C under fidelity assay conditions in a reaction mixture that contained in a final volume of 100 μL increasing amounts of native DNA. Specific activities of [^{3}H]dGTP and [α-^{32}P]dTTP were 36×10^3 and 100 dpm/pmol, respectively. Incorporation of labeled nucleotide was terminated by heating the reaction mixture at 65 °C for 15 min and by adding a 50-fold excess of unlabeled dGTP, dATP, and dTTP. The reaction mixtures were treated with restriction nucleases and proteinase K as diagramed in Chart I and filtered through a column of Bio-Gel A-0.5m. The ratio of ^{3}H to ^{32}P was measured in acid-preciptable material excluded from the columns. The results shown are the average of duplicates which were similar within ±2.5%.

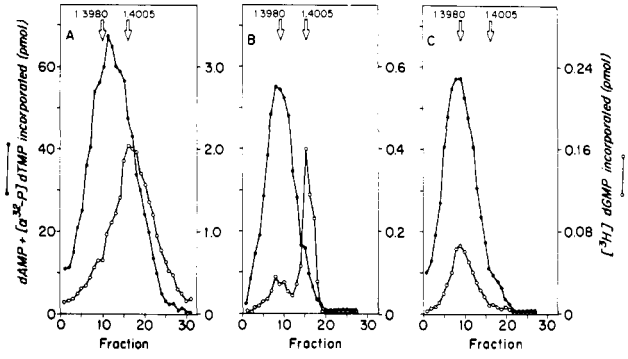

FIGURE 1: Neutral cesium chloride density gradient centrifugation of poly[d(A-T)] synthesized by mouse liver cell chromatin. Poly[d(A-T)] was copied by liver cell chromatin in the fidelity assay with [α-^{32}P]dTTP (77 cmp/pmol) and [^{3}H]dGTP (12 080 cpm/pmol) serving as correct and incorrect labeled substrates, respectively. After polymerization was terminated, the differentially processed samples were centrifuged and analyzed in cesium chloride equilibrium gradients as described under Methods. Arrows mark the positions and refractive indices of marker poly[d(A-T)] and natural DNA which were centrifuged in parallel. (A) Poly[d(A-T)] synthesized by chromatin in the absence of actinomycin D, acid precipitated, washed, and analyzed by cesium chloride gradient centrifugation. (B) Poly[d(A-T)] synthesized by chromatin in the presence of actinomycin D and separated on Bio-Gel A-0.5m column without prior treatment with restriction nucleases and proteinase K. (C) Poly[d(A-T)] synthesized by chromatin and isolated as detailed in Chart I.

groups were centrifuged on neutral cesium chloride gradients which separate DNA from poly[d(A-T)] by virtue of their different densities. Representative results are shown in Figure 1. It is clear from the gradient profile shown in Figure 1A that, in the absence of actinomycin D and with no gel filtration, most of the tritium is found at a density corresponding to that of DNA and no peak is discernible in the poly[d(A-T)] region. When actinomycin D is present during polymerization and a step of gel filtration is included, separate peaks of tritium at the densities of poly[d(A-T)] and DNA are resolved (Figure 1B). However, a significant amount of DNA is still mixed with poly[d(A-T)] in the material which is excluded from the molecular sieve. On the other hand, when the reaction mixture is subjected to nucleolytic and proteolytic digestion prior to gel filtration, both ^{3}H and ^{32}P labels band coincidentally on a cesium chloride gradient at a density corresponding to that of poly[d(A-T)] (Figure 1C). In more than 30 independent

Table V: Nearest-Neighbor Analysis of the Product of Poly[d(A–T)] Synthesis Catalyzed by Mouse Liver Chromatin[a]

	nearest-neighbor sequence (cpm)[c]			
expt[b]	ApX	CpX	TpX	GpX
I	66	20	562	96
II	48	43	342	42
III	79	76	323	48

[a] Experimental conditions for synthesis of poly[d(A–T)] and its subsequent hydrolysis and electrophoretic analysis of the products were as described under Materials and Methods. [b] Each experiment represents analysis of poly[d(A–T)] synthesized by a separately isolated preparation of mouse liver chromatin. [c] A uniform background of 16 cpm was subtracted from the fractionated labeled nucleotides.

cesium chloride gradient centrifugations carried out on material excluded from Bio-Gel columns, we have failed to detect measurable amounts of tritium at a density corresponding to that of DNA. Moreover, in all these experiments the ratio or ^{3}H to ^{32}P was found to be uniform throughout the gradients. That the double-labeled polymer obtained after elimination of DNA indeed represents poly[d(A-T)] was further demonstrated by nearest-neighbor analysis of this material. Poly[d(A-T)] was copied by liver chromatin under the standard fidelity assay conditions except that the labeled substrates were [^{3}H]dATP at a low specific activity and [α-^{32}P]dGTP at a very high specific activity. After synthesis, poly[d(A-T)] was isolated as described in Chart I, and material excluded from Bio-Gel was subjected to extensive digestion by micrococcal nuclease and spleen phosphodiesterase (see Materials and Methods). The resulting 3′-deoxyribonucleoside monophosphates were isolated, concentrated, and separated by electrophoresis on cellulose plates as previously described (Shearman & Loeb, 1979). Quantitation of the radioactivity in the various nucleotides is presented in Table V. It is apparent from the data obtained that ~70% of the label that originated from [α-^{32}P]dGTP had been transferred to dTMP. Only residual activity is found in dCTP which is the only base purely derived from DNA. It seems, therefore, reasonable to conclude that the dGMP in material purified through the standard procedure is mainly in poly[d(A-T)]. This base is erroneously incorporated in place of dAMP into the synthetic template and is predominantly present as a single-base substitution. The restricted distribution of label in dTMP also indicates that incorporation with chromatin is not catalyzed by deoxynucleotidyl terminal transferase. Had such activity predominated, ^{32}P should have been found in association with all four bases at an equal frequency. Last, including [^{3}H]-dATP together with [α-^{32}P]dGTP in the reaction mixture allows independent verification of the fidelity of poly[d(A-T)] synthesis by measuring the ratio of ^{3}H to ^{32}P in the separated nucleotides. Using this technique, we find that the frequency of misincorporation in reactions catalyzed by liver cell chromatin polymerase ranges between 1:950 to 1:1400 (data not shown). The observed low accuracy of the poly[d(A-T)] copying may result from degradation of one of the deoxyribonucleoside triphosphates in the reaction mixture. However, increasing the dNTP concentration 2-fold to 50 μM did not alter the rate or fidelity of the chromatin-directed poly[d(A-T)] synthesis (results not shown).

Fidelity of Chromatin-Associated DNA Polymerase β. After establishment of the adequacy of the procedure for determination of the fidelity of poly[d(A-T)] synthesis by chromatin-bound DNA polymerase β, accuracy of this reaction was compared for chromatin of several cell types (Table VI). Chromatin-associated DNA polymerase β from both normal

Table VI: Fidelity of Poly[d(A–T)] Synthesis Directed by Chromatin-Associated DNA Polymerase β of Various Cell Types[a]

source of chromatin	expt	incorporation [α-^{32}P]dTMP + dAMP	incorporation [^{3}H]dGMP	error rate
normal mouse liver	I	119.8	0.112	1/1 070
	II	454.4	0.371	1/1 225
	III	382.5	0.309	1/1 240
	IV	374.5	0.266	1/1 410
normal human lymphocytes	I	233.6	0.150	1/1 490
	II	159.0	0.116	1/1 370
transformed human lymphocytes	I	209.1	1.398	1/150

[a] Fidelity reactions were carried out with actinomycin D, and the poly[d(A–T)] product was isolated by the use of restriction enzymes and gel filtration. Specific activities of [α-^{32}P]gTTP and [^{3}H]dGTP were 200 and 49 000 dpm/pmol, respectively.

lymphocytes and liver cells copy poly[d(A-T)] at a relatively low fidelity of about 1/1000 to 1/1500 incorrect over correct nucleotides incorporated. Chromatin derived from Molt-4 transformed lymphocytes is even more error prone and leads to insertion of one erroneous nucleotide for every 150 correct bases incorporated. However, it is possible that terminal deoxynucleotidyl transferase found in this chromatin (Table I) is responsible for this apparent high error rate (Srivastava & Minowada, 1973). The low fidelity of chromatin-bound DNA polymerase β as observed here is in discord with a previous report on the high accuracy of DNA polymerase β purified from human placenta (Seal et al., 1979). To exclude the possibility that the observed low fidelity of chromatin-associated polymerase is related to the procedure for isolation of poly[d(A-T)], we have determined the accuracy of crude DNA polymerase β by an alternative independent method. Proteins associated with mouse liver chromatin were solubilized by extraction with salt. Endogenous DNA was eliminated from the system by high-speed centrifugation, batch ion-exchange chromatography, and hydrolysis with micrococcal nuclease (for details, see legend to Table VII). That the resultant crude extract was free of a DNA template was demonstrated by failure to detect incorporation of highly radioactive dGTP into acid-insoluble material in the absence of exogenous template. The only DNA polymerase activity detected with poly[d(A-T)] in this crude extract was DNA polymerase β, as shown by its complete resistance to MalNEt and full sensitivity to 2,3-ddTTP (data not shown). The crude extracts served to catalyze synthesis of poly[d(A-T)] in a fidelity assay system. After termination of polymerization, the product was washed exhaustively as previously described (Battula & Loeb, 1974), and the ratio of ^{3}H to ^{32}P was determined. Table VII summarizes results of a typical experiment. The DNA polymerase β dissociated from chromatin retains the same low accuracy as when it was bound to it. Also, as shown for other polymerases, fidelity is not affected by the presence of actinomycin D in the assay system. We conclude, therefore, that DNA polymerase β, bound to or dissociated from chromatin but not previously purified, is highly error prone in copying poly[d(A-T)] in vitro.

Discussion

The principal object of this study has been to determine the accuracy of DNA synthesis catalyzed by DNA polymerases associated with proteins that function in DNA replication and repair. Isolated native chromatin offers several advantages which make it a logical system of choice for such studies. Chromatin-associated DNA polymerases are able to copy defined exogenous DNA templates at high efficiency (Kaftory & Fry, 1978). This copying of exogenous templates by chromatin requires ATP; its rate of chain elongation is related to the in vivo rate of DNA replication, and the rate of the reaction can be specifically modulated by nuclear proteins (Kaftory & Fry, 1978; Kaftory et al., 1979; Weisman-Shomer et al., 1979). Thus, certain properties of the in vivo replicative machinery are better reflected by chromatin than by isolated DNA polymerases. Despite the complexity of chromatin, it is possible to identify which DNA polymerase is responsible for copying particular exogenous templates by chromatin. DNA polymerase α is the only enzyme in chromatin that is able to effectively utilize poly(dC), poly(dT) (unpublished

Table VII: Accuracy of DNA Polymerase β Dissociated from Native Chromatin[a]

expt[b]	conditions	assay	incorporation [α-^{32}P]dTTP + dAMP	incorporation [^{3}H]dGMP	error rate
I	−actinomycin D	I	48.7	0.040	1/1 220
		II	47.4	0.044	1/1 080
		III	49.2	0.039	1/1 260
	+actinomycin D	IV	52.4	0.043	1/1 220
II	−actinomycin D	I	51.6	0.043	1/1 200
		II	50.6	0.045	1/1 120
		III	44.5	0.039	1/1 140
	+actinomycin D	IV	39.5	0.036	1/1 100
III	−actinomycin D	I	46.2	0.049	1/940
		II	49.2	0.049	1/1 000
	+actinomycin D	IV	40.5	0.038	1/1 070

[a] For solubilization of chromatin-associated proteins, 0.1 volume of 5 M NaCl was added to chromatin suspensions (0.5–1.0 mg as DNA/mL). Salt at this concentration releases most of the DNA polymerase activity conjoined with chromatin (Böhm et al., 1977; Schlaeger et al., 1978). The chromatin solution was subjected to three 10-s cycles of ultrasonication (60 cycles, output 6-Benson Sonic Co.), and the extract was then centrifuged at 28000g for 60 min. The bulk of chromatin DNA was pelleted by this centrifugation. For further removal of contaminating DNA, the supernatant was passed through a 1.5-mL column of DE-52 equilibrated with 20 mM potassium phosphate buffer (pH 7.5), 0.5 M NaCl, 0.5 mM dithiothreitol, and 20% glycerol. The column was washed with 1.5 mL of the same high-salt buffer, and the eluted extract was dialyzed overnight against 500 volumes of 20 mM potassium phosphate buffer (pH 7.5), 0.5 mM dithiothreitol, and 40% glycerol. The remaining DNA contaminant was hydrolyzed by incubating the extract at 37 °C for 30 min with 1 μg/mL micrococcal nuclease in the presence of 1 mM $CaCl_2$. The nuclease reaction was terminated by binding the Ca^{2+} ions with 3 mM EGTA. Proteins extracted from different chromatin preparations were incubated at 37 °C for 60 min with poly[d(A–T)], [α-^{32}P]dTTP (25 cpm/pmol), dATP, [^{3}H]dGTP (7935 cpm/pmol), and 5 mM Mg^{2+} as a metal activator under fidelity assay conditions. The reaction product was washed 5 times, and its ratio of ^{3}H to ^{32}P was determined. Assay systems in which poly[d(A–T)] was omitted incorporated <0.004 pmol of [^{3}H]dGTP. [b] Each experiment refers to a separately isolated preparation of extract from mouse liver chromatin.

experiments), and heat-denaturated DNA as templates (Kaftory & Fry, 1978; Table I, this work). By contrast, even when both DNA polymerases α and β are present in chromatin, it is primarily the latter which copies poly[d(A-T)] (Table I). The reason for the failure of chromatin-bound DNA polymerase α to copy poly[d(A-T)] is not clear. It is possible that association of this enzyme with chromatin components affects its template preferences. Poly(dC) and poly(dT) are copied by chromatin DNA polymerase α. However, the impurity of these polymers which are prepared by terminal transferases may be prohibitive for their use in assessing the accuracy of synthesis directed by DNA polymerase α. Hence, in this study, we were able to measure only the fidelity of copying of poly[d(A-T)] by chromatin-bound DNA polymerase β.

The main impediment to usage of native chromatin in fidelity assays is the presence of excessive amounts of genomic DNA. The standard way to measure the accuracy of poly[d(A-T)] synthesis is to assess the frequency of misincorporation of radioactively labeled dGMP into the copied heteropolymer (Agarwal et al., 1979; Seal et al., 1979). However, since chromatin DNA also serves as the template for endogenous replication (Knopf & Weisbach, 1977; Kaftory & Fry, 1978), radioactivity incorporated into it prohibitively obscures the accuracy of poly[d(A-T)] duplication. A new procedure was developed for elimination of DNA from the chromatin system. First, introduction of actinomycin D into the fidelity assay selectively suppresses endogenous DNA synthesis without affecting fidelity in copying poly[d(A-T)] (Tables II and III, Figure 1A,B). We did find, however, that actinomycin D is able to completely suppress endogenous incorporation only when relatively low amounts of DNA are present in the extract. Hence, addition of this drug may be instrumental in suppressing endogenous synthesis in crude enzyme preparations which contain low amounts of contaminating DNA. Since chromatin contains large amounts of genomic DNA, additional steps to remove it are required. After poly[d(A-T)] polymerization, chromatin DNA is selectively hydrolyzed by restriction enzymes, and the resulting DNA fragments are separated from the high molecular weight poly[d(A-T)] by gel filtration (Chart I). The adequacy of this method in obtaining DNA-free poly[d(A-T)] has been verified by several criteria. The accuracy of synthesis of poly[d(A-T)] by DNA polymerase I was shown to be unaffected by the addition of up to 200 μg of native DNA/mL when the reaction system was processed by this protocol (Table IV). Cesium chloride gradient analysis of material synthesized by chromatin and purified as described fails to demonstrate measurable quantitites of remaining copied DNA. The distribution of incorporated incorrect labeled and correct nucleotides on the gradient are superimposable and both possess the same density as marker poly[d(A-T)] (Figure 1C). Moreover, nearest-neighbor analysis of the purified poly[D(A-T)] demonstrates that DNA contaminant constitutes only a small proportion of the isolated nucleic acid and most of the dGMP is incorporated in place of dAMP into poly[d(A-T)] (Table V). This method enables, therefore, the isolation of pure poly[d(A-T)] after it is copied by crude native chromatin. To further verify results on fidelity of copying of poly[d(A-T)] by chromatin-associated DNA polymerase, we determined the accuracy of this reaction by an entirely independent procedure. Chromatin-associated proteins were extracted by salt, and contaminating DNA was removed by batch ion-exchange chromatography and by digestion with micrococcal nuclease. We find that fidelity of poly[d(A-T)] synthesis catalyzed by these crude extracts is very similar to that determined in native chromatin by use of the protocol described above (Table VII).

Results obtained for chromatin from three cell types indicate that copying of poly[d(A-T)] by DNA polymerase β conjoined with chromatin is highly error prone. Only with DNA polymerase in chromatin from transformed human lymphocytes (Table VI) can this high error rate be attributed to terminal transferase. The observed inaccuracy of crude DNA polymerase β from chromatin stands in contrast with a previous report on the relatively high fidelity of purified DNA polymerase β from calf thymus (Chang et al., 1972) and human placenta (Seal et al., 1979). In a recent paper, Krauss & Linn (1980) have isolated several distinct forms of DNA polymerase β which showed diverse fidelities in copying various synthetic templates. One of the enzymes, designated DNA polymerase β_1, copied poly[d(A-T)] at low accuracy, which is comparable to that of the crude enzyme as reported here. The apparent disaccord among the various measurements of accuracy of purified and chromatin-associated DNA polymerase β can be interpreted in several ways. It is possible that distinct forms of DNA polymerase β indeed have diverse fidelities and that the least accurate enzyme is associated with chromatin. Indirect evidence suggests that DNA polymerase β participates in DNA repair functions (Weissbach, 1977; Sarngadharan et al., 1978). It will be surprising if repair in vivo is as error prone as chromatin-directed copying of poly[d(A-T)] is. However, it is possible that the overall fidelity of in vivo repair is increased by factors other than the DNA polymerase β itself and that the activity of these factors was not detected with poly[d(A-T)] as a template. An alternative explanation to the dichotomy between the high fidelity of purified DNA polymerase β and the accuracy of the chromatin-associated enzyme is that the latter interacts with proteins which reduce its inherent high accuracy.

The described technique for removal of genomic DNA and isolation of synthetic polynucleotide after it has been copied can serve as a general experimental tool for measurement of accuracy of polynucleotide copying by DNA polymerases that are contaminated with DNA. This approach may enable detection of factors which affect accuracy in DNA synthesis that are usually removed during purification of DNA polymerases.

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